SENSORY ECOLOGY OF PLANT-POLLINATOR INTERACTIONS

EDITED BY: Casper J. Van Der Kooi, Sara Diana Leonhardt and Johannes Spaethe

PUBLISHED IN: Frontiers in Ecology and Evolution

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88976-910-0
DOI 10.3389/978-2-88976-910-0

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

SENSORY ECOLOGY OF PLANT-POLLINATOR INTERACTIONS

Topic Editors:
Casper J. Van Der Kooi, University of Groningen, Netherlands
Sara Diana Leonhardt, Technical University of Munich, Germany
Johannes Spaethe, Julius Maximilian University of Würzburg, Germany

Citation: Van Der Kooi, C. J., Leonhardt, S. D., Spaethe, J., eds. (2022). Sensory Ecology of Plant-Pollinator Interactions. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88976-910-0

Table of Contents

frontiers | Frontiers in Ecology and Evolution

TYPE Editorial
PUBLISHED 02 December 2022
DOI 10.3389/fevo.2022.1101114

OPEN ACCESS

EDITED AND REVIEWED BY
Monica Medina,
The Pennsylvania State University (PSU), United States

*CORRESPONDENCE
Casper J. van der Kooi
C.J.van.der.Kooi@rug.nl
Johannes Spaethe
johannes.spaethe@uni-wuerzburg.de
Sara Diana Leonhardt
sara.leonhardt@tum.de

SPECIALTY SECTION
This article was submitted to Coevolution, a section of the journal Frontiers in Ecology and Evolution

RECEIVED 17 November 2022
ACCEPTED 22 November 2022
PUBLISHED 02 December 2022

CITATION
van der Kooi CJ, Spaethe J and Leonhardt SD (2022) Editorial: Sensory ecology of plant-pollinator interactions.
Front. Ecol. Evol. 10:1101114.
doi: 10.3389/fevo.2022.1101114

Editorial: Sensory ecology of plant-pollinator interactions

Casper J. van der Kooi[1]*, Johannes Spaethe[2]* and Sara Diana Leonhardt[3]*

[1]Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, Netherlands, [2]Department of Behavioral Physiology and Sociobiology, University of Würzburg, Würzburg, Germany, [3]Plant-Insect Interactions Group, TUM School of Life Sciences, Technical University of Munich, Freising, Germany

KEYWORDS

pollination, vision, olfaction, flower, color, scent, evolution

Editorial on the Research Topic
Sensory ecology of plant-pollinator interactions

What explains the bewildering diversity of flowers in the natural world? This question has fascinated humans for centuries. Pollinator-mediated selection on floral traits is generally assumed to be the main driver of floral phenotypic divergence (Barth, 1991; Dyer et al., 2012; Schiestl and Johnson, 2013). Indeed, flowers could be considered "sensory billboards" (sensu Raguso, 2004), because they advertise their presence to pollinators *via* an enormous diversity in color, patterns, odor and shape. Pollinators perceive these signals *via* visual, olfactory and/or tactile mechanisms. How floral traits are produced and how they are perceived by pollinators hence is a central aspect in plant and pollination biology.

This Research Topic brings together a suite of papers on the sensory ecology of plant-pollinator interactions Figure 1. The papers can be categorized in the following groups, each of which we will discuss below: (i) inter- and intraspecific variation in floral traits; (ii) perception and learning of floral traits used as signals by pollinators; (iii) use of traits for deception by plants and pollinators, and (iv) variation in floral traits and perception as basis for the evolution of novel interactions.

Intra- and interspecific variation in floral traits

The extraordinary diversity in flower color is a quintessential visualization of plant diversity. Diversity in color is primarily created by different floral pigments, which differ in their absorption spectra and so create different colors. Narbona et al. investigated how three classes of floral pigment determine the visibility of flowers to different pollinators. They found that different pigment classes create colors that occupy separate parts of the visual space, and differences in visual conspicuousness between pigment classes were largely similar among pollinator groups. They also showed that carotenoids and the rarer aurones-chalcones create a higher contrast than the ubiquitous anthocyanins. In addition to the type of pigment, the amount of floral pigment is important for the degree of

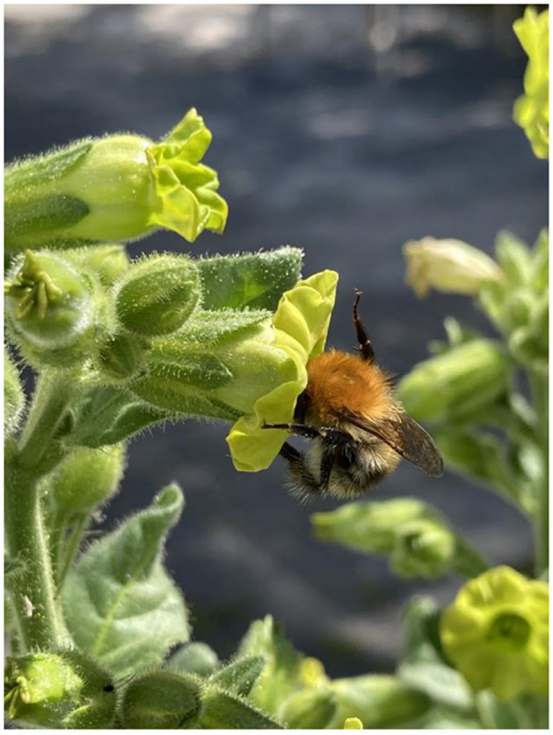

FIGURE 1
A *Bombus pascuorum* bee visiting a *Nicotiana rustica* flower. Image credit: Marjan Kraaij.

modulation of the reflected light. Combining an optical model of flowers and established vision models, van der Kooi showed that more pigment does not necessarily translate into a higher visibility to insect pollinators. Low amounts of pigment yield pale colors with low contrast, moderate amounts of pigment yield high contrast, but with high amounts of pigment, the flower's contrast to the background decreases. These findings dovetail with earlier work showing that bees prefer stimuli with intermediate pigment concentrations (Papiorek et al., 2013), and pave the way for explorations as to how the amount of floral pigment in natural species relates to the theoretical optimum of that species.

Several contributions investigated intraspecific floral trait variation and its consequences for the attraction of pollinators. Palmqvist et al. compared color and scent for diploid and polyploid *Chamerion angustifolium* plants. Flower reflectance was slightly different between cytotypes, but still similar to bee pollinators. Scent profiles, however, differed enough to enable discrimination by pollinator, which has potential implications for cytotype divergence. The South-African plant *Gerbera aurantiaca* showcases more salient flower color polymorphism. To uncover what determines the polymorphism, Johnson et al. charted the natural distribution of color morphs and characterized soil type, climate and color preference of the main pollinator, a hopliine scarab beetle. A 5-year common garden experiment revealed that flower color is not plastic. Intriguingly, they found no clear association between morph color and any of the studied parameters. This contrasts with the case of color-polymorphic South-African *Drosera cistiflora* that is pollinated by related hopliine beetles (Johnson et al., 2020; von Witt et al., 2020). It seems unlikely that the color polymorphism in *G. aurantiaca* is purely coincidental, but what other factor(s) (e.g., floral scent) may determine the geographic pattern remains a question that begs to be answered. Bing et al. measured local adaptation in a suite of floral traits of a wild tobacco plant, *Nicotiana attenuata*. This is an interesting species, because it is primarily pollinated by hawkmoths, but it exhibits high floral trait divergence among populations. The authors showed that this divergence and the ratio of outcrossing vs. selfing can be partly ascribed to local adaptation to different pollinator fauna.

Floral trait divergence that is linked to different pollinator fauna is even more apparent in different varieties of the orchid *Neotinea ustulata* – a deceptive orchid from Central Europe, studied by Martel et al.. Bees and flies pollinate one variety, whereas only flies pollinate the other variety. They found that the two varieties differ in color, morphology and scent. Furthermore, the varieties are different in their emission of alkene scent compounds, which may mean that this species is not only food deceptive, but also (quasi-)sexually deceptive. Together, these works on *C. angustifolium*, *G. aurantiaca*, *N. attenuata* and *N. ustulata* add to the growing body of literature that highlights the vastness of trait variation within species and their implications for the interaction with pollinators and for trait evolution (e.g., Eisen et al., 2022; Venjakob et al., 2022).

A central question in pollination biology is how floral signals scale with rewards for pollinators. Signal "honesty" can occur at different ecological levels. It can occur at the species level, as has been shown in, for example, *Brassica rapa* (Knauer and Schiestl, 2015) and *Dalechampia* spp. (Armbruster et al., 2005; Pélabon et al., 2012), but it can also occur at the community level, when a set of co-flowering species with a shared trait (e.g. flower color or shape) are similarly rewarding for a certain pollinator. That "community-level honesty" was studied by Streinzer et al. in the Austrian alps. They found that blue flowers produce comparatively much nectar, but because pollinators learn that blue flowers are extra rewarding, they visit them disproportionally more, which cancels out the association in the field. The authors also investigated the importance of color contrast of the flower against the (green) background, which currently is considered the most realistic proxy for flower conspicuousness to pollinators (van der Kooi and Spaethe, 2022). They found that with increasing color contrast, flowers were less rewarding, which suggests that very conspicuous flowers get away with investing less into reward without negative impacts on fitness.

Perception and learning of floral traits used as signals by pollinators

Producing a signal is only one side of the coin of attracting to pollinators. To perceive floral stimuli, pollinators need to be equipped with appropriate sensory systems. Pollinators can sometimes exhibit a behavioral preference for a specific cue, such as a specific scent or color. The widespread (and sometimes notorious crop pest) *Pieris rapae* butterfly is an example of a species for which it was commonly assumed that it uses color vision to locate flowers, but nobody had explicitly tested that assumption. Arikawa et al. show that this butterfly indeed uses color vision to locate flowers, and that they innately prefer blue and yellow. Innate (color) preferences occur in numerous flower-visiting species (Lunau and Maier, 1995; Dyer et al., 2019) and can be a significant element in flower (color) evolution. Such a presence of a "hard-wired" flower template in the insect brain is also shown by Howard et al., who provided evidence of an innate bias toward visual stimuli with flower-like configurations. Both naïve and experienced honey bees (*Apis mellifera*) readily learn not only flower color but also flower shape. In addition to the chromatic component, the achromatic component (i.e., "brightness") conveys visual information. Behavioral experiments by van der Kooi and Kelber show that naïve hawkmoths prefer bright over dim stimuli, and a literature review suggests that achromatic contrast between flowers and their background may be more important for flies, butterflies and bees than commonly assumed. The ways *via* which floral pigments and structure determine the achromatic signal on different backgrounds is dissected using optical modeling. Particularly for pollinators foraging under dim light conditions, such as nocturnal and crepuscular bees, olfactory information plays an important role complementing or even replacing visual information (Wright and Schiestl, 2009), as highlighted by Martinez-Martinez et al..

Besides bees, flies and butterflies, beetles have played a pivotal role in the radiation of angiosperm and gymnosperm plants (Labandeira et al., 2007; Ollerton, 2017). Beetles are atypical pollinators, because although many species use color vision to locate flowers, they are generally dichromatic (they lack a blue photoreceptor type), unlike most other pollinator groups that are tri- or tetrachromatic. Sharkey et al. found that gene duplications in visual genes (opsins) underlying putative tri- and tetrachromatic color vision may be relatively common among beetles that strongly depend on floral resources and these gene duplications have evolved independently in multiple beetle lineages. Although duplications do not necessarily imply new photoreceptor sensitivities, the authors showed a marked increase in gene duplication in obligate flower visitors (74%) vs. non-flower visitors (28%).

In the noisy natural world, it probably requires a substantial amount of time and brainpower to perceive and process visual, olfactory and taste stimuli simultaneously presented by flowers. This may explain why several pollinators use only specific sensory stimuli during specific foraging tasks. For example, Sculfort et al. found that *Bombus terrestris* bumblebees were unable to perceive three potentially toxic plant secondary metabolites (amygdalin, scopolamine, and sinigrin) in sugar solutions. Similarly, Ruedenauer et al. showed that honeybees focus taste perception on restricted nutrient groups, i.e., amino acids and fatty acids, but ignore others, i.e., sterols, when faced with complex chemical profiles as represented by pollen. Brandt et al. provided evidence for the evolutionary adaptation of olfactory perception in scent collecting male euglossine bees. The authors demonstrated that male bees showed highly species-specific patterns of antennal responses to various scent bouquets. More closely related species were more similar in their responses, indicating adaptation to those chemical compounds that typically occur in scent bouquets of their preferred perfume flowers.

Adaptation of sensory systems can result in marked differences between the actual and perceived stimulus and explain why final perception is highly species-, context- and occasionally individual-specific. Animals can use various sensory strategies to counteract limitations imposed by the physiological properties of their sensory system. For example, some animals, e.g., birds, increase the signal-to-noise ratio under dim illumination (Warrant, 1999). Taking into account the interaction between environmental complexity and species-specific sensory properties to understand behavioral patterns is very challenging, as highlighted by Garcia et al.. The authors applied a modeling function that takes into account psychophysics data to model how birds use color information in (visually) complex environments to make meaningful choices. The ability to process and learn olfactory stimuli in complex environments appears to be essential for pollinators not only to detect flowers and make appropriate choices, but also to navigate in foraging habitats, as demonstrated by Evans et al. for *B. terrestris*. Together, these contributions highlight the importance of evolving appropriate sensory modalities for foraging pollinators and the complex interplay between floral stimuli, pollinator sensory modalities and foraging behavior. These works further suggest that we should be cautious with usage of pesticides that targets insect sensory systems, such as neonicotinoids. In this context, Straub et al. showed that treating bees with a field-realistic dose of the neonicotinoid clothianidin decreased the antennal sensitivity to a common floral odor compound (2-phenylethanol) in the mason bee *Osmia bicornis* and in *B. terrestris*. Clothianidin also negatively affected the foraging behavior of *O. bicornis*.

Use of traits for deception by plants and pollinators

Pollinators are deceived by plants *via* mimicry, where a mimic resembles a model, and/or the exploitation of perceptual biases. In the latter case, the pollinator has a sensory or cognitive

bias for a trait, such as a certain odor or color pattern, and that bias is co-opted by the plant to achieve pollination without offering any reward. Traits involved can address different sensory modalities, like vision, olfaction, taste or touch, and can target different pollinator needs, like food, mating partners or breeding sites. In their field study, Rupp et al. investigated deceptive pollination in *Aristolochia microstoma*, which belongs to a genus that is known for its fly-deceptive pollination strategy. The authors recorded a wide diversity of arthropod flower visitors, but only dipterans of the family Phoridae were found to carry pollen during the female phase of the flowers, indicating that they are the exclusive pollinator of *A. microstoma*. The authors also found that floral scent was strongly dominated by oligosulphides, which are widespread among plants pollinated by carrion-flies and bats. Taking both findings together, the authors hypothesized that *A. microstoma* is a fly-deceptive plant that mimics brood-sites of invertebrate carrion.

In flowering plants, pollen contains the male gametes and its transfer thus is essential for outcrossing, but pollen may also function as a reward for pollinators. To reduce consumption of pollen, plants have evolved multiple strategies. For example, in some monoecious plants, the unrewarding female flowers mimic the pollen-rewarding male flowers. Russell et al. set out to investigate whether pollinators can learn to discriminate between rewarding and unrewarding flowers to maximize their foraging efficiency. They investigated whether flower size variation in the monoecious *Begonia odorata*, where unrewarding female flowers are on average 30% larger than male flowers, affects the discrimination ability of *B. impatiens*. The authors found that the bees quickly learned to avoid unrewarding female flowers and then choose the rewarding male flowers, independent of size variation.

Nectar is produced by flowers to attract potential pollinators, but nectar-robbing bees bypass the reproductive organs by entering the flower from a different direction, e.g., through biting a hole in the corolla. Whereas the impact of nectar robbing on plant fitness is well-studied, less is known about the behavioral and cognitive processes underlying robbing. Richman et al. reviewed the literature about the sensory and cognitive processes involved in nectar robbing and highlight open questions, such as differences in the degree of an innate preference for nectar robbing (i.e., the underlying motor patterns) between flower visiting species or the role of previous experiences, e.g., encounters of open vs. (still) closed flowers during the initial foraging flight.

Variation in floral traits and perception as basis for the evolution of new interactions?

Many studies, including several in this Research Topic, have demonstrated the high degree of inter- and intraspecific variation in flower traits or behavior of pollinators (Füssel et al., 2007; Palmer-Young et al., 2019; Sapir et al., 2021; van der Kooi et al., 2021). Such variation is pivotal for the local adaptation of both interaction partners as well as for their resilience against environmental change (Bolnick et al., 2011). It likely also provides the basis for the evolution of novel interactions, provided that pollinators show sufficient flexibility in their sensory systems. For example, Burger et al. showed that naïve *Chelostoma rapunculi* bees were equally attracted by olfactory cues of two non-host plant species (*Malva moschata* and *Geranium sanguineum*. This attractiveness may be explained by shared olfactory and visual stimuli. However, each plant species still has its individual scent profile, indicating that in particular naïve *C. rapunculi* bees show relatively large sensory flexibility or a high degree of generalization, which may eventually enable its plant host expansion or switch. Conversely, if pollinators show an innate preference for specific signals, e.g., color, and thus rather low sensory flexibility, plants may only exploit them as pollinators if they adapt their trait phenotypes to the pollinators' sensory range. A neat experiment by Byers and Bradshaw investigated flower color preferences of *Mimulus* monkeyflowers. The authors manipulated two flower color loci and tested the attractiveness of the resulting four color phenotypes (red, yellow, pink, and white) to hawkmoths. They demonstrated that hawkmoths strongly preferred derived (yellow, pink, and white) over ancestral (red) colors. Owing the simple flower color genetics, the authors could follow an elegant prospective approach to understand plant diversification.

In summary, the mesmerizing diversity of flowers is in large part driven by the sensory ecology of pollinators. This Research Topic covered a very small part of the enormous body of work on this most fascinating topic, and included some important aspects such as perception and learning by pollinators, deception by plants, and inter- and intraspecific trait variation. No doubt the future will bring much more illuminating research that will stimulate the senses and thinking of (sensory) ecologists.

Author contributions

CK and SL drafted the editorial, with specific input from JS. All authors agree to the final version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Armbruster, W. S., Antonsen, L., and Pélabon, C. (2005). Phenotypic selection on Dalechampia blossoms: honest signaling affects pollination success. *Ecology* 86, 3323–3333. doi: 10.1890/04-1873

Barth, F. G. (1991). *Insects and Flowers: The Biology of a Partnership*. Princeton: Princeton University Press.

Bolnick, D. I., Amarasekare, P., Araújo, M. S., Bürger, R., Levine, J. M., Novak, M., et al. (2011). Why intraspecific trait variation matters in community ecology. *Trends Ecol. Evol.* 26, 183–192. doi: 10.1016/j.tree.2011.01.009

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G., Simonov, V., and Wong, B. B. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. B* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Dyer, A. G., Boyd-Gerny, S., Shrestha, M., Garcia, J. E., van der Kooi, C. J., and Wong, B. B. M. (2019). Colour preferences of Tetragonula carbonaria stingless bees for colour morphs of the Australian native orchid Caladenia carnea. *J. Compar. Physiol. A* 205, 347–361. doi: 10.1007/s00359-019-01346-0

Eisen, K. E., Ma, R., and Raguso, R. A. (2022). Among- and within-population variation in morphology, rewards, and scent in a hawkmoth-pollinated plant. *Am. J. Bot.* 109, 1794–1810. doi: 10.1002/ajb2.16030

Füssel, U., Dötterl, S., Jürgens, A., and Aas, G. (2007). Inter- and intraspecific variation in floral scent in the genus Salix and its implication for pollination. *J. Chem. Ecol.* 33, 749–765. doi: 10.1007/s10886-007-9257-6

Johnson, S. D., von Witt, C. G., and Anderson, B. (2020). Floral color variation in Drosera cistiflora is associated with switches in beetle pollinator assemblages. *Front. Plant Sci.* 11, 606259. doi: 10.3389/fpls.2020.606259

Knauer, A. C., and Schiestl, F. P. (2015). Bees use honest floral signals as indicators of reward when visiting flowers. *Ecol. Lett.* 18, 135–143. doi: 10.1111/ele.12386

Labandeira, C. C., Kvaček, J., and Mostovski, M. B. (2007). Pollination drops, pollen, and insect pollination of Mesozoic gymnosperms. *Taxon* 56, 663–695. doi: 10.2307/25065852

Lunau, K., and Maier, E. J. (1995). Innate colour preferences of flower visitors. *J. Compar. Physiol. A* 177, 1–19. doi: 10.1007/BF00243394

Ollerton, J. (2017). Pollinator diversity: distribution, ecological function, and conservation. *Annu. Rev. Ecol. Evol. Syst.* 48, 353–376. doi: 10.1146/annurev-ecolsys-110316-022919

Palmer-Young, E. C., Farrell, I. W., Adler, L. S., Milano, N. J., Egan, P. A., Junker, R. R., et al. (2019). Chemistry of floral rewards: intra- and interspecific variability of nectar and pollen secondary metabolites across taxa. *Ecol. Monogr.* 89, e01335. doi: 10.1002/ecm.1335

Papiorek, S., Rohde, K., and Lunau, K. (2013). Bees' subtle colour preferences: how bees respond to small changes in pigment concentration. *Naturwissenschaften* 100, 633–643. doi: 10.1007/s00114-013-1060-3

Pélabon, C., Thöne, P., Hansen, T. F., and Armbruster, W. S. (2012). Signal honesty and cost of pollinator rewards in Dalechampia scandens (Euphorbiaceae). *Ann. Bot.* 109, 1331–1340. doi: 10.1093/aob/mcs091

Raguso, R. A. (2004). Flowers as sensory billboards: Progress towards an integrated understanding of floral advertisement. *Curr. Opin. Plant Biol.* 7, 434–440. doi: 10.1016/j.pbi.2004.05.010

Sapir, Y., Gallagher, M. K., and Senden, E. (2021). What maintains flower colour variation within populations? *Trends Ecol. Evol.* 36, 507–5219. doi: 10.1016/j.tree.2021.01.011

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

van der Kooi, C. J., and Spaethe, J. (2022). Caution with colour calculations: spectral purity is a poor descriptor of flower colour visibility. *Ann. Bot.* 130, 1–9. doi: 10.1093/aob/mcac069

van der Kooi, C. J., Vallejo-Marín, M., and Leonhardt, S. D. (2021). Mutualisms and (a)symmetry in plant-pollinator interactions. *Curr. Biol.* 31, R91–R99. doi: 10.1016/j.cub.2020.11.020

Venjakob, C., Ruedenauer, F. A., Klein, A. -M., and Leonhardt, S. D. (2022). Variation in nectar quality across 34 grassland plant species. *Plant Biol.* 24, 134–144. doi: 10.1111/plb.13343

von Witt, C. G., Anderson, B., Durbach, I. N., and Johnson, S. D. (2020). Breeding systems of floral colour forms in the Drosera cistiflora species complex. *Plant Biol.* 22, 992–1001. doi: 10.1111/plb.13159

Warrant, E. J. (1999). Seeing better at night: Life style, eye design and the optimum strategy of spatial and temporal summation. *Vision Res.* 39, 1611–1630. doi: 10.1016/S0042-6989(98)00262-4

Wright, G. A., and Schiestl, F. P. (2009). The evolution of floral scent: the influence of olfactory learning by insect pollinators on the honest signalling of floral rewards. *Funct. Ecol.* 23, 841–851. doi: 10.1111/j.1365-2435.2009.01627.x

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 22 March 2021
doi: 10.3389/fevo.2021.650069

Foraging Small White Butterflies, *Pieris rapae*, Search Flowers Using Color Vision

Kentaro Arikawa, Yoshihiro Nakatani, Hisaharu Koshitaka and Michiyo Kinoshita*

Laboratory of Neuroethology, SOKENDAI, The Graduate University for Advanced Studies, Hayama, Japan

We demonstrate that the small white butterfly, *Pieris rapae*, uses color vision when searching flowers for foraging. We first trained newly emerged butterflies in a series of indoor behavioral experiments to take sucrose solution on paper disks, colored either blue, green, yellow, or red. After confirming that the butterflies were trained to visit a certain colored disk, we presented all disks simultaneously. The butterflies selected the disk of trained color, even among an array of disks with different shades of gray. We performed the training using monochromatic lights and measured the action spectrum of the feeding behavior to determine the targets' *Pieris*-subjective brightness. We used the subjective brightness information to evaluate the behavioral results and concluded that *Pieris rapae* butterflies discriminate visual stimuli based on the chromatic content independent of the intensity: they have true color vision. We also found that *Pieris* butterflies innately prefer blue and yellow disks, which appears to match with their flower preference in the field, at least in part.

Keywords: insect, photoreceptor, spectral sensitivity, action spectrum, chromaticity, subjective brightness

OPEN ACCESS

Edited by:
Johannes Spaethe, Julius Maximilian University of Würzburg, Germany

Reviewed by:
Adam James Blake, Simon Fraser University, Canada
Balamurali G. S., Indian Institute of Science Education and Research, Thiruvananthapuram, India

****Correspondence:***
Kentaro Arikawa
arikawa@soken.ac.jp

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *06 January 2021*
Accepted: *04 March 2021*
Published: *22 March 2021*

Citation:
Arikawa K, Nakatani Y, Koshitaka H and Kinoshita M (2021) Foraging Small White Butterflies, Pieris rapae, Search Flowers Using Color Vision. Front. Ecol. Evol. 9:650069. doi: 10.3389/fevo.2021.650069

INTRODUCTION

Color vision is the ability to discriminate visual stimuli based on the chromatic content irrespective of the brightness. Since the first demonstration of color vision in flower-visiting honeybees (Turner, 1910; Frisch, 1914), it has become a central topic of insect behavioral neuroscience. Foraging honeybee uses a trichromatic system based on the UV, blue, and green-sensitive photoreceptors in their compound eyes (Menzel and Backhaus, 1989; Wakakuwa et al., 2005).

Flowers attract many other insects for pollination, including lepidopterans, dipterans, hemipterans, and coleopterans (Chittka and Thomson, 2001). Their visual systems are not necessarily the same as that of the honeybee. For example, the Japanese yellow swallowtail, *Papilio xuthus*, has six spectral receptor classes comprising a UV, violet, blue, green, red, and broadband class (Arikawa, 2003). As in the honeybee, *Papilio* uses color vision for finding flowers (Kelber and Pfaff, 1999; Kinoshita et al., 1999), but it covers a wider spectral range. The wavelength discrimination property of *Papilio xuthus* exhibits three highly sensitive wavelength regions, indicating that the system is tetrachromatic, with a UV, blue, green, and red channel (Koshitaka et al., 2008).

While *Papilio*'s color vision has been studied intensively among butterflies, that of the small white, *Pieris rapae*, another important butterfly species for biology because of its abundance and impact on crops, is poorly understood. The spectral organization of the *Pieris* compound eye differs from that of *Papilio*: most notably, *Pieris rapae* has three classes of red receptors (Blake et al., 2019).

Presumably, such a species-specific eye organization creates ecologically essential differences in the color discrimination ability.

The visual characteristics of the cosmopolitan pest species *Pieris rapae* have been studied particularly well in relationship with its reproductive behavior (Traynier, 1984; Kelber, 2001; Wakakuwa et al., 2004; Blake et al., 2020). Males of the Japanese subspecies *Pieris rapae crucivora* search for potential mates using the wings' UV reflection. This is one of the earliest examples of how UV light affects the visual behavior of an insect (Obara and Hidaka, 1968; Obara and Majerus, 2000).

As adults, *Pieris* butterflies are nectar feeders. They presumably find and discriminate nectar-providing flowers by their colors (Kolb and Scherer, 1982; Scherer and Kolb, 1987; Goulson and Cory, 1993; Kandori and Ohsaki, 1996; Kelber, 2001). However, the previous studies do not conclusively demonstrate color vision *per se*, because the experiments were not designed to do so. Here we focus on this particular point and establish color vision in foraging *Pieris rapae*.

MATERIALS AND METHODS

Materials

We used summer-form virgin females of the small white butterfly *Pieris rapae crucivora* Boisduval (Lepidoptera, Pieridae) from a laboratory culture derived from eggs laid by females caught around the campus of Sokendai, Hayama, Japan. The hatched larvae were fed on fresh kale leaves at 28°C under a 16 h:8 h light:dark cycle. Pupae were allowed to emerge in a plastic box. We defined the day of emergence as post-emergence day 1.

Experimental Setup

We performed experiments using freely flying butterflies and colored disks in a cage covered with nylon net (W80 × D60 × H45 cm, see Kinoshita et al., 1999). The cage was illuminated with eight 300 W halogen lamps. The reflection spectrum of a MgO-coated surface, placed on the cage floor and measured using a calibrated spectrometer (HSU-100S, Asahi Spectra, Japan), represented the illumination spectrum (**Figure 1A**, spectrum *a*). The room temperature was set at 28°C. The cage floor was covered with black cardboard.

Stimuli were ø 5 cm disks of colored paper (Training color 120, Nihon Shikisai Co. Ltd., Tokyo Japan) centrally arranged on the cage floor. **Figures 1B–D** show reflectance spectra of the papers. We presented the disks in four patterns, as shown in **Figures 1E–H**. The 4-color pattern had disks of four basic colors (blue, green, yellow, and red). The 13-color pattern had the disks of seven other colors (**Figure 1C**) in addition to the basic four colors (**Figure 1B**) plus two grays (Gray 2 and 3, **Figure 1D**). For patterns with more than one disk (**Figures 1F–H**), we randomized the disks' relative position during tests. We covered the patterns with anti-reflection glass to protect the patterns from unwanted contamination and suppress specular reflection that might affect the visual perception of the stimuli.

We performed experiments using tethered butterflies and monochromatic lights on a bench setup (**Figure 2A**), a modified version of what we used previously (Koshitaka et al., 2008). Briefly, the benchtop had a piece of frosted quartz glass as the back-projection screen, which was illuminated from below with monochromatic light of varying wavelength and intensity produced by a 500 W xenon arc, a monochromator, and a set of quartz neutral density filters. We measured the photon flux of the monochromatic lights using a radiometer (Model 470D, Sanso, Tokyo, Japan). The benchtop was illuminated by four 300 W halogen lamps (**Figure 1A**, spectrum *b*). The room temperature was set at 28°C.

Training and Tests Using Colored Disks

During the training phase of free-flying butterflies, we released only one butterfly in the cage with a training pattern (**Figure 1E**), having a few drops of 6% sucrose solution on the disk. We defined a visit as a positive response when the butterfly landed and extended its proboscis toward the colored disk.

To check whether or not the foraging behavior exhibits any bias, we recorded the color of the disk that naïve butterflies visited for the first time. On post-emergence day 4, we released a butterfly, which was never fed after emergence, in the cage with a four-color pattern but no sucrose solution (**Figure 1F**). We changed the relative position of the four disks for each individual to eliminate any possible effects of disk position.

For testing color vision, we trained butterflies to visit disks of a certain color to forage. On post-emergence day 3, the starved butterflies were individually trained by letting them take 6% sucrose solution on the colored disk of a training pattern (**Figure 1E**). If they did not visit the disk spontaneously, we manually placed the butterflies on the disk. We repeated the training once a day for 6 days. We quantified the training effect by performing the color learning tests with a four-color pattern (**Figure 1F**) immediately before the day's training session. We released a butterfly in the cage and let the butterfly visit the disks five times, and counted the number of visits to each color. If the butterfly visited the disks less than five times within10 min, we finished the test and recorded the performed visits. We randomly changed the array of colors on the pattern after each visit to eliminate a possible positional effect.

We evaluated the final effect of training on post-emergence day 9. We let them visit 10 times on the 13-color pattern (**Figure 1G**) and five times for the gray-scale pattern (**Figure 1H**) and then counted the number of visits to each disk. We appropriately changed the disks' position during the tests.

Training and Tests Using Monochromatic Lights

During the training phase, we covered the frosted quartz screen with a black plastic plate having a window of 1.0 × 1.0 cm^2 in the center (**Figure 2B**) through which the butterflies saw the light of a particular wavelength. The training started on post-emergence day 3, using starved and tethered butterflies with clipped wings. We brought a tethered butterfly close to the illuminated window and fed it on 6% sucrose solution for a few seconds: the butterflies took sucrose solution using the extended proboscides. We repeated such a brief feeding until the

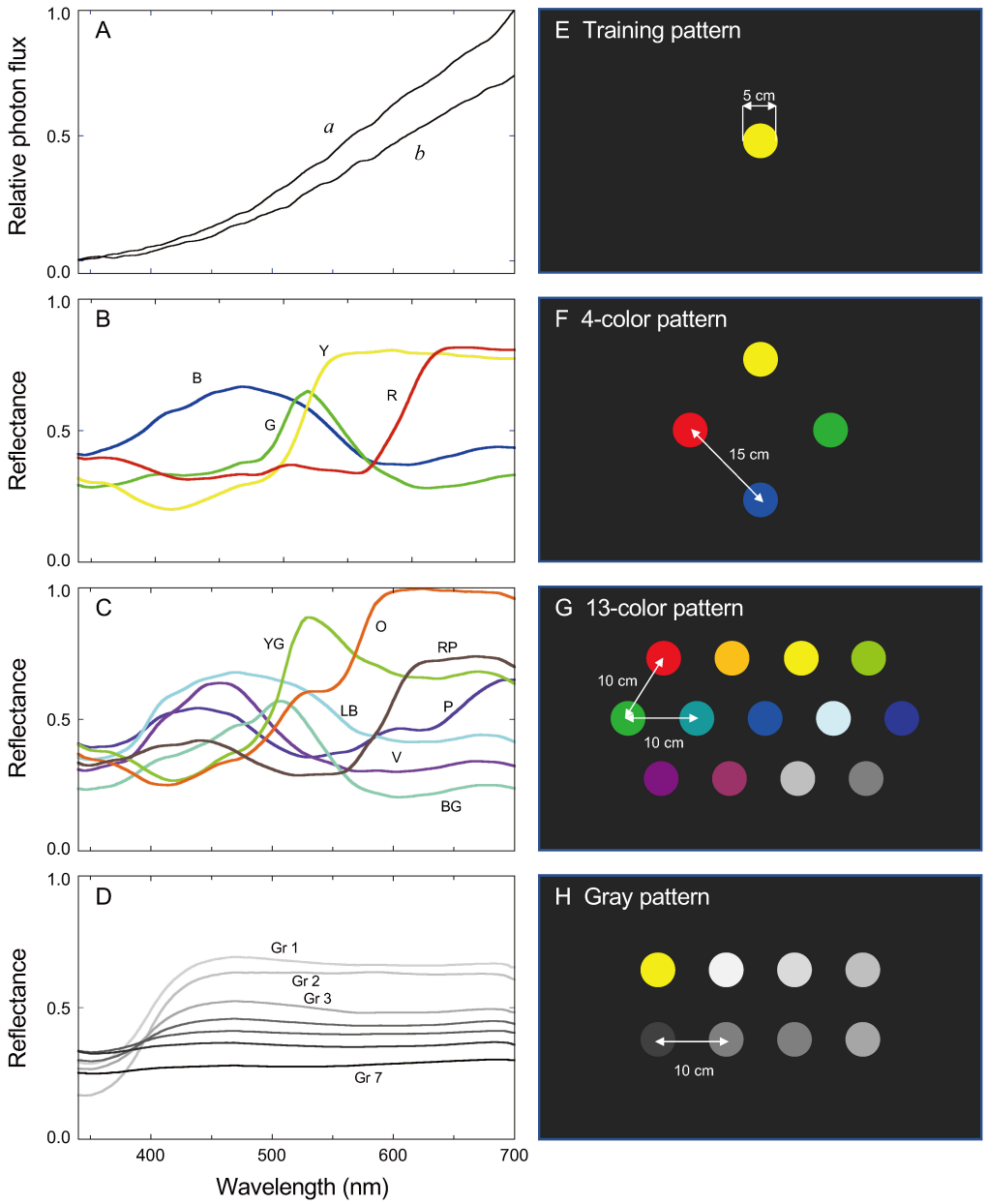

FIGURE 1 | (A) Illumination spectra of the experimental arena: *a*, eight 300 W halogen lamps for freely flying butterflies; *b*, four 300 W halogen lamps for tethered butterflies. **(B)** Reflectance spectra of colored papers used for training. **(C)** Reflectance spectra of colored papers used in the 13-color pattern (see **Figure 1D**) together with those shown in **(B)**. **(D)** Reflectance spectra of papers of seven different shades of gray. **(E)** Training pattern. **(F)** 4-color pattern. **(G)** 13-color pattern. **(H)** Gray pattern. The subjective brightness of each colored paper, B_i, is listed in **Table 1**. B, blue; BG, blue-green; G, green; Gr, gray; LB, light-blue; O, orange; P, purple; R, red; RP, red-purple; V, violet; Y, yellow; YG yellow-green.

butterfly became satiated. We trained butterflies every day until they showed proboscis extension without reward upon seeing the training monochromatic light.

For measuring the butterflies' detection threshold for monochromatic lights, we covered the quartz screen with a black plastic plate having two 1.0 × 1.0 cm^2 windows separated by a 5 mm gap (**Figure 2C**). To be strict that the butterflies responded not to windows but the light, we presented two windows, one illuminated with the training wavelength of varying intensity, and the other kept unilluminated. We presumed that the trained butterflies extend the proboscis toward the illuminated window only when the light intensity was above the detection threshold. We changed the position of the illuminated window randomly.

We measured the detection thresholds in the wavelength range from 340 to 680 nm at intervals of 20 or 40 nm using the trained tethered butterflies. **Figure 3** shows the protocol of the

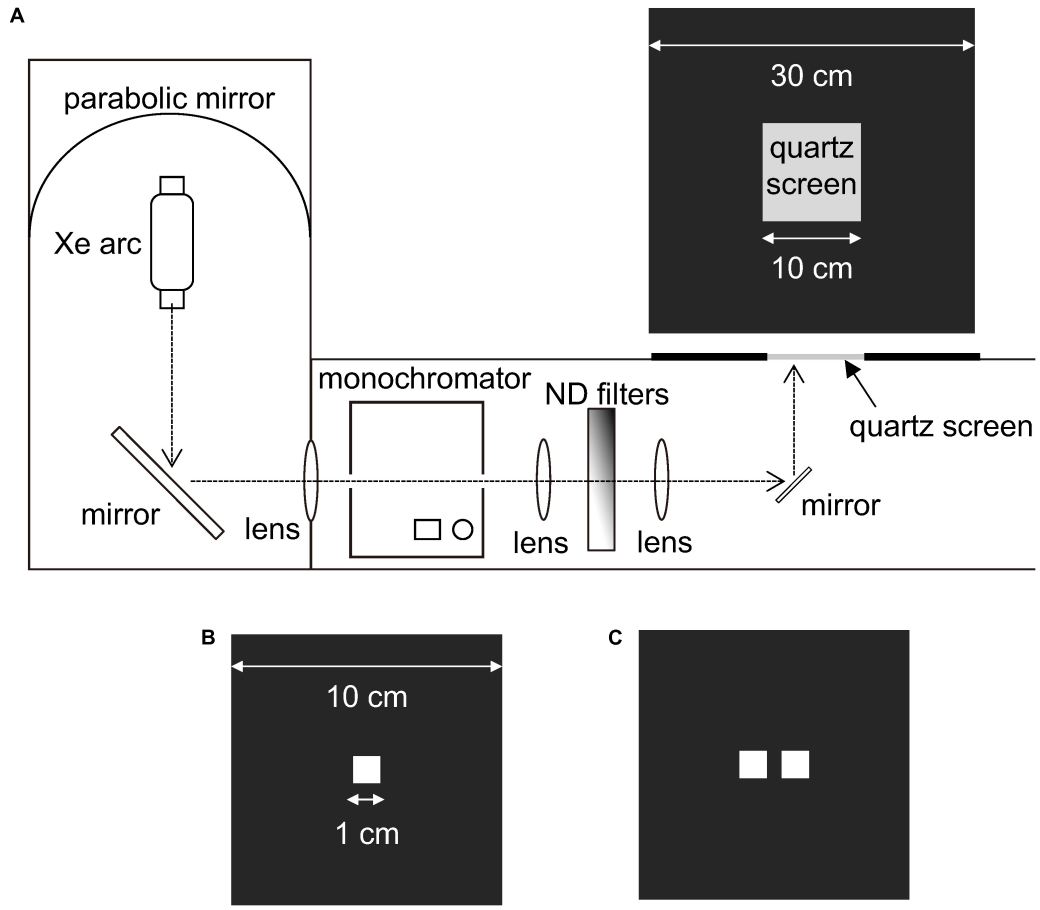

FIGURE 2 | Experimental setup for presenting monochromatic lights. **(A)** Light from a Xe arc was directed into a monochromator via a mirror. The monochromatic light, whose intensity was attenuated with ND filters, was reflected upwards to a quartz screen. **(B)** A single-windowed plate used to cover the quartz screen during training sessions. **(C)** A double-windowed plate used to cover the quartz screen during test sessions.

behavioral experiment. We checked the butterflies' motivation every time they did not respond to the presented stimulus: the motivation check (*MC*) was executed by confirming whether the maximum intensity of a given wavelength elicited proboscis extension (**Figure 3A**). On the first day of the test, we checked whether the butterflies could respond to the training wavelength at 4, 3, 2, or 1 log unit weaker than the training intensity (**Figure 3B**). On the next day and later, we started the day's test at the lowest intensity to which the individual responded on the previous day (**Figure 3C**). We thus determined the threshold intensity at the sampling rate of 0.25 log unit for each individual. We plotted the average number of responding individuals against the photon flux. The photon flux required for eliciting criterion response plotted vs. wavelength yielded the action spectrum of foraging behavior.

Statistical Analysis

We used Statcel4 add-in for Excel (OMS publishers, Tokyo, Japan) for the analysis and set an alpha value of $P < 0.05$ as statistically significant. We applied a multinomial test to check the randomness of data distribution. We checked the statistical significance of the innate preference data using the Tukey-Kramer test. To evaluate the learning process of color X, we aggregated data of a particular day (color X vs. the other colors) and performed the Wilcoxon signed-rank test. We applied the same to evaluate the final effect of learning. **Supplementary Tables S1–S3** summarize the *P*-values.

RESULTS

Innate Preference

We tested nine naïve female adults. Five butterflies visited the blue disk, and four visited the yellow disk as the first feeding place in their lives, while none visited the red and green disks (**Figure 4** and **Supplementary Table S1**). The distribution of visits was not random (Multinomial test, $P < 0.05$).

Learning Process

We collected data of butterflies that visited any disk more than five times a day for 5 days: 24 individuals met the criterion. The butterflies trained to the blue disk visited the

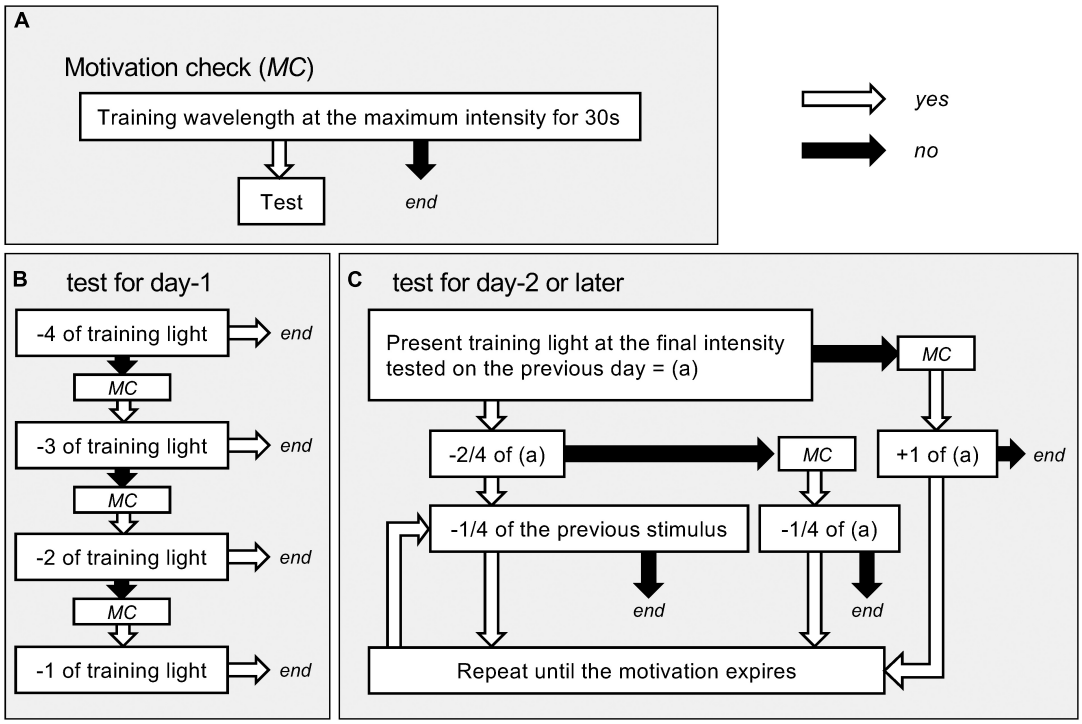

FIGURE 3 | Flow charts of the behavioral experiments. **(A)** The protocol of checking motivation. The motivation check (*MC*) was done whenever necessary during the tests. Only when we confirmed the motivation, we continued tests. **(B)** Test for day-1. **(C)** Test for day-2 or later. Numbers with plus or minus (e.g., −4, +1/4) mean changes in light intensity in log unit (e.g., 4 log unit darker or 0.25 unit brighter; comparison with the previous test light otherwise specified).

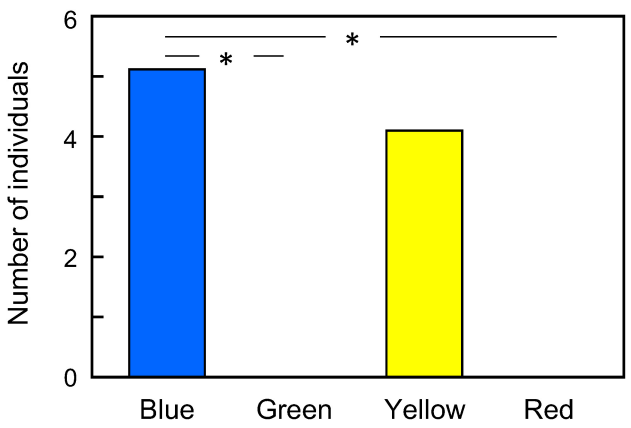

FIGURE 4 | First color choice of naïve *Pieris rapae* on a 4-color pattern. The data distribution was non-random (Multinomial test, $P < 0.05$). *$P < 0.05$ (Tukey-Kramer test, **Supplementary Table S1**).

blue disk exclusively after a single training (**Figure 5A**). The butterflies trained to the yellow disk exhibited a similar pattern. On the other hand, those trained to the red or green disk preferred the yellow and blue disks after the first training. Preference to the disk of trained color significantly increased day by day, reaching almost maximum on post-emergence day 7 for green-trained butterflies and day 6 for red-trained ones ($P < 0.05$, Wilcoxon signed-rank test, **Supplementary Table S2**).

Learning Results

Figure 6 shows the cumulative results of the trained butterflies tested on the 13-color pattern. The stacked bars are arranged with the training colors on the leftmost. The yellow-trained butterflies visited yellow, the training color, most frequently as expected ($P < 0.05$, Wilcoxon signed-rank test). However, those trained to green and blue, respectively, chose preferentially blue-green and violet, followed by the training colors. The red-trained butterflies visited orange most frequently, followed by purple and then by red. The 13-color pattern had two gray disks, gray 2 and gray 3 (**Figures 1D,G**), but no butterflies visited them. We also tested the trained butterflies on a gray-scale pattern together with a colored disk of respective training (**Figure 1H**). We found all butterflies visited the colored disk: none visited any of the gray disks.

Action Spectrum of Proboscis Extension

We could successfully train 3–6 butterflies to 1 of the 14 test wavelengths ranging from 340 to 680 nm. **Figure 7A** shows the response-log intensity functions for the proboscis extension response. The solid curves are the best fits of sigmoidal function for each wavelength. Reciprocals of the photon number eliciting a criterion response give an action spectrum. As the curves' slopes vary, we took the criteria at 20, 50, and 80% response,

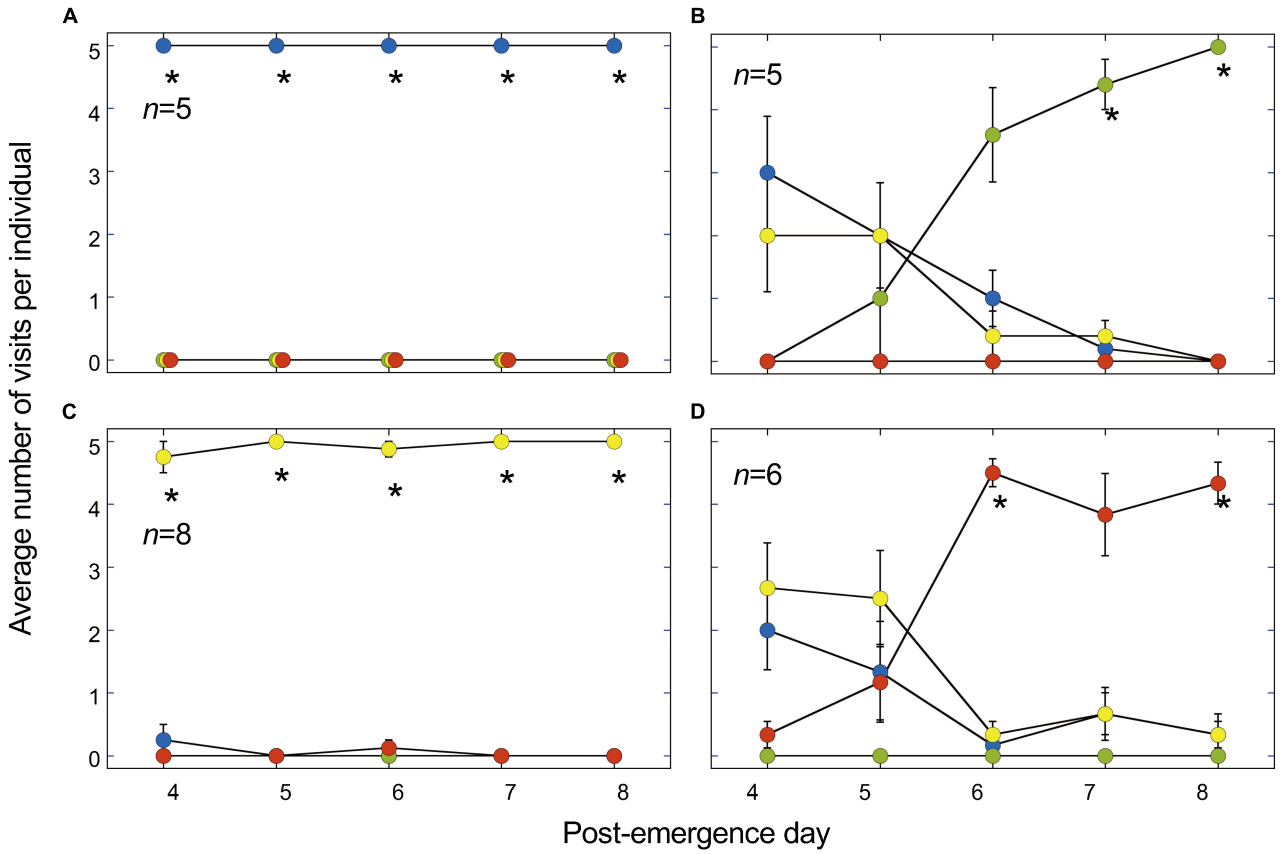

FIGURE 5 | Visit numbers after a training period lasting 5 days on a disk of a particular color. **(A)** Blue-training. **(B)** Green-training. **(C)** Yellow-training. **(D)** Red-training. Asterisks (*) indicate that the training color was selected significantly more often than the sum of other colors. $P < 0.05$ (Wilcoxon signed-rank test, **Supplementary Table S2**). *n*, number of tested individuals. Values are means ± S.E.M.

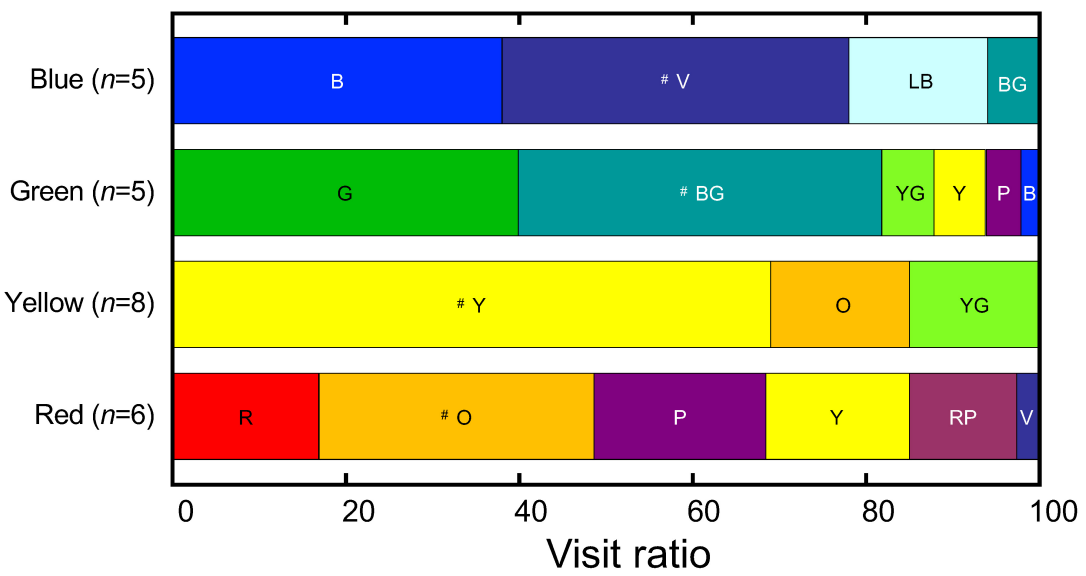

FIGURE 6 | The visit ratio after the 5-day training, measured on day 6 using the 13-color pattern. For the reflection spectrum, the subjective brightness, and the abbreviation of each color, see **Figure 1** and **Table 1**. The symbol # indicates the most frequently visited color in each group. No butterflies selected Gray 2 and 3. *n*, the number of tested individuals. For the results of statistical analysis, see **Supplementary Table S3**.

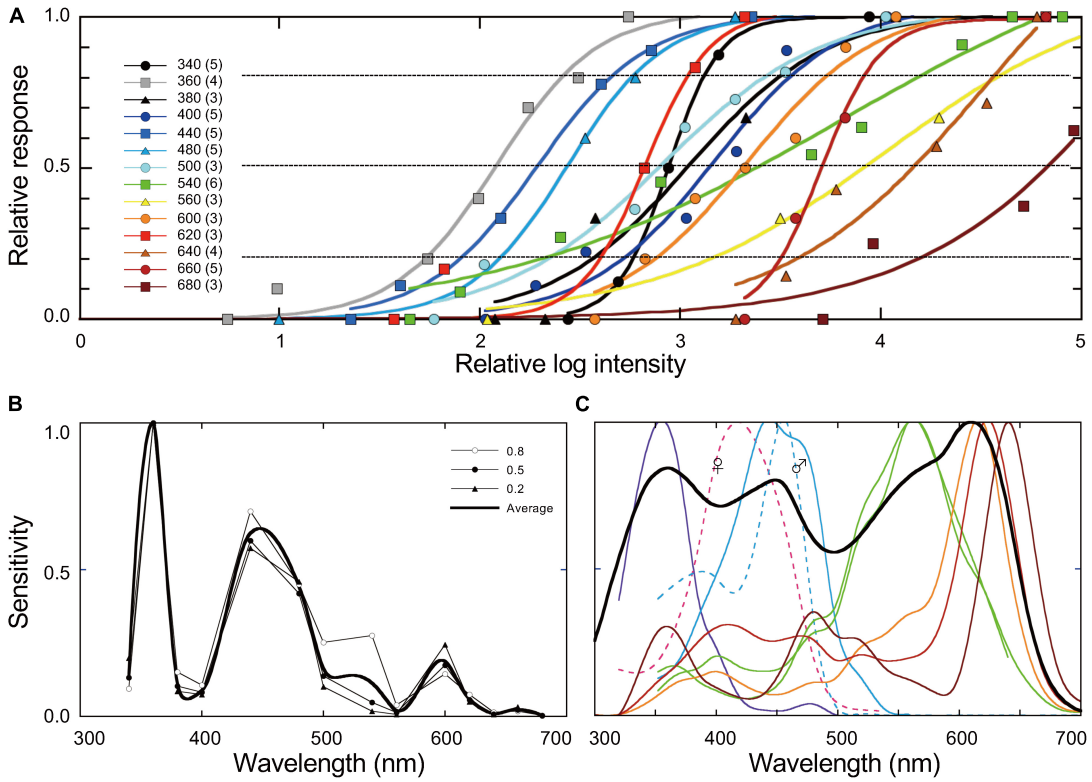

FIGURE 7 | (A) Dependency of proboscis extension response on the intensity of monochromatic light. The inset shows the tested wavelengths with the number of individuals in parentheses. Response values of 1.0 indicate where all individuals extended their proboscis toward the stimulus light. Solid curves are the best fits of a sigmoidal function. **(B)** Action spectra based on A at 80, 50, and 20% as the criteria. The bold curve is the average of three spectra. **(C)** The photoreceptor spectral sensitivities (colored curves, spline-interpolated) and the estimated spectral sensitivity of the entire compound eye of female *Pieris rapae* (bold curve, the sum of the ommatidial absorptance spectra in Stavenga and Arikawa (2011), weighted by the ommatidial occupation ratio). The dashed curves show the sex-specific receptors.

yielding three similar spectra, with only slight variations in the long-wavelength region (**Figure 7B**).

DISCUSSION

Color Vision

For a convincing demonstration of color vision in an animal, one has to demonstrate that the animal can visually discriminate "colored" stimuli, either objects or light sources, based on their spectral contents irrespective of the intensity. Here, the intensity is crucial because we need to know the intensity for the animal. The animal-subjective brightness can be obtained as the product of the physical intensity of the stimuli and the animal's sensitivity to spectral lights.

The spectral sensitivity of compound eyes can be fairly easily measured by electroretinography (Belušič, 2011). Such a spectral sensitivity may be acceptable as the first approximation for this purpose, but in reality, the sensitivity often differs depending on the behavioral context. For example, the UV-violet, blue, and green-yellow region of the spectrum, respectively, elicits the escape, feeding, and oviposition behavior in *Pieris* butterflies (Scherer and Kolb, 1987; Kelber, 2001). With this in mind, we measured the intensity dependency of the proboscis extension for foraging toward monochromatic lights using trained butterflies (**Figure 7A**). The resulting action spectrum of proboscis extension, $A(\lambda)$, exhibits higher sensitivity in the short wavelength region with the maximum at 360 nm. We used the average of three behaviorally determined action spectra (**Figure 7B**, bold curve) to evaluate the subjective brightness of the stimuli for *Pieris rapae*.

We calculated the *Pieris*-subjective brightness of colored paper i, B_i, under the present experimental condition by:

$$B_i = \int_{340}^{680} I(\lambda)\, R_i(\lambda)\, A(\lambda)\, d\lambda,$$

where $I(\lambda)$ is the irradiance spectrum of illumination (**Figure 1A**, spectrum *a*), $R_i(\lambda)$ is the reflectance spectrum of colored paper i (**Figures 1B–D**), and λ is the wavelength. **Table 1** shows the relative B_i values of all colored papers normalized to the brightest gray's value. For the colors used in the 4-color pattern, the B_i values are 0.80 (blue), 0.79 (yellow), 0.70 (red), and 0.57 (green).

Naïve butterflies selected a blue or yellow disk as the feeding site for the first time in their lives. Butterflies tend to select brighter stimuli (Kinoshita et al., 2011), which may be related to the positive phototaxis often observed in insects (Nouvian and Galizia, 2020). For *Pieris*, the blue and yellow disks are

TABLE 1 | *Pieris*-subjective intensity, B_i, of each colored paper under the illumination of the spectrum *a* in **Figure 1A**.

Color	B_i	Color	B_i	Color	B_i
Blue	0.80	Purple	0.70	Gray 1	1.00
Green	0.57	Light-blue	0.84	Gray 2	0.93
Yellow	0.79	Violet	0.68	Gray 3	0.75
Red	0.70	Blue-green	0.55	Gray 4	0.66
		Yellow-green	0.82	Gray 5	0.61
		Orange	0.91	Gray 6	0.54
		Red-purple	0.67	Gray 7	0.42

equally bright, so the butterflies may have selected these disks because they appear brighter than the red and green disks and not necessarily because of an innate color preference.

The innate preference for "blue" is often reported in lepidopteran species (Kelber, 1997; Yoshida et al., 2015; Satoh et al., 2016). However, the terminology of "innate color preference" needs to be carefully re-addressed. The innate preference can be easily modified by further training. **Figure 5** shows how the training on the 4-color pattern proceeded in time in the present study. Clearly, there are two distinct patterns. The selection pattern of the butterflies trained to the blue or yellow disk persisted throughout the test (**Figures 5A,C**): the training with the innately preferred disks was extremely effective. On the other hand, it was necessary to have two and three training sessions to learn green and red as the feeding site (**Figures 5B,C**): after the first training, the butterflies selected the yellow or blue disk, presumably depending on their innate preference.

Did butterflies make selections solely based on the colors in the 4-color pattern? Indeed, *Papilio xuthus* could learn to forage on a darker disk while they innately prefer brighter stimuli. If *Pieris* butterflies use brightness as the cue, they will confuse a gray disk sharing the brightness with the training disk. We considered that the confusion could occur when we presented the training disk with all grays, particularly between the following combinations: blue (subjective brightness = 0.80)/yellow (0.79) vs. gray 3 (0.75), green (0.57) vs. gray 6 (0.54), and red (0.70) vs. gray 4 (0.66) (see **Table 1**). However, any confusion never happened when tested on the gray pattern (**Figure 1G**). We thus conclude that *Pieris rapae* has true color vision and uses it when searching for food.

Implications of the Action Spectrum

The colored solid curves in **Figure 7C** are photoreceptor spectral sensitivities recorded in the compound eyes of female *Pieris rapae*: they are of the UV (λ_{max} = 350 nm), violet (420 nm), blue (450 nm), green (560 nm), dual-peaked green (560 nm), orange (610 nm), red (620 nm), and deep-red (640 nm) classes (Blake et al., 2019). The dashed curves represent the spectral sensitivities of sex-specific receptors (Arikawa et al., 2005). The photoreceptors are embedded in ommatidia, each containing nine photoreceptor cells in three different combinations, making the eye a mesh of three spectrally distinct types of ommatidia. For example, a type I ommatidium has one UV, one blue, two green, and five orange receptors, each bearing a rhodopsin-containing rhabdomere to form a photoreceptive structure along the ommatidial axis.

Using the thorough anatomical and optical information about the compound eye of *Pieris rapae*, we previously calculated the absorptance spectrum for each ommatidial type (Stavenga and Arikawa, 2011). We also know that the ratio of type I, II, and III ommatidia is approximately 2:1:1 (Qiu et al., 2002). Here we calculated the sum of the ommatidial absorptance spectra, weighted by the ommatidial occupation ratio, as an estimation of the entire eye's spectral sensitivity (**Figure 7C**).

The estimated eye sensitivity is broad with the maximum in the red wavelength range (**Figure 7C**), while the behavioral action spectrum exhibits some prominent peaks with the maximum at 360 nm (**Figure 7B**). The difference in these spectra indicates that not all photoreceptors equally contribute to the present behavioral context, i.e., the floral foraging. The contribution of the UV, violet, and blue receptors appears to be much stronger than that of the green and red receptors. Green receptors, which exist in all ommatidia (Blake et al., 2019), make a complete hexagonal lattice, presumably serving motion and shape vision. In fact, a set of green receptors feeds information to the channel for motion vision in the *Papilio* visual system (Stewart et al., 2015). However, it is difficult to assume that green and red receptors do not contribute to color vision at all in *Pieris rapae*. The receptors may contribute more to color vision in other behaviors such as searching for mates, egg-laying, or even escaping: color information processing may be context-dependent.

Animals often exhibit spontaneous reactions to the light of specific wavelengths, known as the wavelength-specific or hard-wired behaviors, which do not require learning (Menzel, 1979). The function for feeding reaction of *Pieris brassicae* exhibits a major sensitivity band peaking at 450 nm and another lower peak at 600 nm (see Figure 8 of Scherer and Kolb, 1987). These peaks perfectly match those of our action spectrum except for the largest peak in the UV (**Figure 7B**). If not a species difference, the difference must be attributed to the experimental design: while Scherer and Kolb (1987) recorded hard-wired reactions of non-trained individuals, we used trained butterflies with monochromatic lights. The trained butterflies presumably learned the UV light as a certain color connected to the food because foraging butterflies use color vision, as discussed in this study. In other words, the action spectrum demonstrates the subjective brightness as well as the detection threshold of monochromatic lights as colors. Both spectra are informative and valuable, but it is important to be aware of the difference between them when evaluating and/or using them.

Comparative Aspects

Honeybee compound eyes contain UV, B, and G receptors, which serve as the basis of their trichromatic color vision (Menzel and Backhaus, 1989). This spectral organization is shared by many bee species (Peitsch et al., 1992).

Recent studies on butterfly eyes have revealed much more diversity in this respect (van der Kooi et al., 2021). The Japanese yellow swallowtail, *Papilio xuthus*, has six classes (UV, violet, blue, green, red, broadband) of receptors (Arikawa, 2003), four of

which serve in the UV-B-G-R tetrachromatic vision (Koshitaka et al., 2008). *Pieris rapae* has eight receptor classes, including three varieties of red-sensitive photoreceptors (**Figure 7C**). Such species-specific eye organization may create a difference in the color discrimination ability among butterfly species, which will be ecologically significant.

In the well-trained butterflies, we evaluated the color discrimination ability on the 13-color pattern. The butterflies visited the training colors and some other colors: most likely, the visited colors appeared similar to the butterflies. The confusion was most prominent in red-trained butterflies, where red was less preferred than orange and purple (**Figure 6**): none of the visited disks by the red-trained butterflies attracted significantly more individuals than others (**Supplementary Table S3**). However, we did not see such confusion in red in *Papilio xuthus* where the red training resulted in the most robust effect in the test using a 13-color pattern (Kinoshita et al., 1999).

These results suggest that the discrimination ability in the red range is better in *Papilio xuthus* than in *Pieris rapae*. This somehow matches with a field study, where Tanaka (1991) analyzed the color of flowers visited by 61 diurnal insect species (23 Hymenoptera, 16 Lepidoptera, 12 Coleoptera, 10 Diptera) for foraging. The field study demonstrates that the flower-visiting behavior of three species belonging to the genus *Papilio*, i.e., *P. xuthus*, *P. machaon*, and *P. bianor*, is strongly biased to red flowers. On the other hand, no other species, including *Pieris rapae*, exhibited such a strong red preference: *Pieris rapae* visited yellow flowers most frequently (Tanaka, 1991). The less frequent visits to red flowers of *Pieris rapae* is rather enigmatic because it has a wide variety of red receptors, with distinct spectral sensitivities, and the overall eye sensitivity is high in the red wavelength range (**Figure 7C**). We have assumed that the multiple red receptors of *Pieris rapae* may contribute to enhance contrast sensitivity and/or color discrimination in the long-wavelength spectral region (Qiu and Arikawa, 2003). However, the present study on virgin females contradicts this assumption. The results may be different in males and/or even in mated females. Multiple red receptors also exist in another pierid species, *Colias erate*, which does not exhibit a red preference either (Tanaka, 1991). In the case of *Colias erate*, the peak wavelengths of the red receptors' spectral sensitivity are well-separated only in females (Ogawa et al., 2013). The female-specific multiple red receptors of *Colias erate* may therefore be adaptive for assessing the quality of clover leaves for oviposition.

Sexual dimorphism in photoreceptor spectral sensitivities appears common among butterflies (Sison-Mangus et al., 2006; McCulloch et al., 2016), including *Pieris rapae*. In *Pieris rapae*, the "violet" receptors exist in fluorescing ommatidia in males. The fluorescing material absorbs the violet light and converts the violet receptors into double-peaked blue (dB) receptors (**Figure 7C**). When we discovered the dimorphism in *Pieris rapae crucivora*, we assumed that the male-specific dB receptors would help males to locate the UV-reflecting females efficiently upon mating (Arikawa et al., 2005). However, we recently found the same sexual dimorphism in the eyes of *Pieris rapae rapae*, the European subspecies, where the female-specific UV reflection is absent (Blake et al., 2019). It thus raises a question of whether or not the violet receptors in females and the dB receptors in males affect the color discrimination upon floral foraging. The biological significance of sexual dimorphism in vision requires further attention in future studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KA, YN, and MK designed the research and wrote the manuscript. YN and HK performed the behavioral experiments and optical measurements. YN, HK, and KA performed the data analysis. All authors read, commented, and approved the final version of the manuscript.

FUNDING

This work was supported by the JSPS KAKENHI (18H05273 to KA and 17K07485 to MK) and the SOKENDAI Advanced Sciences Synergy Program (SASSP) to MK.

ACKNOWLEDGMENTS

We thank Doekele Stavenga for the critical reading of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.650069/full#supplementary-material

REFERENCES

Arikawa, K. (2003). Spectral organization of the eye of a butterfly. *Papilio*. *J. Comp. Physiol. A* 189, 791–800. doi: 10.1007/s00359-003-0454-7

Arikawa, K., Wakakuwa, M., Qiu, X., Kurasawa, M., and Stavenga, D. G. (2005). Sexual dimorphism of short-wavelength photoreceptors in the small white butterfly, *Pieris rapae crucivora*. *J. Neurosci.* 25, 5935–5942. doi: 10.1523/JNEUROSCI.1364-05.2005

Belušič, G. (2011). *Electroretinograms*. Norderstedt: BoD–Books on Demand.

Blake, A. J., Hahn, G. S., Grey, H., Kwok, S. A., McIntosh, D., and Gries, G. (2020). Polarized light sensitivity in *Pieris rapae* is dependent on both color and intensity. *J. Exp. Biol.* 223(Pt 13):jeb220350. doi: 10.1242/jeb.220350

Blake, A. J., Pirih, P., Qiu, X., Arikawa, K., and Gries, G. (2019). Compound eyes of the small white butterfly *Pieris rapae* have three distinct classes of red photoreceptors. *J. Comp. Physiol. A* 205, 553–565. doi: 10.1007/s00359-019-01330-8

Chittka, L., and Thomson, J. D. (2001). *Cognitive ecology of Pollination: Animal Behavior and Floral Evolution*. Cambridge: Cambridge University Press.

Frisch, K. V. (1914). Der farbensinn und formensinn der biene. *Zool. Jb. Physiol* 37, 1–238.

Goulson, D., and Cory, J. S. (1993). Flower constancy and learning in foraging preferences of the green-veined white butterfly *Pieris napi*. *Ecol. Entomol.* 18, 315–320. doi: 10.1111/j.1365-2311.1993.tb01107.x

Kandori, I., and Ohsaki, N. (1996). The learning abilities of the white cabbage butterfly, *Pieris rapae*, foraging for flowers. *Res. Popul. Ecol.* 38, 111–117. doi: 10.1007/bf02514977

Kelber, A. (1997). Innate preferences for flower features in the hawkmoth *Macroglossum stellatarum*. *J. Exp. Biol.* 200, 827–836.

Kelber, A. (2001). Receptor based models for spontaneous colour choices in flies and butterflies. *Entomol. Exp. Appl.* 99, 231–244. doi: 10.1046/j.1570-7458.2001.00822.x

Kelber, A., and Pfaff, M. (1999). True colour vision in the orchard butterfly, *Papilio aegeus*. *Naturwissenschaften* 86, 221–224. doi: 10.1007/s001140050601

Kinoshita, M., Shimada, N., and Arikawa, K. (1999). Colour vision of the foraging swallowtail butterfly *Papilio xuthus*. *J. Exp. Biol.* 202, 95–102.

Kinoshita, M., Yamazato, K., and Arikawa, K. (2011). Polarization-based brightness discrimination in the foraging butterfly, *Papilio xuthus*. *Phil. Trans. R. Soc. B* 366, 688–696. doi: 10.1098/rstb.2010.0200

Kolb, G., and Scherer, C. (1982). Experiments on wavelength specific behavior of *Pieris brassicae* L. during drumming and egg-laying. *J. Comp. Physiol. A* 149, 325–332. doi: 10.1007/bf00619148

Koshitaka, H., Kinoshita, M., Vorobyev, M., and Arikawa, K. (2008). Tetrachromacy in a butterfly that has eight varieties of spectral receptors. *Proc. Biol. Sci.* 275, 947–954. doi: 10.1098/rspb.2007.1614

McCulloch, K. J., Osorio, D., and Briscoe, A. D. (2016). Sexual dimorphism in the compound eye of *Heliconius erato*: a nymphalid butterfly with at least five spectral classes of photoreceptor. *J. Exp. Biol.* 219, 2377–2387. doi: 10.1242/jeb.136523

Menzel, R. (1979). "Spectral sensitivity and color vision in invertebrates," in *Invertebrate Photoreceptors*, ed. H. Autrum (Berlin: Springer-Verlag), 503–580. doi: 10.1007/978-3-642-66999-6_9

Menzel, R., and Backhaus, W. (1989). "Color vision in honey bees: phenomena and physiological mechanisms," in *Facets of Vision*, eds D. G. Stavenga and R. C. Hardie (Berlin: Springer-Verlag), 281–297. doi: 10.1007/978-3-642-74082-4_14

Nouvian, M., and Galizia, C. G. (2020). Complexity and plasticity in honey bee phototactic behaviour. *Sci. Rep.* 10:7872. doi: 10.1038/s41598-020-64782-y

Obara, Y., and Hidaka, T. (1968). Recognition of the female by the male, on the basis of ultra-violet reflection, in the white cabbage butterfly, *Pieris rapae* crucivora Boisduval. *Proc. Jap. Acad.* 44, 829–832. doi: 10.2183/pjab1945.44.829

Obara, Y., and Majerus, M. E. N. (2000). Initial mate recognition in the British cabbage butterfly, *Pieris rapae rapae*. *Zool. Sci.* 17, 725–730. doi: 10.2108/zsj.17.725

Ogawa, Y., Kinoshita, M., Stavenga, D. G., and Arikawa, K. (2013). Sex-specific retinal pigmentation results in sexually dimorphic long-wavelength-sensitive photoreceptors in the Eastern Pale Clouded Yellow butterfly, *Colias erate*. *J. Exp. Biol.* 216, 1916–1923. doi: 10.1242/jeb.083485

Peitsch, D., Fietz, A., Hertel, H., Desouza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* 170, 23–40. doi: 10.1007/bf00190398

Qiu, X., and Arikawa, K. (2003). Polymorphism of red receptors: Sensitivity spectra of proximal photoreceptors in the small white butterfly, *Pieris rapae crucivora*. *J. Exp. Biol.* 206, 2787–2793. doi: 10.1242/jeb.00493

Qiu, X., Vanhoutte, K. A., Stavenga, D. G., and Arikawa, K. (2002). Ommatidial heterogeneity in the compound eye of the male small white butterfly, *Pieris rapae crucivora*. *Cell Tissue Res.* 307, 371–379. doi: 10.1007/s00441-002-0517-z

Satoh, A., Kinoshita, M., and Arikawa, K. (2016). Innate preference and learning of colour in the male cotton bollworm *Helicoverpa armigera*. *J. Exp. Biol.* 219, 3857–3860. doi: 10.1242/jeb.148064

Scherer, C., and Kolb, G. (1987). Behavioral experiments on the visual processing of color stimuli in *Pieris brassicae* L. (Lepidoptera). *J. Comp. Physiol. A* 160, 645–656. doi: 10.1007/bf00611937

Sison-Mangus, M. P., Bernard, G. D., Lampel, J., and Briscoe, A. D. (2006). Beauty in the eye of the beholder: the two blue opsins of lycaenid butterflies and the opsin gene-driven evolution of sexually dimorphic eyes. *J. Exp. Biol.* 209, 3079–3090. doi: 10.1242/jeb.02360

Stavenga, D. G., and Arikawa, K. (2011). Photoreceptor spectral sensitivities of the Small White butterfly *Pieris rapae crucivora* interpreted with optical modeling. *J. Comp. Physiol. A* 197, 373–385. doi: 10.1007/s00359-010-0622-5

Stewart, F. J., Kinoshita, M., and Arikawa, K. (2015). The butterfly *Papilio xuthus* detects visual motion using chromatic contrast. *Biol. Lett.* 11:20150687. doi: 10.1098/rsbl.2015.0687

Tanaka, H. (1991). Floral colours preferred by insects. *Insectarium* 28, 12–16. (in Japanese)

Traynier, R. M. M. (1984). Associative learning in the ovipositional behaviour of the cabbage butterfly, *Pieris rapae*. *Physiol. Entomol.* 9, 465–472. doi: 10.1111/j.1365-3032.1984.tb00789.x

Turner, C. H. (1910). Experiments on color-vision of the honey bee. *Biol. Bull.* 19, 257–279. doi: 10.2307/1536088

van der Kooi, C. J., Stavenga, D. G., Arikawa, K., Belušič, G., and Kelber, A. (2021). Evolution of insect color vision: from spectral sensitivity to visual ecology. *Ann. Rev. Entomol.* 66, 435–461. doi: 10.1146/annurev-ento-061720-071644

Wakakuwa, M., Kurasawa, M., Giurfa, M., and Arikawa, K. (2005). Spectral heterogeneity of honeybee ommatidia. *Naturwissenschaften* 92, 464–467. doi: 10.1007/s00114-005-0018-5

Wakakuwa, M., Stavenga, D. G., Kurasawa, M., and Arikawa, K. (2004). A unique visual pigment expressed in green, red and deep-red receptors in the eye of the small white butterfly, *Pieris rapae* crucivora. *J. Exp. Biol.* 207, 2803–2810. doi: 10.1242/jeb.01078

Yoshida, M., Itoh, Y., Ômura, H., Arikawa, K., and Kinoshita, M. (2015). Plant scents modify innate colour preference in foraging swallowtail butterflies. *Biol. Lett.* 11:20150390. doi: 10.1098/rsbl.2015.0390

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

BRIEF RESEARCH REPORT
published: 29 March 2021
doi: 10.3389/fevo.2021.658710

Rational Design of a Novel Hawkmoth Pollinator Interaction in *Mimulus* Section *Erythranthe*

*Kelsey J. R. P. Byers**† and H. D. Bradshaw Jr.*

Department of Biology, University of Washington, Seattle, WA, United States

Diversification of the *ca*. 275,000 extant flowering plant species has been driven in large part by coevolution with animal pollinators. A recurring pattern of pollinator shifts from hummingbird to hawkmoth pollination has characterized plant speciation in many western North American plant taxa, but in the genus *Mimulus* (monkeyflowers) section *Erythranthe* the evolution of hawkmoth pollination from hummingbird-pollinated ancestors has not occurred. We manipulated two flower color loci and tested the attractiveness of the resulting four color phenotypes (red, yellow, pink, and white) to naïve hawkmoths (*Manduca sexta*). Hawkmoths strongly prefer derived colors (yellow, pink, white) over the ancestral red when choosing an initial flower to visit, and generally preferred derived colors when total visits and total visit time were considered, with no hawkmoth preferring ancestral red over derived colors. The simple flower color genetics underlying this innate pollinator preference suggests a potential path for speciation into an unfilled hawkmoth-pollinated niche in *Mimulus* section *Erythranthe*, and the deliberate design of a hawkmoth-pollinated flower demonstrates a new, predictive method for studying pollination syndrome evolution.

Keywords: *Mimulus*, floral color, *Manduca sexta*, experimental evolution, pollination, reproductive isolation, speciation

OPEN ACCESS

Edited by:
Johannes Spaethe, Julius Maximilian University of Würzburg, Germany

Reviewed by:
Klaus Lunau, Heinrich Heine University of Düsseldorf, Germany
Matthew Koski, Clemson University, United States
Joaquín Goyret, University of Tennessee at Martin, United States

****Correspondence:***
Kelsey J. R. P. Byers
kelsey.byers@jic.ac.uk

†Present address:
Kelsey J. R. P. Byers, John Innes Centre, Norwich, United Kingdom

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *26 January 2021*
Accepted: *11 March 2021*
Published: *29 March 2021*

Citation:
Byers KJRP and Bradshaw HD Jr (2021) Rational Design of a Novel Hawkmoth Pollinator Interaction in Mimulus Section Erythranthe. Front. Ecol. Evol. 9:658710. doi: 10.3389/fevo.2021.658710

INTRODUCTION

Darwin called the dramatic radiation of the *ca*. 275,000 flowering plant species "an abominable mystery," though he recognized the potential role of the strong coevolutionary relationships between plants and their pollinators (Darwin, 1862). It is now clear that animal pollination is responsible for high rates of speciation in the flowering plants (Coyne and Orr, 2004; van der Niet and Johnson, 2012). Shifts between pollinator guilds (e.g., bumblebees, hummingbirds, hawkmoths, bats) often coincide with plant speciation events (Whittall and Hodges, 2007; Forest et al., 2014), and each pollinator guild is attracted by a different suite of floral traits (e.g., color, scent, pattern, shape, nectar reward, anthesis time) collectively known as a pollination syndrome (Fenster et al., 2004). Although some controversy around the validity of these syndromes exists (Ollerton et al., 2009; Wang et al., 2020), evidence suggests they are valid in the broad taxonomic sense (Rosas-Guerrero et al., 2014) as well as in specific taxonomic groups (Murúa and Espíndola, 2014). Extensive work has identified pollination syndromes among various plant families (Fenster et al., 2004), but the detailed genetics of traits involved in pollinator shift-driven plant speciation remain largely unresolved outside of a few key systems (Yuan et al., 2013; Fattorini and Glover, 2020). Have we learned enough about the genetic basis of the origin of flowering plant species to *engineer*

a shift in pollinator guilds? Borrowing from Gould's metaphor of the "tape of life," (Gould, 1989) can we *anticipate* (rather than recapitulate) evolutionary trajectories, and, instead of replaying the tape of life, run the tape in fast forward? Can we predict, and then produce, a pollinator shift into a previously unfilled niche within a specific group, and is this shift genetically simple enough to potentially occur in the wild?

A recurring pattern of pollinator shifts from hummingbird to hawkmoth pollination has characterized plant speciation in many western North American taxa (e.g., *Aquilegia, Ipomopsis*, and *Mimulus* section *Diplacus*: Grant, 1993; Whittall and Hodges, 2007) and more globally (Rosas-Guerrero et al., 2014), but in the genus *Mimulus* (monkeyflowers) section *Erythranthe* (*sensu* Lowry et al., 2019) the evolution of hawkmoth pollination from hummingbird-pollinated ancestors has not occurred. "Hawkmoth flowers" share several characteristics with "hummingbird flowers," including a large volume of dilute nectar and a long tubular corolla (Martins and Johnson, 2013; Johnson et al., 2016). But most hummingbird flowers are red (Grant, 1966; Rodríguez-Gironés and Santamaría, 2004; Lunau et al., 2011), hence not easily visible to hawkmoths, whose visual sensitivity does not extend into the longer wavelengths (Cutler et al., 1995). Hawkmoth flowers are usually white (or pale) and highly reflective in the visual wavelengths while lacking UV reflection (Grant, 1993; Goyret et al., 2008; Martins and Johnson, 2013; Johnson et al., 2016), adapted for detection by crepuscular and nocturnal hawkmoths.

Our goal is to design and synthesize a new *Mimulus* species (*sensu* Duffy et al., 2007; Villa et al., 2019), pollinated by hawkmoths and reproductively isolated from its red-flowered, hummingbird-pollinated ancestor, *M. cardinalis*. Several traits already present in *Mimulus*, including nocturnal anthesis, large nectar volume in *M. cardinalis*, and floral scent, suggest that the evolution of hawkmoth pollination in section *Erythranthe* should be genetically tractable and require few mutational changes. We set out to determine if the minimal combination of only two flower color changes—loss of anthocyanins and loss of carotenoids, either separately or together, both of which are found in wild *M. lewisii* and *M. cardinalis*—(Vickery, 1992; Wu et al., 2013) would be necessary and sufficient to change the behavior of a model hawkmoth, *Manduca sexta*. Given the extensive existing data on hawkmoth color preferences (White et al., 1994; Kelber, 1997; Goyret et al., 2008; Kuenzinger et al., 2019; and others), largely demonstrating a naïve preference for blue colors but the potential for training to prefer the more common hawkmoth-pollinated white colors (Goyret et al., 2008), we predicted that hawkmoths would prefer flowers with two mutational steps from the "ancestor" (white flowers, with the loss of both anthocyanins and carotenoids) over the ancestral state (red flowers). Moths might also show an intermediate preference for single mutational steps (yellow or pink flowers, with the loss of anthocyanins and carotenoids, respectively).

As a first step, we manipulated two flower color loci in *M. cardinalis* and tested the attractiveness of the resulting four color phenotypes (red, yellow, pink, white; **Figure 1A**) to naïve hawkmoths. If we are able to demonstrate a potential pro-hawkmoth change in *Mimulus* section *Erythranthe* via color shift, this suggests that a transition into the hawkmoth niche not yet fulfilled in this section might be a potential future evolutionary trajectory in the group resulting in a novel species.

METHODS

Genetic Stocks

The red color of *M. cardinalis* flowers is produced by the combination of high concentrations of anthocyanin (pink) and carotenoid (yellow) pigments (Hiesey et al., 1971). *Mimulus cardinalis* Douglas ex Benth. (inbred line CE10, derived by single seed descent from a plant collected along the South Fork of the Tuolumne River, Yosemite, CA) was crossed to *Mimulus lewisii* Pursh (inbred LF10 line derived in the same way from the same area) homozygous for a recessive EMS-induced mutation at the *BOO1* locus (Pince, 2009), producing an anthocyanin-less flower. *M. lewisii* is homozygous for a dominant suppressor of carotenoid pigmentation (*YUP*: Bradshaw and Schemske, 2003), and the mutant has the genotype *boo1/boo1 YUP/YUP*. *M. cardinalis* is homozygous for the alternative alleles (*BOO1/BOO1 yup/yup*). A resulting pink-flowered F_1 offspring (*BOO1/boo1 yup/YUP*) was selfed to produce the segregating F_2 study population (n = 500). Flowers of four colors were selected (**Figure 1A**), corresponding to the four combinations of alleles at the two flower color loci: red, similar in color to the "ancestral" *M. cardinalis* (*BOO1 yup*); pink (*BOO1 YUP*); yellow (*boo1 yup*); and white (*boo1 YUP*). Three F_2 plants of each color were selected based on similarity of flower size, shape including petal reflexing, and nectar volume. Neither *M. lewisii* nor *M. cardinalis* reflect in ultraviolet wavelengths (Vickery, 1992; Owen and Bradshaw, 2011), meaning the only visible signals to hawkmoths (whose visual receptors peak at 357, 450, and 520 nm: UV, blue, and green respectively, Cutler et al., 1995) should be in the visible spectrum.

Experimental Animals

Carolina hawkmoths (*Manduca sexta*) were raised on artificial diet (Bell and Joachim, 1976) under controlled conditions at the University of Washington. As this typical diet is lacking in vitamin A, these moths likely differ in their visual sensitivity compared with wild moths or those reared on a complete diet, which might affect our behavioral results (Goyret et al., 2009). Hawkmoths were eclosed in full artificial lighting and were not fed or light-cycled prior to the experimental runs. Female hawkmoths eclosed four to six days prior to the experiment were used in all experiments, as this is within the approximate range where feeding motivation is highest (Goyret et al., 2007).

Test Chamber Experiments

Hawkmoths were tested in a 1 m × 1 m × 70 cm chamber constructed of black Coroplast (Coroplast, Dallas, TX) with a clear Plexiglas top for observation. The chamber was located within a darkroom illuminated with red safelight; the chamber itself was illuminated with a single blue-white LED emitting 2 lumens mounted on the Plexiglas top. One flower, including pedicel, of each color was mounted at a height of 50 cm on a long side of the symmetrical chamber using matte black tape.

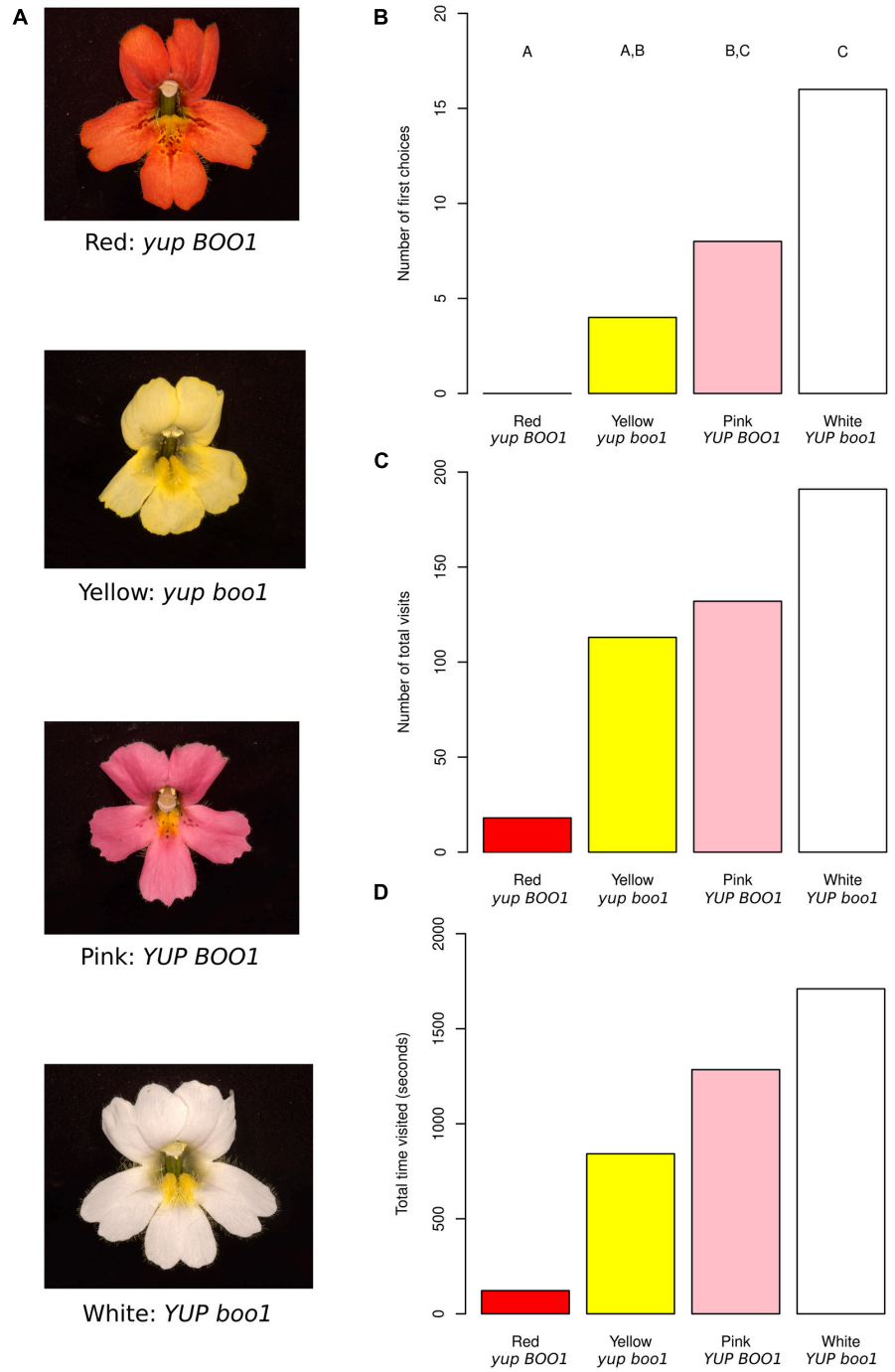

FIGURE 1 | *Mimulus* color mutants and behavioral results. **(A)** Red, yellow, pink, and white *Mimulus* flowers from a single F_2 population with their inferred genotypes. **(B)** First choices to the flowers across all moths, with letters indicating statistically significant differences between colors. **(C)** Total visits to the flowers across all moths. **(D)** Total time each flower color was visited across all moths. Data from individual moths were not pooled for statistics for **(C,D)** and therefore no statistical differences are shown (see section "Methods").

Each run was randomized for both flower color at each position and one of three parent plants for each color. As hawkmoths are able to see color in dim starlight (Kelber et al., 2002), the black Coroplast and blue-white LED may not fully reflect the visual environment and background contrast these hawkmoths would encounter in the wild; the darker background may have instead increased contrast between the color morphs and might affect preference (Kuenzinger et al., 2019).

Each hawkmoth was observed until an initial naïve choice—defined as proboscis extension and contact with the floral surface

(Raguso and Willis, 2003)—was made. At that point, number of visits and time for each visit were recorded until nectar exhaustion or hawkmoth exhaustion. Nectar exhaustion was defined as a visit of one second or less and hawkmoth exhaustion as the hawkmoth becoming unwilling to fly. At this point the hawkmoth was removed and the flowers replaced before a new hawkmoth was introduced. Each moth was used for only one experiment.

Statistical Analysis

Initial visit data (first choice) were analyzed using a chi-square goodness-of-fit and individual ranking was done with pairwise chi-square tests with a sequential Bonferroni correction (Rice, 1989). Visit profiles were examined to rule out initial preference having an effect on further visits (e.g., moths preferring only their first flower color once they discover it is rewarding). Of 28 moths, 22 visited all three non-red colors, distributed evenly with initial preference ($X^2 = 1.375$, $p = 0.503$, df = 2); additionally, 17 of the 28 moths visited another color as often as or more than their initial choice.

Chi-square goodness-of-fit tests were considered for total visit numbers and visit time, but a two-way chi-square test on the individual moth data showed that moths differed in their behavior for total visit time ($X^2 = 1449.638$, $p = 1.286 \times 10^{-249}$, df = 81), and a Fisher's exact test (chosen instead of chi-square due to the expected values violating the chi-square assumptions) showed that moths differed in their behavior for total visit number ($p = 5 \times 10^{-4}$) as well. Therefore, these data were not pooled across moths. Instead, visit time and visit number data from individual moths that made over 20 visits (7 of 28 moths) were analyzed using chi-square goodness-of-fit tests with sequential Bonferroni correction separately for each moth.

RESULTS

Using naïve captive-bred female hawkmoths (*Manduca sexta*) in a dimly lit flight chamber with one flower of each color (**Supplementary Figure 1**), we counted their first choices, the total number of pollinator visits to each flower color, and the time spent on each color (**Supplementary Table 1**). A total of 28 hawkmoths were observed, resulting in a total of 454 visits with between 3 and 40 visits per moth. First choices differed significantly between color morphs (**Figure 1B**; $n = 28$, $X^2 = 20$, df = 3, $p = 1.70 \times 10^{-4}$), with hawkmoths preferring white and pink morphs equally, also visiting yellow morphs equally to pink, and ignoring red morphs. These results indicate that hawkmoths are attracted to flowers with at least one allele substitution step (yellow or pink) from the red flower color characteristic of the ancestral hummingbird-pollinated *M. cardinalis*.

Since hawkmoths differed in their visitation profiles between moths (see section "Methods"), we present statistics for individual moths instead of pooling them when discussing total visit count (**Figure 1C** and **Supplementary Figure 2A**; $n = 28$) and total visit time (**Figure 1D** and **Supplementary Figure 2B**; $n = 28$). Five out of seven moths making more than 20 visits differed in their visit numbers between color morphs ($p < 0.05$), while one moth trended toward a difference ($p = 0.0534$) and one moth showed no difference ($p = 0.572$). All seven moths making more than 20 visits differed in their visit times to different colors ($p < 0.05$). Sequential Bonferroni correction for each moth demonstrated some general trends. When total visits were considered, all significant comparisons between red and another color showed higher visitation to the other color (white vs. red: 4 of 5 moths significant; pink vs. red: 3 of 5 moths significant; yellow vs. red: 2 of 5 moths significant). The same was true for total time, i.e., red flowers were visited for a shorter time overall by most moths (white vs. red: 6 of 7 moths significant; pink vs. red: 5 of 7 moths significant; yellow vs. red: 5 of 7 moths significant).

We also considered the transitions between flower morphs in the array (**Figure 2**). Nearly 20% of transitions were from the white flower back to the white flower, while same-flower transitions were rarer to pink (4.7%) and yellow (5.6%) flowers and absent for red flowers. As suggested by visitation numbers, transitions to red flowers were rare (1.4% from all three other colors). Transition rates were similar in both directions, i.e., transitions from white to pink were similar in frequency (12.7%) to transitions from pink to white (12.0%). As suggested by visit numbers, transitions to/from white and pink were more frequent than transitions to/from white and yellow, with transitions to/from pink and yellow intermediate. When accounting for the position of flowers in the array (**Supplementary Figure 3**), we see that red flowers were never visited when more than two positions away in the array, suggesting that moths could not see the red flowers very well from a distance, while white flowers were frequently visited when three positions away in the array, suggesting they are more conspicuous at a distance than any of the other colors. Anecdotally, most visits to red flowers seemed to occur after accidental contact between the moth and the flower, while most visits to white, pink, or yellow flowers appeared to be the result of more deliberate navigation, most likely due to the known visual receptor sensitivities of *Manduca sexta*, peaking in the UV to green range (Cutler et al., 1995) in combination with the higher brightness of the other color morphs. When moths did visit red flowers, however, they did not appear to visit for shorter amounts of time (white: 12.1 s/visit; pink: 9.2 s/visit; yellow: 8.1 s/visit; red: 9.7 s/visit), suggesting that they found red flowers equally rewarding once they were encountered.

DISCUSSION

When given the choice between four color phenotypes (red, yellow, pink, and white) representing four genotypes at two genetic loci, hawkmoths preferred the "derived" non-red colors for their initial choice, and generally preferred these colors to red (or in a few cases treated them equally) when total visits and total time were considered. Red flowers seemed less conspicuous in the flight chamber than other colors, consistent with the lack of visual sensitivity at these wavelengths (Cutler et al., 1995), while white flowers, with the highest brightness, were frequently visited even when at the opposite end of the array from the previous flower. These results are in agreement

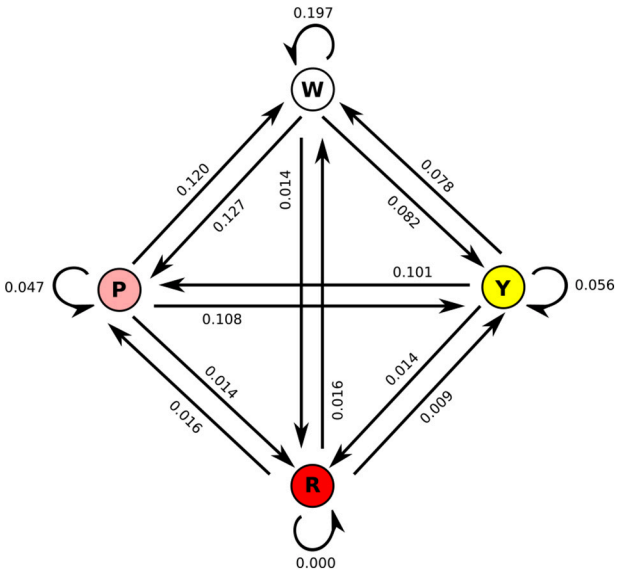

FIGURE 2 | State change diagram for the four floral colors, with values averaged across all moths. All values in the diagram sum to 1, i.e., the numbers are the proportion of all total changes, not of changes to/from each color.

with existing data on hawkmoth color preferences (White et al., 1994; Kelber, 1997; Goyret et al., 2008; Kuenzinger et al., 2019; and others), though they may have been influenced by the experimental diet's deficiency in beta-carotenes (see section "Methods").

Testing these four flower color phenotypes with naïve hawkmoths in an experimental chamber has established the remarkably simple genetic basis (two mutational steps) of phenotypic change required to initiate a potential pollinator guild shift from hummingbirds to hawkmoths. Observations of pollinator preference and pollen movement in the native environment of *M. cardinalis* using near-isogenic lines for the *YUP* and *boo1* alleles will be needed for a definitive assessment of reproductive isolation between the hummingbird-pollinated ancestral *M. cardinalis* and the rationally designed hawkmoth-pollinated derivatives with yellow, pink, or white flowers. Of note, many hummingbird-pollinated flowers are also white (Lunau et al., 2011), and thus our potential pollinator shift would reflect a "pro-hawkmoth" rather than an "anti-hummingbird" trait shift (*sensu* Castellanos et al., 2004).

Although not measured in this floral study population, floral scent and anthesis time are known to be important characteristics in the hawkmoth pollination syndrome (Faegri and van der Pijl, 1979). *Mimulus lewisii* and *M. cardinalis*, the two parent species of our test population, both emit moderate amounts of terpene volatiles (Byers et al., 2014a) that provoke electroantennographic responses in the hawkmoths *Hyles lineata* (Raguso et al., 1996) and *Sphinx perelegans* (Raguso and Light, 1998). In addition, *Mimulus lewisii* demonstrates nocturnal anthesis (**Supplementary Video 1**). In combination, the scent, nocturnal anthesis, and potential color shift (including a lack of UV reflection, White et al., 1994) would argue that a hawkmoth niche shift is possible in *Mimulus* section *Erythranthe*. Although *Manduca sexta* largely feeds on members of the Solanaceae (the nightshade family), we expect that other local hawkmoths (for which we are here using *Manduca* as a proxy) would be potential pollinators of these novel color variants should they arise in nature.

The classical approach to understanding plant speciation by pollinator shift is *retrospective*—sister taxa with different pollinators are analyzed for differences in key floral traits, often with known effects on pollinator preference (e.g., Bradshaw and Schemske, 2003; Streisfeld and Kohn, 2007; Whittall and Hodges, 2007; Byers et al., 2014a; Wessinger et al., 2014), and their underlying alleles (e.g., Schlüter et al., 2011; Hermann et al., 2013; Streisfeld et al., 2013; Byers et al., 2014b) to infer the evolutionary history of divergence from their common ancestor. But perhaps the most stringent test of our understanding of flowering plant diversification is the *prospective* approach we have used here. Darwin famously predicted that the Malagasy star orchid (*Angraecum sesquipedale*), which has a white flower and *ca.* 35cm nectar spur, must be pollinated by a (then-undiscovered) hawkmoth with a *ca.* 35 cm proboscis (Darwin, 1862). Building on similar predictions, and backed by experimental evidence, we have shown that critical steps toward the origin of a new, human-designed, hawkmoth-pollinated plant species can, likewise, be simple and predicted based upon a fundamental knowledge of pollination syndromes and genetics.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

HB conceived the study and provided seed stocks. KB performed experiments and analyzed data and wrote the initial manuscript. Both authors contributed to manuscript editing and designed the study and methodology.

ACKNOWLEDGMENTS

We are indebted to T. Daniel, A. Mountcastle, D. Haak, T. Schuldiner-Harpaz, C. Miller, and K. Brandt for advice and assistance. Greenhouse and darkroom resources for this work were provided by the University of Washington Department of Biology and hawkmoths were kindly provided by T. Daniel. The manuscript has also benefited from feedback by KL, JG, and MK. This manuscript has previously appeared online at bioRxiv (Byers and Bradshaw, 2017).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.658710/full#supplementary-material

Supplementary Figure 1 | Carolina hawkmoth (*Manduca sexta*) feeding from white *Mimulus* mutant in flight chamber.

Supplementary Figure 2 | Visit and visitation time data for individual moths. Individuals are presented in order of experiments, which were randomized, and the order is the same for parts **(A,B)**. **(A)** Total number of visits broken down by floral color for each moth (from bottom to top of each bar: white, pink, yellow, red). **(B)** Total visit time broken down by floral color for each moth [as in **(A)**].

Supplementary Figure 3 | Moth transitions to flowers of each color based on distance flown to that flower. "Transition index" is the ratio of the percentage of time moths flew to a given color to the percentage of time that color was *n* positions away from the origin color, where a higher transition index indicates a larger percentage of the time that a given color was flown to.

Supplementary Table 1 | Experimental design and raw data from hawkmoth visitation experiments.

Supplementary Video 1 | *Mimulus lewisii* flowers open during nighttime, with full anthesis present at approximately 02:00 a.m.

REFERENCES

Bell, R. A., and Joachim, F. G. (1976). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. Ent. Soc. Amer.* 69, 365–373. doi: 10.1093/aesa/69.2.365

Bradshaw, H. D. Jr., and Schemske, D. W. (2003). Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* 426, 176–178. doi: 10.1038/nature02106

Byers, K. J. R. P., and Bradshaw, H. D. Jr. (2017). Rational design of a novel pollinator interaction. *bioRxiv [preprint]* doi: 10.1101/2020.06.26.173286

Byers, K. J. R. P., Bradshaw, H. D. Jr., and Riffell, J. A. (2014a). Three floral volatiles contribute to differential pollinator attraction in monkeyflowers (*Mimulus*). *J. Exp. Biol.* 217, 614–623. doi: 10.1242/jeb.092213

Byers, K. J. R. P., Vela, J. P., Peng, F., Riffell, J. A., and Bradshaw, H. D. Jr. (2014b). Floral volatile alleles can contribute to pollinator-mediated reproductive isolation in monkeyflowers (*Mimulus*). *Plant J.* 80, 1031–1042. doi: 10.1111/tpj.12702

Castellanos, M. C., Wilson, P., and Thomson, J. D. (2004). 'Anti-bee' and 'pro-bird' changes during the evolution of hummingbird pollination in *Penstemon* flowers. *J. Evol. Biol.* 17, 876–885. doi: 10.1111/j.1420-9101.2004.00729.x

Coyne, J. A., and Orr, H. A. (2004). *Speciation*. Sunderland, MA: Sinauer Associates.

Cutler, D. E., Bennett, R. R., Stevenson, R. D., and White, R. H. (1995). Feeding behavior in the nocturnal moth *Manduca sexta* is mediated mainly by blue receptors, but where are they located in the retina? *J. Exp. Biol.* 198, 1909–1917.

Darwin, C. R. (1862). *On the Various Contrivances by Which British and Foreign Orchids are Fertilized by Insects, and on the Good Effects of Intercrossing*. London: J. Murray.

Duffy, S., Burch, C. L., and Turner, P. E. (2007). Evolution of host specificity drives reproductive isolation among RNA viruses. *Evolution* 61, 2614–2622. doi: 10.1111/j.1558-5646.2007.00226.x

Faegri, K., and van der Pijl, L. (1979). *The Principles of Pollination Ecology*. Pergamon: Oxford.

Fattorini, R., and Glover, B. J. (2020). Molecular mechanisms of pollination biology. *Annu. Rev. Plant Biol.* 71, 487–515. doi: 10.1146/annurev-arplant-081519-040003

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., and Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.* 35, 375–403. doi: 10.1146/annurev.ecolsys.34.011802.132347

Forest, F., Goldblatt, P., Manning, J. C., Baker, D., Colville, J. F., Devey, D. S., et al. (2014). Pollinator shifts as triggers of speciation in painted petal irises (*Lapeirousia*: iridaceae). *Ann. Bot.* 113, 357–371. doi: 10.1093/aob/mct248

Gould, S. J. (1989). *Wonderful Life: The Burgess Shale and the Nature of History*. New York, NY: W.W. Norton and Company.

Goyret, J., Kelber, A., Pfaff, M., and Raguso, R. A. (2009). Flexible responses to visual and olfactory stimuli by foraging *Manduca sexta*: larval nutrition affects adult behaviour. *Proc. Royal Soc. B: Biol. Sci.* 276, 2739–2745. doi: 10.1098/rspb.2009.0456

Goyret, J., Markwell, P. M., and Raguso, R. A. (2007). The effect of decoupling olfactory and visual stimuli on the foraging behavior of *Manduca sexta*. *J. Exp. Biol.* 210, 1398–1405. doi: 10.1242/jeb.02752

Goyret, J., Pfaff, M., Raguso, R. A., and Kelber, A. (2008). Why do *Manduca sexta* feed from white flowers? Innate and learnt colour preferences in a hawkmoth. *Naturwissenschaften* 95, 569–576. doi: 10.1007/s00114-008-0350-7

Grant, K. (1966). A hypothesis concerning the prevalence of red coloration in California hummingbird flowers. *Am. Nat.* 100, 85–97.

Grant, V. (1993). Origin of floral isolation between ornithophilous and sphingophilous plant species. *Proc. Natl. Acad. Sci.* 90, 7729–7733. doi: 10.1073/pnas.90.16.7729

Hermann, K., Klahre, U., Moser, M., Sheehan, H., Mandel, T., and Kuhlemeier, C. (2013). Tight genetic linkage of prezygotic barrier loci creates a multifunctional speciation island in *Petunia*. *Curr. Biol.* 23, 873–877. doi: 10.1016/j.cub.2013.03.069

Hiesey, W. M., Nobs, M. A., and Bjorkman, O. (1971). *Experimental Studies on the Nature of Species. V. Biosystematics, Genetics, and Physiological Ecology of the Erythranthe* Section of *Mimulus*. Washington, DC: Carnegie Institute of Washington.

Johnson, S. D., Moré, M., Amorim, F. W., Haber, W. A., Frankie, G. W., Stanley, D. A., et al. (2016). The long and the short of it: a global analysis of hawkmoth pollination niches and interaction networks. *Func. Ecol.* 31, 101–115. doi: 10.1111/1365-2435.12753

Kelber, A. (1997). Innate preferences for flower features in the hawkmoth *Macroglossum stellatarum*. *J. Exp. Biol.* 200, 827–836.

Kelber, A., Balkenius, A., and Warrant, E. J. (2002). Scotopic colour vision in nocturnal hawkmoths. *Nature* 419, 922–925. doi: 10.1038/nature01065

Kuenzinger, W., Kelber, A., Weesner, J., Travis, J., Raguso, R. A., and Goyret, J. (2019). Innate colour preferences of a hawkmoth depend on visual context. *Biol. Lett.* 15:20180886. doi: 10.1098/rsbl.2018.0886

Lowry, D. B., Sobel, J. M., Angert, A. L., Ashman, T.-L., Baker, R. L., Blackman, B. K., et al. (2019). The case for the continued use of the name *Mimulus* for all monkeyflowers. *Taxon* 68, 617–623. doi: 10.1002/tax.12122

Lunau, K., Papiorek, S., Eltz, T., and Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *J. Exp. Biol.* 214, 1607–1612. doi: 10.1242/jeb.052688

Martins, D. J., and Johnson, S. D. (2013). Interactions between hawkmoths and flowering plants in East Africa: polyphagy and evolutionary specialization in an ecological context. *Biol. J. Linn. Soc.* 110, 199–213. doi: 10.1111/bij.12107

Murúa, M., and Espíndola, A. (2014). Pollination syndromes in a specialized plant-pollinator interaction: does floral morphology predict pollinators in *Calceolaria*? *Plant Biol.* 17, 551–557. doi: 10.1111/plb.12225

Ollerton, J., Alarcón, R., Waser, N. M., Price, M. V., Watts, S., Cranmer, L., et al. (2009). A global test of the pollination syndrome hypothesis. *Ann. Bot.* 103, 1471–1480. doi: 10.1093/aob/mcp031

Owen, C. R., and Bradshaw, H. D. (2011). Induced mutations affecting pollinator choice in *Mimulus lewisii* (Phrymaceae). *Arth.-Plant Interact.* 5, 235–244. doi: 10.1007/s11829-011-9133-8

Pince, C. R. (2009). *Evolution and Genetics of Floral Form in Mimulus*. Ph.D. thesis, University of Washington, Seattle, WA.

Raguso, R. A., and Light, D. M. (1998). Electroantennogram responses of male *Sphinx perelegans* hawkmoths to floral and 'green-leaf volatiles'. *Entomol. Exp. Et Appl.* 86, 287–293. doi: 10.1046/j.1570-7458.1998.00291.x

Raguso, R. A., Light, D. M., and Pichersky, E. (1996). Electroantennogram responses of *Hyles lineata* (Sphingidae: lepidoptera) to volatile compounds from *Clarkia breweri* (Onagraceae) and other moth-pollinated flowers. *J. Chem. Ecol.* 22, 1735–1766. doi: 10.1007/BF02028502

Raguso, R. A., and Willis, M. A. (2003). "Hawkmoth pollination in Arizona's Sonoran Desert: behavioral responses to floral traits," in *Butterflies: Ecology and*

Evolution Taking Flight, eds C. Boggs, W. B. Watt, and P. R. Ehrlich (Chicago, IL: University of Chicago Press), 43–66.

Rice, W. R. (1989). Analyzing tables of statistical tests. *Evolution* 43, 223–225. doi: 10.2307/2409177

Rodríguez-Gironés, M. A., and Santamaría, L. (2004). Why are so many bird flowers red? *PLoS Biol.* 2:e350. doi: 10.1371/journal.pbio.0020350

Rosas-Guerrero, V., Aguilar, R., Martén-Rodríguez, S., Ashworth, L., Lopezaraiza-Mikel, M., Bastida, J. M., et al. (2014). A quantitative review of pollination syndromes: do floral traits predict effective pollinators? *Ecol. Lett.* 17, 388–400. doi: 10.1111/ele.12224

Schlüter, P. M., Xu, S., Gagliardini, V., Whittle, E., Shanklin, J., Grossniklaus, U., et al. (2011). Stearoyl-acyl carrier protein desaturases are associated with floral isolation in sexually deceptive orchids. *Proc. Natl. Acad. Sci. U.S.A.* 108, 5696–5701. doi: 10.1073/pnas.1013313108

Streisfeld, M. A., and Kohn, J. R. (2007). Environment and pollinator-mediated selection on parapatric floral races of *Mimulus aurantiacus*. *J. Evo. Bio.* 20, 122–132. doi: 10.1111/j.1420-9101.2006.01216.x

Streisfeld, M. A., Young, W. N., and Sobel, J. M. (2013). Divergent selection drives genetic differentiation in an R2R3-MYB transcription factor that contributes to incipient speciation in *Mimulus aurantiacus*. *PLoS Genet.* 9:e1003385. doi: 10.1371/journal.pgen.1003385

van der Niet, T., and Johnson, S. D. (2012). Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends Ecol. Evol.* 27, 353–361. doi: 10.1016/j.tree.2012.02.002

Vickery, R. K. Jr. (1992). Pollinator preferences for yellow, orange, and red flowers of *Mimulus verbenaceus* and *M. cardinalis*. *Great Basin Nat.* 52, 145–148.

Villa, S. M., Altuna, J. C., Ruff, J. S., Beach, A. B., Mulvey, L. I., and Poole, E. J. (2019). Rapid experimental evolution of reproductive isolation from a single natural population. *Proc. Natl. Acad. Sci. U.S.A.* 116, 13440–13445. doi: 10.1073/pnas.1901247116

Wang, X., Wen, M., Qian, X., Pei, N., and Zhang, D. (2020). Plants are visited by more pollinator species than pollination syndromes predicted in an oceanic island community. *Sci. Rep.* 10:13918. doi: 10.1038/s41598-020-70954-7

Wessinger, C. A., Hileman, L. C., and Rausher, M. D. (2014). Identification of major quantitative trait loci underlying floral pollination syndrome divergence in *Penstemon*. *Phil. Trans. Roy. Soc. B.* 369:20130349. doi: 10.1098/rstb.2013.0349

White, R. H., Stevenson, R. D., Bennett, R. R., and Cutler, D. E. (1994). Wavelength discrimination and the role of ultraviolet vision in the feeding behavior of hawkmoths. *Biotropica* 26, 427–435. doi: 10.2307/2389237

Whittall, J. B., and Hodges, S. A. (2007). Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* 447, 706–709. doi: 10.1038/nature05857

Wu, C. A., Streisfeld, M. A., Nutter, L. I., and Cross, K. A. (2013). The genetic basis of a rare flower color polymorphism in *Mimulus lewisii* provides insight into the repeatability of evolution. *PLoS One* 8:e81173. doi: 10.1371/journal.pone.0081173

Yuan, Y.-W., Byers, K. J. R. P., and Bradshaw, H. D. Jr. (2013). The genetic control of flower-pollinator specificity. *Curr. Op. Plant Biol.* 16, 422–428. doi: 10.1016/j.pbi.2013.05.004

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 21 May 2021
doi: 10.3389/fevo.2021.658441

Flowers of Deceptive *Aristolochia microstoma* Are Pollinated by Phorid Flies and Emit Volatiles Known From Invertebrate Carrion

Thomas Rupp[1], Birgit Oelschlägel[2], Katharina Rabitsch[1,3], Hafez Mahfoud[2], Torsten Wenke[2], R. Henry L. Disney[4], Christoph Neinhuis[2], Stefan Wanke[2] and Stefan Dötterl[1]**

[1] Plant Ecology, Department of Biosciences, Paris Lodron University of Salzburg, Salzburg, Austria, [2] Institut für Botanik, Technische Universität Dresden, Dresden, Germany, [3] Institute of Biology, Universität Graz, Graz, Austria, [4] Department of Zoology, University of Cambridge, Cambridge, United Kingdom

OPEN ACCESS

Edited by:
Sara Diana Leonhardt, Technical University of Munich, Germany

Reviewed by:
Manfred Ayasse, University of Ulm, Germany
Renate A. Wesselingh, Catholic University of Louvain, Belgium

***Correspondence:**
Stefan Wanke
stefan.wanke@tu-dresden.de
Stefan Dötterl
Stefan.Doetterl@sbg.ac.at

Specialty section:
This article was submitted to Chemical Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: 25 January 2021
Accepted: 26 April 2021
Published: 21 May 2021

Citation:
Rupp T, Oelschlägel B, Rabitsch K, Mahfoud H, Wenke T, Disney RHL, Neinhuis C, Wanke S and Dötterl S (2021) Flowers of Deceptive Aristolochia microstoma Are Pollinated by Phorid Flies and Emit Volatiles Known From Invertebrate Carrion. Front. Ecol. Evol. 9:658441. doi: 10.3389/fevo.2021.658441

Deceptive flowers decoy pollinators by advertising a reward, which finally is not provided. Numerous deceptive plants are pollinated by Diptera, but the attractive cues and deceptive strategies are only identified in a few cases. A typical fly-deceptive plant genus is *Aristolochia*, which evolved sophisticated trap flowers to temporarily capture pollinators. Though rarely demonstrated by experimental approaches, *Aristolochia* species are believed to chemically mimic brood sites, food sources for adult flies, or utilize sexual deception. Indeed, for most species, studies on scent composition and attractive signals are lacking. In this study, we focused on *Aristolochia microstoma*, a peculiar Greek endemic with flowers that are presented at ground level in the leaf litter or between rocks and are characterized by a unique morphology. We analyzed flower visitor and pollinator spectra and identified the floral scent composition using dynamic headspace and gas chromatography coupled to mass spectrometry (GC/MS). Female and male phorid flies (Phoridae) are the exclusive pollinators, although the flowers are also frequently visited by Sciaridae, as well as typical ground-dwelling arthropods, such as Collembola and arachnids. The carrion-like floral scent mainly consists of the oligosulphide dimethyldisulfide and the nitrogen-bearing compound 2,5-dimethylpyrazine. These compounds together are known to be released from decomposing insects, and thus, we conclude that pollinators are likely deceived by chemical imitation of invertebrate carrion, a deceptive strategy not described from another plant species so far.

Keywords: Aristolochiaceae, deceptive pollination, dimethyldisulfide, 2,5-dimethylpyrazine, floral scent, Phoridae, sapromyiophily, *Megaselia*

INTRODUCTION

Deceptive pollination evolved in 4–6% of angiosperms (Renner, 2006), and relies on the inability of pollinators to distinguish between a true resource (e.g., mating partners, brood-sites, and food) and the flower/inflorescence that imitates the reward (Brodmann et al., 2008, 2009; Urru et al., 2011). Pollinators are cheated by deceptive flowers through sophisticated olfactory, visual, and tactile

traits (Vogel, 1978; Dafni, 1984; Stensmyr et al., 2002; Schiestl et al., 2003; Schiestl, 2005; Stökl et al., 2010; Woodcock et al., 2014). In such systems, Diptera are common pollinators (Renner, 2006; Woodcock et al., 2014). Fly-deceptive pollination strategies include mimicry of brood-sites (Stensmyr et al., 2002; Urru et al., 2011; Jürgens et al., 2013), food (e.g., Heiduk et al., 2016), and mating partners (Martel et al., 2016). However, the specific signals involved in fly attraction and the deceptive strategies are identified in a few cases only (Stökl et al., 2010; Heiduk et al., 2015, 2016; Oelschlägel et al., 2015).

A prominent example of fly-pollinated deceptive plants is the genus *Aristolochia* (Aristolochiaceae). The different species are visited by a wide range of dipteran families, but often information on the actual pollinators is lacking (reviewed in Berjano et al., 2009). However, there is evidence that each *Aristolochia* species is specialized in just one or few pollinator families (e.g., Phoridae, Drosophilidae, and Chloropidae), and in some species fly attraction is sex-specific (Hime and Costa, 1985; Wolda and Sabrosky, 1986; Hall and Brown, 1993; Rulik et al., 2008; Berjano et al., 2009). *Aristolochia* species are long known for their spectacular, highly derived trap flowers (Knoll, 1929). To assure cross-pollination, the plants have evolved elaborate micro- and macromorphological features, enabling them to trap, retain, and release insects [described in detail by Oelschlägel et al. (2009)]. Pollinators enter the protogynous flower in the female phase through the tube, where downward-bending trichomes lead them to the utricle, and prevent them from escaping during the female flower phase (Oelschlägel et al., 2009). The trapped pollinators are able to deposit pollen, previously picked up from another flower, on the receptive stigmatic lobes before the flower enters the male phase. In the early male phase the pollen is released, but trapping trichomes still block the exit, before they finally shrink and allow pollinators to leave the trap, loaded with pollen (Oelschlägel et al., 2009).

Due to their often obvious and strong scents during the female phase, many authors suggested that *Aristolochia* flowers generally attract their pollinators by floral scent (Vogel, 1978; Hall and Brown, 1993; Bänziger and Disney, 2006; Trujillo and Séric, 2006; Rulik et al., 2008; Martin et al., 2017), which indeed was substantiated by behavioral assays in a few species (Cammerloher, 1923; Daumann, 1971; Oelschlägel et al., 2015). Based on the type of scent released, it is believed that the flowers generally mimic brood-sites of their respective pollinators, such as carrion, feces, decaying plants, or fungi, by chemical deception (Cammerloher, 1923; Vogel, 1978; Proctor et al., 1996; Martin et al., 2017). In some weakly odored species with strongly male- or female-biased pollinator attraction, mimicry of sex pheromones was suggested (Wolda and Sabrosky, 1986; Hall and Brown, 1993). First attempts to identify floral scent compounds in *Aristolochia* date back almost 100 years (Cammerloher, 1923, 1933), but the scent composition was only studied recently in four species by quantitative chemical analytics (Stashenko et al., 2009; Johnson and Jürgens, 2010; Oelschlägel et al., 2015; Martin et al., 2017). Among other compounds (e.g., citral), all these studies identified substances characteristic of brood-site deceptive plants (e.g., dimethyldisulfide), with one exception. Oelschlägel et al. (2015) mainly identified aliphatic hydrocarbons and esters in the Mediterranean *A. rotunda*. More detailed physiological and behavioral analyses with the pollinators of this species rejected brood-site deception and discovered a novel pollination strategy in plants, called kleptomyiophily (Oelschlägel et al., 2015). *A. rotunda* deceives its chloropid pollinators by mimicking alarm pheromones of preyed-upon mirid bugs, which are a food source of these kleptoparasitic adult flies (Oelschlägel et al., 2015).

Most of the approximately 500 *Aristolochia* species are native to tropical and subtropical regions, but about 50 species occur in the Mediterranean and adjacent Near East (Nardi, 1984, 1991; Neinhuis et al., 2005; Wanke et al., 2006). Among those, *Aristolochia microstoma* BOISS. & SPRUNER, a species endemic to Greece, stands out due to its unique perianth morphology and flower presentation (Wanke, 2006). The limb of the small, purplish-brownish flowers (**Figures 1A–C**) is reduced to a small beak or missing, and the entrance into the floral tube is reduced to a small pore, responsible for the name of the species (Nardi, 1991). While most *Aristolochia* species display their often showy flowers above the ground, *A. microstoma* flowers are presented close to or partly buried in the ground, among leaf litter (**Figures 1A,B**) or between rocks (**Figures 1C,D**), often hidden from above (Nardi, 1991; Wanke, 2006). Another unusual feature is the more or less horizontal orientation of the floral tube, which is vertical in other species. Pollinators were hypothesized to be small arthropods living near the ground or in leaf litter (Nardi, 1991; Wanke, 2006). So far, the flower visitors and pollinators, the reproductive biology, and the floral scent of *A. microstoma* remained unknown.

In this study, we recorded flower visitors and pollinators, and analyzed floral scents in three natural populations of *A. microstoma*. Specifically, we asked: (1) Are the flowers, as in congeners, also pollinated by flies, or by other ground-dwelling arthropods, and is the pollinator spectrum similar among populations? (2) Do the flowers produce scent, and if so, what is the composition, and is it similar among populations? Based on the obtained data, we discuss possible deceptive strategies of this unusual flower.

MATERIALS AND METHODS

Study Sites

Aristolochia microstoma is endemic to Central Greece and Northern Peloponnesus, where it colonizes dry, stony, calcareous places in open woodlands, garrigue, and macchia (Nardi, 1991). Samples were collected during field trips in March 2019 and 2020, around the peak of flowering, in "Egaleo" (Athens, Mt. Egaleo, 37.999377N, 23.641652E, 225 m a.s.l.), "Arachneo" (surroundings of Arachneo, near Moní Panagías Talantíou, 37.6714204N, 22.9114465E, 420 m a.s.l.), and "Methana" (Methana peninsula, south of Kypseli, 37.6002630N, 23.4050652E, 65 m a.s.l.). Voucher specimens of the plants are deposited at Herbarium Dresdense (DR).

FIGURE 1 | *Aristolochia microstoma* and its flowers in their natural habitat in Greece (Athens, Mt. Egaleo): The small flowers (length = 2 cm to 3 cm) are presented on ground-level, either well hidden in the leaf litter **(A,B)**, or between rocks **(C)**, where they are not visible from above (**D**; rock removed to see the flowers).

Flower Visitor and Pollinator Collection and Identification

A total of 1,457 flowers (1,044 female phase, 413 male phase) were randomly collected at the three study sites. As a rhizome may or may not produce several shoots (Nardi, 1991), it was difficult to identify a plant individual. Thus, we recorded the visitors at the level of populations. The flowers were opened and checked for trapped arthropods in the field or stored in 80% isopropanol for later processing in the lab. For each flower, we recorded the flowering phase (female or male), and the number of trapped arthropods with and without pollen. Applying the most conservative approach, only arthropods collected from female phase flowers that carried *Aristolochia* pollen were treated as pollinators (Rulik et al., 2008; Oelschlägel et al., 2015). The inaperturate exine is characteristic of *Aristolochia*-pollen (unpublished data), and since no other *Aristolochia* species were co-flowering at the study sites, we assumed that all *Aristolochia* pollen belonged to *A. microstoma*. Collected arthropods were conserved in 80% isopropanol and identified to the order level. Diptera were further identified to family level. The sex of visitors was determined in the two main visitor families, i.e., Phoridae and Sciaridae (see section "Results"). Morphological determinations were performed mainly with the help of Disney (1994) and Oosterbroek (2006). Voucher specimens of the collected arthropods are deposited at the Department of Biosciences, University of Salzburg.

Molecular Analyses of Pollinators

In addition to the identification based on morphological characters, pollinators were characterized by molecular data as well. DNA was extracted from all specimens carrying pollen in female stage flowers (total 25 individuals). In order to preserve the specimens as intact as possible for subsequent morphological identification only one single hind leg of each isopropanol conserved fly was used for DNA isolation.

Genomic DNA was extracted using the NucleoSpin® Tissue kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's protocol. The extracted DNA samples were stored at −20°C until use. The quality and quantity of each extracted DNA sample was assessed using Invitrogen™ Qubit 3 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, United States).

The barcoding marker COI, a 658 bp fragment of cytochrome oxidase I, was amplified using the two primer pairs COI-Dip-F5 (CWACWAAYCAYAARGATATTGG)/COI-Dip R3 (TNGTRATAAAATTWACDGCNCC) and COI-Dip-F7 (CWATTATAATTGGDGGDTTYGG)/COI-Dip-R4 (CCAAARAATCARAATARRTGTTG), respectively (newly designed for this study). The PCR reactions were performed in a total volume of 20 μL containing 5.5 μL ddH_2O, 4 μL 1 × GoTaq Flexi buffer, 0.1 μL GoTaq G2 Flexi DNA polymerase (Promega, Fitchburg, MA, United States), 3.2 μL dNTPs (1,25 mM each), 1.2 μL 25mM $MgCl_2$, 1 μL of 10mM F- and R-primer, and 4 μL of 1:10 diluted genomic DNA. Amplification was performed in Biometra

T3000 thermocycler (Analytic Jena, Jena, Germany) according to the following protocol: initial denaturation for 30 s at 98°C, 35 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 10 s and extension at 72°C for 60 s, and a final extension step at 72°C for 5 min. Quality of PCR products was assessed by gel electrophoresis employing a 1% agarose gel. 5 μL of each PCR, 1.5 μL Gelstar (Bio-RAD Laboratories Inc., Hercules, CA, United States), 2 μL 6× loading dye was run at 80V and amplicons were visualized under UV light (Biometra BioDoc, Analytic Jena, Jena, Germany).

PCR products were purified employing the NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL, Düren, Germany). The manufacturers protocol was followed. Samples were diluted in 30 μL elution buffer and directly sequenced using the Macrogen Europe sequencing service (Amsterdam, Netherlands). Sequence quality and trimming was done by eye accessing the pherograms. Forward and reverse sequences for each PCR product were aligned manually using PhyDE® – Phylogenetic Data Editor version 0.9971[1]. Thus, each COI region for each fly individual was amplified and sequenced in two broadly overlapping parts resulting in up to 4x coverage for each nucleotide. Quality controlled sequences were submitted to NCBI nucleotid blast (megablast) as well as BOLD. First 100 hits were checked for query coverage, and percentage identity. Only BLAST search results with at least 90% query coverage and >95% identity were considered.

[1]http://www.phyde.de/

Floral Scent Sampling

The volatiles emitted by single female phase flowers were collected by dynamic headspace methods (Dötterl et al., 2005) in the field during daytime (11:00–17:30) at the three study sites (Egaleo: $n = 7$; Arachneo: $n = 10$; Methana: $n = 6$). Due to their short fragile stems and hidden position, it was often necessary to cut the flowers for scent sampling. The effect of cutting was found to be minor, as scent collected *in situ* from still attached flowers ($n = 4$) yielded the same compounds in comparable ratios (see section "Results," **Table 1** and **Figure 2**). Therefore, cut and uncut flower samples were pooled for further analyses. Single flowers were inserted into oven bags (10 × 5 cm; Toppits®, Minden, Germany), and scent collection was initiated immediately after bagging. The air containing the volatiles was sucked through an adsorbent tube for 30 min at a flow rate of 200 ml min^{-1} by a membrane pump (G12/01 EB; Rietschle Thomas Inc., Puchheim, Germany). Adsorbent tubes consisted of a microvial (ChromatoProbe quartz microvials; Varian Inc., Palo Alto, CA, United States: length 15 mm, inner diameter 2 mm) filled with 3 mg of a 1:1 mixture of Tenax-TA (mesh 60–80) and Carbotrap

TABLE 1 | Median relative (%) and total absolute (ng/h) amounts of scent (compounds) emitted by single female phase *Aristolochia microstoma* flowers, collected at three natural sites in Greece: Egaleo, Arachneo, and Methana.

		Egaleo ($n = 7$)		Arachneo ($n = 10$)		Methana ($n = 6$)	
KRI	**Relative amounts of scent compounds (%)**	**Median (min – max)**					
Sulfur-bearing compounds							
746	Dimethyldisulfide	78.6	(15.9–97.2)	54.7	(10.4–76.9)	40.4	(12.5–62.8)
979	Dimethyltrisulfide	1.2	(0.0–6.6)	5.4	(1.5–14.7)	3.8	(tr–6.3)
Nitrogen-bearing compounds							
824	2-methylpyrazine	0.0	(0.0–0.6)	0.0	(0.0–0.2)	0.1	(0.0–0.6)
912	2,5-dimethylpyrazine	7.5	(0.4–76.8)	25.8	(11.0–64.8)	46.9	(36.1–76.8)
1140	2-isobutyl-3-methylpyrazine	0.0	(0.0–7.2)	0.0	(0.0–24.3)	0.0	(0.0–0.1)
C5-branched chain compounds							
731	3-methyl-1-butanol	1.4	(0.0–7.9)	0.3	(0.0–1.6)	0.1	(0.0–2.0)
876	3-methylbutyl acetate	0.0	(0.0–1.5)	0.0	(0.0–0.0)	0.0	(0.0–0.1)
Irregular terpenes							
1233	β-cyclocitral	0.0	(0.0–3.9)	0.0	(0.0–0.5)	0.2	(0.0–0.7)
Aromatic compounds							
1598	Methyl-3,4-dimethoxybenzoate	0.3	(0.0–7.9)	0.5	(0.0–1.9)	0.1	(0.0–2.5)
Unknown compounds							
702	Unknown (m/z: 45, 77, 59, 81, 43, and 44)	0.0	(0.0–2.7)	0.0	(0.0–23.6)	0.0	(0.0–6.2)
809	Unknown (m/z: 92, 45, 77, 57, 47, and 44)	tr	(0.0–0.2)	0.1	(0.1–0.3)	0.2	(tr–0.3)
1048	Unknown (m/z: 121, 108, 136, 135, 69, and 83)	0.0	(0.0–0.0)	tr	(0.0–2.2)	0.1	(0.0–0.7)
1068	Unknown (m/z: 47, 126, 63, 79, 64, and 46)	tr	(0.0–0.2)	2.3	(0.3–7.4)	0.3	(tr–3.2)
1133	Unknown (m/z: 122, 121, 135, 108, 150, and 39)	0.0	(0.0–tr)	tr	(0.0–3.8)	tr	(0.0–1.4)
1139	Unknown (m/z: 61, 43, 138, 95, 123, and 85)	0.0	(0.0–0.2)	0.1	(0.0–0.5)	0.1	(tr–1.0)
1283	Unknown (m/z: 79, 108, 93, 99, 127, and 155)	0.0	(0.0–1.4)	0,0	(0.0–0.0)	0.0	(0.0–0.0)
	Total amount of scent per flower (ng/h)	84.2	(2.9–108.9)	48.3	(10.5–139.4)	33.6	(24.1–145.3)

Compounds are sorted by compound class, and within class by the Kovats retention index (KRI). tr, compounds occurring only in trace amounts (<0.05%); m/z, mass-to-charge ratio of unknown compounds.

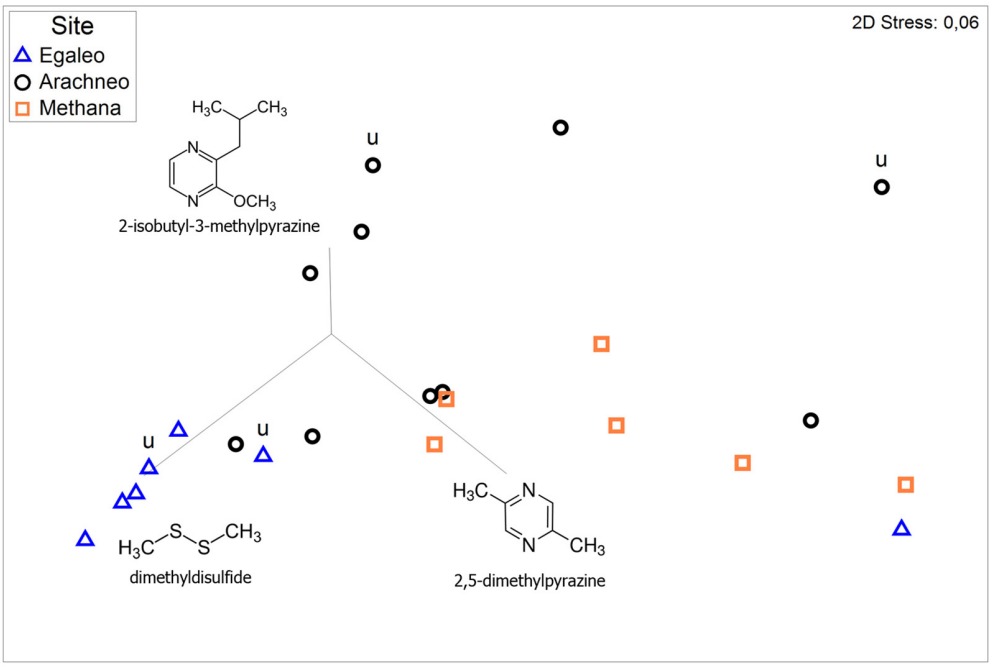

FIGURE 2 | Non-metric multidimensional scaling (NMDS) to visualize semi-quantitative dissimilarities among individual floral scent samples from three *Aristolochia microstoma* populations in Greece: Egaleo (*n* = 7); Arachneo (*n* = 10); and Methana (*n* = 6). The ordination is based on pairwise Bray-Curtis similarities. The vectors depict the volatiles most correlating with the axes. "u" marks samples collected from uncut flowers, all other samples were collected from cut flowers.

B (mesh 20–40) (both Supelco, Bellefonte, PA, United States) fixed by glass wool plugs (Heiduk et al., 2015). Subsequently to sampling, the flowers were dissected to determine their sexual phase. To unambiguously identify compounds as floral volatiles, control samples of leaves, as well as ambient air, were sampled in a similar way. Samples were stored at 4°C during fieldwork and at –20°C in the laboratory before GC/MS analyses.

Gas Chromatography Coupled to Mass Spectrometry (GC/MS)

The adsorbent tubes containing the trapped volatiles were analyzed by GC/MS on an automatic thermal desorption (TD) system (TD-20, Shimadzu, Japan) coupled to a Shimadzu GC/MS-QP2010 Ultra equipped with a ZB-5 fused silica column (5% phenyl polysiloxane; length = 60 m, inner diameter = 0.25 mm, film thickness = 0.25 µm, Phenomenex), as described by Heiduk et al. (2015). At a consistent helium carrier gas flow of 1.5 ml/min, the samples were processed at a split ratio of 1:1. The GC oven temperature started at 40°C, then increased by 6°C/min to 250°C and was held for 1 min. The MS interface worked at 250°C. Mass spectra were taken at 70 eV (EI mode) from m/z 30 to 350. GC/MS data were analyzed using the GCMSolution package, Version 4.41 (Shimadzu Corporation 1999-2015). Compounds were tentatively identified by comparison of Kovats retention indices (KRI, based on a series of n-alkanes) and mass spectra to data available in the databases ADAMS, ESSENTIALOILS-23P, FFNSC 2, and W9N11. All compound identities were confirmed by authentic reference standards available at the Plant Ecology lab of the University of Salzburg. Compounds also detected in leaf and ambient air controls were excluded from the analyses. Total scent emission was estimated by injecting known amounts of alkane standards.

Statistical Analyses

We used chi-square tests to compare sex-ratios in Phoridae and Sciaridae among populations. Similarities and dissimilarities in scent bouquets among the samples were visualized by non-metric multidimensional scaling (NMDS), based on pairwise Bray-Curtis dissimilarities calculated on the relative amounts of compounds. We performed analyses of similarities (ANOSIM; 10,000 permutations) to test for differences in floral scent among study sites, and PERMDISP (Anderson et al., 2008) to test for differences in dispersion among populations (10,000 permutations). All multivariate statistical analyses were performed with the software PRIMER 6.1.0.5 (Clarke and Gorley, 2006).

RESULTS

Flower Visitors and Pollinators

The 248 flower visitors recorded in this study (**Table 2**) originated from 11 to 17% of investigated female-phase and 13–20% of male-phase flowers, respectively, depending on the study site. The majority of flowers had no visitors. Females and males of the dipteran families Phoridae (99 individuals in total) and

Sciaridae (52) were the most abundant flower visitors at all study sites (**Table 2** and **Figure 3**). In flower-visiting Phoridae, sex ratios differed between sites (chi-square$_2$ = 10.3; P = 0.006). They were female-biased at Arachneo and Methana and balanced at Egaleo. In contrast, Sciaridae were male-biased at all sites (chi-square$_2$ = 2.65; P = 0.265). Further visitors included other Diptera (Sphaeroceridae, Drosophilidae), and a range of other arthropods, such as Collembola, Acari, Myriapoda, Isopoda, and Coleoptera. The only visitors that carried pollen in female- and/or male-phase flowers were Diptera. In all flies, pollen was attached dorsally to the thorax, mainly around the wing-base, only occasionally on the front and central parts (see **Figure 3B**). Only Phoridae (25 individuals) carried pollen in female-phase flowers (**Figures 3A,B**), and thus classified as the exclusive pollinators at all three study sites (**Table 2**). At the sites Egaleo and Arachneo, Phoridae of both sexes were recorded as pollinators, at Methana only females. Additional pollen-carrying Phoridae of both sexes (10 females and 4 males in total) were found in male-phase flowers at each site. Morphological identification showed that all but one pollinator belonged to the genus *Megaselia*, mostly the *Megaselia angusta/longicostalis* complex, and *Megaselia scalaris* (Loew, 1866). The BLAST hits on NCBI and BOLD confirmed the presence of species of the *Megaselia angusta/longicostalis* complex [three individuals, each up to >99.7% identity with GenBank accessions of *Megaselia longicostalis* (Wood, 1912)], of *M. scalaris* (one individual; 99.7% identity to GenBank accession HM399356), and also suggests the presence of *Conicera similis* (Haliday, 1833) (one individual) as pollinator (95 to 97% identity with GenBank accessions). COI sequences of the pollinators are provided as a **Supplementary Data Sheet 1**.

Although frequent visitors, Sciaridae never carried pollen in female phase flowers (**Figures 3C,D**), however, pollen grains were found on three individuals collected from male phase flowers at Egaleo.

TABLE 2 | Flower visitors of *Aristolochia microstoma* collected at the three study sites in Greece (Egaleo, Arachneo, and Methana), shown overall (sum) and per site.

	sum	Egaleo	Arachneo	Methana
# female phase flowers	1,044 [13%]	366 [13%]	251 [17%]	427 [11%]
# male phase flowers	413 [15%]	189 [13%]	71 [20%]	153 [15%]
Taxa				
Arachnida				
Acari	16	8	4	4
Araneae	1	0	1	0
Pseudoscorpiones	4	0	1	3
Crustacea				
Isopoda	6	6	0	0
Insecta				
Coleoptera	5	2	3	0
Collembola	26	7	12	7
Diptera				
Drosophilidae	1	0	0	1
Phoridae ♀	66 (19/10)	19 (2/6)	22 (5/2)	25 (12/2)
Phoridae ♂	30 (6/4)	19 (5/2)	5 (1/1)	6 (–/1)
Phoridae unknown sex	3	1	1	1
Sciaridae ♀	11 (–/1)	8 (–/1)	0	3
Sciaridae ♂	41 (–/2)	21 (–/2)	7	13
Sphaeroceridae	15	1	5	9
Hemiptera	3	0	1	2
Hymenoptera	4	3	1	0
Thysanoptera	1	1	0	0
Unidentified larvae	7	3	4	0
Myriapoda	8	1	7	0

Arthropod individuals carrying pollen are given in brackets (in female/male phase flowers), as a subset of visiting individuals. Sexes were determined only in Phoridae and Sciaridae. The total numbers of flowers sampled per site are given, with the percentage of flowers containing arthropods in square brackets. ♀ mean females, ♂ means males.

Floral Scent

Aristolochia microstoma flowers emitted an unpleasant, carrion-like scent, which was typically well noticeable by the human nose from a few centimeters distance to the flowers. The total scent emission per flower varied considerably among flowers (2.9–145.3 ng/h), with a median between 34 and 84 ng/h, depending on the study site. A total of 16 compounds was found (**Table 1**), including nitrogen-bearing (3 compounds), sulfur-bearing (2) and C5-branched chain compounds (2), one aromatic compound, one irregular terpene, and seven unknown substances. The main compounds were dimethyldisulfide, with a median relative amount between 40 and 79%, and 2,5-dimethylpyrazine (8–47%), followed by dimethyltrisulfide (1–5%). Those three compounds were present in all samples, except dimethyltrisulfide, which was not detected in one sample. All further compounds, such as 3-methyl-1-butanol and methyl-3,4-dimethoxybenzoate, were minor. Interestingly, the nitrogen-bearing compound 2-isobutyl-3-methylpyrazine, as well as an unknown compound, were particularly strong in some flowers (both up to 24%), although absent in the majority of samples. The relative amount of compounds differed among sites (**Figure 2**) (ANOSIM: R = 0.826; P = 0.004) and cannot be explained by differences in dispersion among populations (PERMDISP: $F_{2,20}$ = 0.443; P = 0.830). While there were no significant differences between the sites Arachneo and Methana, the site Egaleo differed from both other sites (ANOSIM: R > 0.265; P < 0.019), due to a higher relative amount of dimethyldisulfide (see **Figure 2**).

DISCUSSION

Flower Visitors and Pollinators

Aristolochia microstoma was mainly visited by the dipteran families Phoridae and Sciaridae, and less frequently by Sphaeroceridae and Drosophilidae. Further flower visitors included a range of other arthropods, most frequently members of Collembola, Acari, Myriapoda, Isopoda, and Coleoptera. Among flower visitors, Phoridae were the exclusive pollinators at all study sites. The carrion-like floral scent comprised 16 compounds, and was dominated by the oligosulphides dimethyldisulfide and dimethyltrisulfide, and the nitrogen-bearing compound 2,5-dimethylpyrazine. Absolute and

FIGURE 3 | Two specimens of the two most frequent Diptera families visiting flowers of *Aristolochia microstoma*: **(A)** a pollinating female *Megaselia* sp. (Phoridae) carrying pollen on its thorax **(B)**; and **(C)** a male of an unidentified species of Sciaridae not carrying pollen **(D)**.

relative amounts of the main compounds were variable, and flowers from the site Egaleo differed in scent patterns from Arachneo and Methana.

Our findings show that *A. microstoma* is not pollinated by non-dipteran ground- or litter-dwelling arthropods, as Wanke (2006) hypothesized, but by flies, as all other *Aristolochia* species studied so far (Berjano et al., 2009). To which extent the pollinating phorid flies are ground-associated could not be determined. Of the 25 pollinating phorid flies, 24 belong to the megadiverse genus *Megaselia*, and the remaining individual to the genus *Conicera (C. similis)*, but determination to species level remained difficult. While several COI sequences of the pollinating *Megaselia* specimens showed high accordances to GenBank accessions of the *M. angusta/longicostalis* complex, as well as *M. scalaris*, others did not match any identified accessions. Unfortunately, most individuals of *Megaselia* in BOLD and Genbank are identified to genus level only (if at all). This is due to the difficult identification and the large number of species in the genus *Megaselia*, with the majority of species still undescribed or known from one sex only (Disney, 1994). Therefore, the species mentioned here have to be viewed as provisional, and demand further investigations (ongoing research).

Phoridae are well-documented pollinators and flower visitors in *Aristolochia*. Numerous species in this genus are preferentially or exclusively pollinated by members of this family, including tropical and Mediterranean species, some of them with male or female sex bias (Hime and Costa, 1985; Hall and Brown, 1993; Bänziger and Disney, 2006; Rulik et al., 2008; Berjano et al., 2009; Hipólito et al., 2012; Martin et al., 2017). In our study, the observed sex-ratio in Phoridae could be the result of differing abundances of sexes in the respective fly populations during the collection period. However, a balanced sex-ratio in flower-visiting Phoridae was only found at the site Egaleo, where the floral scent bouquet differed significantly from the other two sites, in which the flower visitors of this family were female-biased. Therefore, it might be possible that these differences in scent lead to sex-biased attractiveness in phorid visitors. In contrast, the flower-visiting Sciaridae were male-biased at all sites, suggesting that the observed differences in floral volatiles did not affect sex-specific attractiveness in this family. Although Sciaridae, and to a lesser extent Sphaeroceridae, were frequently found in the flowers of *A. microstoma*, they were not classified as pollinators. The occurrence of significant numbers of non-pollinating Diptera families is not unusual in *Aristolochia*, since several species attract and trap different Diptera, with only a subset of taxa actually pollinating them (e.g., Cammerloher, 1933; Brantjes, 1980; Hilje, 1984; Burgess et al., 2004; Berjano et al., 2009). The spectrum of flower visitors of *A. microstoma* is remarkably similar to that of *A. pallida* (Rulik et al., 2008), another Mediteranean species. Apart from the pollinating male Phoridae (*Megaselia longicostalis*, *M. pumila*, *M. superciliata*), flowers of *A. pallida* are visited – but not pollinated – predominantly by

Sciaridae of both sexes, and occasionally by other visitors also found in *A. microstoma* flowers, including Sphaeroceridae, Acari, Coleoptera, and Collembola (Rulik et al., 2008; Disney and Rulik, 2012). Preliminary morphometric measures of the narrowest part of the floral tube, and the distance between gynostemium and utricle wall of *A. microstoma* suggest that they are similar to those of *A. pallida* (mean = 1.37 mm and 1.68 mm, respectively; see Rulik et al., 2008). These two major morphological floral filters in *Aristolochia* assure that only visitors sharing a specific body size range – small enough to enter the flower, but big enough to physically interact with the gynostemium – can act as pollinators (Brantjes, 1980; Rulik et al., 2008).

In addition to body size, differences in thoracic bristles could contribute to pollinator specialization, as suggested by Cammerloher (1933). As in other *Aristolochia* species (Bänziger and Disney, 2006; Rulik et al., 2008; Oelschlägel et al., 2015), *A. microstoma* pollen was generally deposited dorsally on the thorax. The majority of the pollen was concentrated around the wing base, where the pollinating Phoridae are covered by pronounced, stiff bristles (**Figure 3B**) that probably facilitate pollen adherence. On the front and central parts of the thorax, where bristles are usually very short, pollen grains were hardly found. The lack of such bristles (**Figure 3D**) might exclude Sciaridae as pollinators of *A. microstoma*, or at least make them less efficient, as three pollen-carrying specimens collected from male flowers indicate. The less frequent dipteran flower visitors of the families Sphaeroceridae and Drosophilidae, which possess thoracic bristles similar to those of Phoridae, were never found with attached pollen. Whether this was due to their low abundance in our samples, or other reasons, i.e., different body size or non-recurrent visitation of flowers, remains unanswered. Anyhow, the importance of thoracic bristles for pollination of *Aristolochia* should be experimentally tested in the future. Non-dipteran arthropods were most likely accidental flower visitors, as reported in other *Aristolochia* species (Cammerloher, 1923, 1933; Trujillo and Séric, 2006; Rulik et al., 2008). Generally, the number of flowers containing visitors was strikingly low across all sites, which could be the result of low pollinator availability, of a low attractiveness of the floral signals, or of a small proportion of attracted animals that entered the flowers through the small pore. This pore might have evolved as a morphological filter, i.e., to limit the number of ground-dwelling animals not appropriate as pollinators, that accidentally fall or crawl into the flower, potentially blocking the flower's reproductive organs.

Floral Scent and Possible Deceptive Strategies

The floral scent of *A. microstoma* was strongly dominated by oligosulphides, which are widespread among plants pollinated by carrion-flies and bats, and alkylpyrazines, which are rare floral volatiles (Knudsen et al., 2006). Especially the high amounts of oligosulphides (dimethyldisulfide and dimethyltrisulfide), suggest a sapromyiophilous pollination strategy, as those compounds are the two most common and characteristic volatiles in carrion and carnivorous dung-mimicking flowers, across several plant families (Jürgens et al., 2006, 2013). In contrast, the second main compound of *A. microstoma*, the alkylpyrazine 2,5-dimethylpyrazine, was rarely found in saprophilous flowers. In lower relative amounts than in the present study, it is emitted by the sapromyiophilous South African stapeliads *Orbea variegata* (11%) and *Stapelia leendertzia* (1%), which also emit high amounts of dimethyldisulfide and dimethyltrisulfide, among other compounds, most prominently indole (Johnson and Jürgens, 2010; Jürgens et al., 2013). Both stapeliads, however, were observed to be visited by flies of the families Calliphoridae and Sarcophagidae, and not by Phoridae (Meve and Liede, 1994; Johnson and Jürgens, 2010). Other pyrazines (3-isopentyl 2,5-dimethylpyrazine and 2,6-dimethyl-3-(2-methyl-1-butyl)-pyrazine) are the main compounds in another stapeliad, *Echidnopsis montana*, the biological function of which remains unclear (Jürgens et al., 2006). Sapromyiophily was proposed for several *Aristolochia* species (Cammerloher, 1923, 1933; Vogel, 1978; Johnson and Jürgens, 2010), but chemical analyses of floral scent remain scarce, limiting comparisons within the genus. Nevertheless, *A. microstoma* shares dimethyldisulfide and dimethyltrisulfide with the sapromyiophilous *A. cymbifera*, which, in cultivation, attracts carrion flies (Johnson and Jürgens, 2010). However, the scent of this species is overall dominated by benzenoids. Dimethyldisulfide is also found in smaller amounts in the neotropical phorid-pollinated *A. gigantea*, the odor of which is dominated by sweet lemon-scented citronella-like compounds (Martin et al., 2017). Compared to those and other *Aristolochia* species, which comprise between 63 and 168 floral scent compounds (Johnson and Jürgens, 2010; Oelschlägel et al., 2015; Martin et al., 2017), the odor of *A. microstoma* with only 16 compounds is strikingly less complex. Although the main floral scent compounds of *A. microstoma* were present throughout all samples, their absolute amounts were variable among individuals, and their relative amounts at the sites Arachnea and Methana differed from the site Egaleo. Such intraspecific variation in floral scent is a widespread phenomenon in both deceptive and rewarding plant species, and can be caused by multiple factors, such as local adaptation and genetic drift (Delle-Vedove et al., 2017). In dichogamous plants or plants with unisexual flowers, floral scents sometimes vary between the sexual phases/flower sexes. Preliminary data of *A. microstoma*, however, suggest that the scent of male-phase flowers is similar in both total amount and composition to that of female-phase flowers (**Supplementary Table 1**), and thus might also attract insects, likely to increase pollen export. In *A. gigantea*, the only *Aristolochia* species with such data available, the scent emission is strongly reduced in the male compared to the female phase, with strong differences in composition between the sexual phases (Martin et al., 2017).

Dimethyldisulfide and dimethyltrisulfide are common volatiles in degrading meat (carcasses and carnivore/omnivore feces), that, however, do not emit 2,5-dimethylpyrazine (Jürgens et al., 2006, 2013). Instead, 2,5-dimethylpyrazine was found in the scent of dead bark beetles (*Ips typographus*), alongside dimethyldisulfide, 3-methyl-1-butanol, and other compounds (Zhang et al., 2003). Future studies have to show whether those compounds are also released from other invertebrate

carrion, e.g., other arthropods, and why 2,5-dimethylpyrazine is obviously not released by decomposing vertebrate carrion (Stutz et al., 1991; Johnson and Jürgens, 2010; Jürgens et al., 2013).

Various pyrazines are important volatiles in animal pheromones, such as urinal pheromones in mammals like mice, voles, and hamsters (Novotny et al., 1986; Boyer et al., 1989; Soini et al., 2005), and sex pheromones of fruit flies (Robacker et al., 2009) and thynnine wasps, the latter being exploited by the sexually deceptive orchid *Drakaea glyptodon* (Bohman et al., 2014). Best known, however, is the role of alkylpyrazines as key volatiles in alarm- and trail pheromones in several genera of ants, including 2,5-dimethylpyrazine (Attygalle and Morgan, 1984; Jackson et al., 1990; Morgan et al., 1992; Hölldobler et al., 2001). Pyrazines are key volatiles in host-localization in specialized myrmecophilous Phoridae (*Pseudacteon* spp.), so called ant-decapitating flies (Sharma et al., 2011; Sharma and Fadamiro, 2013; Ngumbi and Fadamiro, 2014). However, to the best of our knowledge, dimethyldisulfide and dimethyltrisulfide were never reported in context with ant pheromones, and no typical ant-associated (myrmecophilous) phorid genera were found among the pollinators of *A. microstoma*. Although there are also cases of myrmecophilous behavior found in *Megaselia* (Disney, 1994), often described as "one of the largest, most biologically diverse and taxonomically difficult genera in the entire animal kingdom" (Marshall, 2012), the pollinators recorded in the present study are probably unspecifically saprophagous. Larvae and adults of *Conicera similis* and members of the *M. angusta/longicostalis* complex (i.e., *M. longicostalis*) are known to feed on vertebrate (e.g., rabbit) and invertebrate (snail) carrion, decomposing plants, but also fungi (Disney, 1994, 1999; Buck, 1997, 2001). The cosmopolitan *M. scalaris* even utilizes the broadest spectrum of larval substrates known in all insects, including numerous dead and living animals, fungi and plants (reviewed in Disney, 2008). Larvae of Sciaridae, which were frequent flower visitors but not pollinators in *A. microstoma*, are usually feeding on living or decomposing plants and fungi, as well as on herbivore excrements, and are frequently found among detritus and forest litter (Menzel and Mohrig, 2000).

CONCLUSION AND OUTLOOK

The spatial position of *A. microstoma* flowers suggests that the pollinating Phoridae probably search for breeding sites or food close to the ground, in leaf litter, or between rocks. This hidden presentation of the flowers also points toward scent as the attractive cue to lure the pollinators. Our data on pollinators and floral scent indicate that *A. microstoma* deceives its phorid pollinators by employing a sapromyiophilous strategy, as proposed for other *Aristolochia* species. The co-occurrence of high amounts of oligosulphides and 2,5-dimethylpyrazine is novel among plants and suggests a so far undescribed type of sapromyiophilous mimicry. Due to the high similarity to carrion scents of dead beetles, and the absence of either 2,5-dimethylpyrazine or dimethyldisulfide in vertebrate carcasses and carnivorous feces, or ant pheromones, we hypothesize that brood-site mimicry of invertebrate carrion is the most likely deceptive strategy. Studies testing the attractiveness of the scent compounds of *A. microstoma* flowers and different potential substrates to the pollinators are currently carried out to test this hypothesis.

DATA AVAILABILITY STATEMENT

All data used for the study are presented in the manuscript.

AUTHOR CONTRIBUTIONS

BO, CN, SW, and SD designed and planned the study. TR, SW, and BO conducted the field work and collected the samples. TR, KR, BO, and HM processed the flower visitors. KR and TR identified the arthropods to order/family level. HM, BO, TW, and SW performed the molecular lab work and characterization of phorid flies. RD morphologically identified phorids flies. TR performed and SD supported the chemical and statistical analyses. TR drafted the manuscript. All authors contributed to the final manuscript and approved the submitted version.

FUNDING

This research has been funded by the Austrian Science Fund (FWF, I 3722-B29) and the German Research Foundation (DFG, WA 2461/9-1). RD's studies of Phoridae are currently funded by the Balfour-Browne Trust (University of Cambridge).

ACKNOWLEDGMENTS

The collection permit was issued by the Greek General Directorate for Forest and Agriculture (66435/811). We are grateful to Ioanna Oikonomou (Athens) for assistance in field work and for her hospitality, Bernardo Cañiza (TU Dresden) for help in searching for flower visitors, Roman Fuchs (University of Salzburg) for lab support, and Enio Nardi (Florence) for valuable information on finding suitable *A. microstoma* sites. We thank Herbert Braunschmid, Eva Gfrerer, Karin Gross, and Danae Laina (all University of Salzburg) for valuable comments on an earlier version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.658441/full#supplementary-material

Supplementary Table 1 | It contains the floral scent dataset.

Supplementary Data Sheet 1 | It contains the COI sequence data of the pollinators.

REFERENCES

Anderson, M. J., Gorley, R. N., and Clarke, K. R. (2008). *PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods*. Plymouth: PRIMER-E.

Attygalle, A. B., and Morgan, E. D. (1984). Identification of trail pheromone of the ant *Tetramorium caespitum* L. (Hymenoptera: Myrmicinae). *J. Chem. Ecol.* 10, 1453–1468. doi: 10.1007/BF00990315

Bänziger, H., and Disney, R. H. L. (2006). Scuttle flies (Diptera: Phoridae) imprisoned by *Aristolochia baenzigeri* (Aristolochiaceae) in Thailand. *Mitt. Schweiz. Entomol. Gesellschaft* 79, 29–61.

Berjano, R., Ortiz, P. L., Arista, M., and Talavera, S. (2009). Pollinators, flowering phenology and floral longevity in two Mediterranean *Aristolochia* species, with a review of flower visitor records for the genus. *Plant Biol.* 11, 6–16. doi: 10.1111/j.1438-8677.2008.00131.x

Bohman, B., Phillips, R. D., Menz, M. H. M., Berntsson, B. W., Flematti, G. R., Barrow, R. A., et al. (2014). Discovery of pyrazines as pollinator sex pheromones and orchid semiochemicals: implications for the evolution of sexual deception. *New Phytol.* 203, 939–952. doi: 10.1111/nph.12800

Boyer, M. L., Jemiolo, B., Andreolini, F., Wiesler, D., and Novotny, M. V. (1989). Urinary volatile profiles of pine vole, *Microtus pinetorum*, and their endocrine dependency. *J. Chem. Ecol.* 15, 649–662. doi: 10.1007/BF01014708

Brantjes, N. B. M. (1980). Flower morphology of *Aristolochia* species and the consequences for pollination. *Acta Bot. Neerl.* 29, 212–213. doi: 10.1111/j.1438-8677.1986.tb00491.x

Brodmann, J., Twele, R., Francke, W., Hölzler, G., Zhang, Q.-H., and Ayasse, M. (2008). Orchids mimic Green-Leaf volatiles to attract prey-hunting wasps for pollination. *Curr. Biol.* 18, 740–744. doi: 10.1016/j.cub.2008.04.040

Brodmann, J., Twele, R., Francke, W., Yi-Bo, L., Xi-Qiang, S., and Ayasse, M. (2009). Orchid mimics honey bee alarm pheromone in order to attract hornets for pollination. *Curr. Biol.* 19, 1368–1372. doi: 10.1016/j.cub.2009.06.067

Buck, M. (1997). *Untersuchungen zur Ökologischen Einnischung Saprophager Dipteren unter Besonderer Berücksichtigung der Phoridae und Sphaeroceridae (Brachycera/ Cyclorrhapha)*. Ph.D. dissertation. Ulm: Universität Ulm.

Buck, M. (2001). Identification of females of European *Conicera* Meigen, with a discussion of certain features of the reproductive anatomy of female Phoridae (Diptera). *Dtsch. Entomol. Z.* 48, 69–81.

Burgess, K. S., Singfield, J., Melendez, V., and Kevan, P. G. (2004). Pollination biology of *Aristolochia grandiflora* (Aristolochiaceae) in Veracruz, Mexico. *Ann. Mo. Bot. Gard.* 91, 346–356.

Cammerloher, H. (1923). Zur Biologie der Blüte von *Aristolochia grandiflora* Swartz. *Österr. Bot. Ztg.* 72, 180–198. doi: 10.1007/BF01660049

Cammerloher, H. (1933). Die Bestäubungseinrichtungen der Blüten von *Aristolochia lindneri* Berger. *Planta* 19, 351–365. doi: 10.1007/BF01920951

Clarke, K. R., and Gorley, R. N. (2006). *PRIMER v6: User Manual/Tutorial*. Plymouth: PRIMER-E.

Dafni, A. (1984). Mimicry and deception in pollination. *Annu. Rev. Ecol. Syst.* 15, 259–278.

Daumann, E. (1971). Zur Bestäubungsbiologie von *Aristolochia clematitis* L. *Preslia* 43, 105–111.

Delle-Vedove, R., Schatz, B., and Dufay, M. (2017). Understanding intraspecific variation of floral scent in light of evolutionary ecology. *Ann. Bot.* 120, 1–20. doi: 10.1093/aob/mcx055

Disney, R. H. L. (1994). *Scuttle Flies: The Phoridae*. London: Chapman & Hall. doi: 10.1007/978-94-011-1288-8

Disney, R. H. L. (1999). A troublesome sibling species complex of scuttle flies (Diptera: Phoridae) revisited. *J. Nat. Hist.* 33, 1159–1216. doi: 10.1080/002229399299987

Disney, R. H. L. (2008). Natural history of the scuttle fly, *Megaselia scalaris*. *Annu. Rev. Entomol.* 53, 39–60. doi: 10.1146/annurev.ento.53.103106.093415

Disney, R. H. L., and Rulik, B. (2012). Scuttle flies (Diptera: Phoridae) trapped by flowers of *Aristolochia* L. (Aristolochiaceae) in Italy. *Entomol. Mon. Mag.* 148, 234–236.

Dötterl, S., Wolfe, L. M., and Jürgens, A. (2005). Qualitative and quantitative analyses of flower scent in *Silene latifolia*. *Phytochemistry* 66, 203–213. doi: 10.1016/j.phytochem.2004.12.002

Hall, D. W., and Brown, B. V. (1993). Pollination of *Aristolochia littoralis* (Aristolochiales: Aristolochiaceae) by males of *Megaselia* spp. (Diptera: Phoridae). *Ann. Entomol. Soc. Am.* 86, 609–613.

Heiduk, A., Brake, I., von Tschirnhaus, M., Göhl, M., Jürgens, A., Johnson, S. D., et al. (2016). *Ceropegia sandersonii* mimics attacked honeybees to attract kleptoparasitic flies for pollination. *Curr. Biol.* 26, 2787–2793. doi: 10.1016/j.cub.2016.07.085

Heiduk, A., Kong, H., Brake, I., Von Tschirnhaus, M., Tolasch, T., Tröger, A. G., et al. (2015). Deceptive *Ceropegia dolichophylla* fools its kleptoparasitic fly pollinators with exceptional floral scent. *Front. Ecol. Evol.* 3:66. doi: 10.3389/fevo.2015.00066

Hilje, L. (1984). Fenología y ecología floral de *Aristolochia grandiflora* Swartz (Aristolochiaceae) en Costa Rica. *Brenesia* 22, 1–44.

Hime, N. C, and Costa, E. L (1985). Sobre *Megaselia* (M.) aristolochiae n. sp. (Diptera, Phoridae) cujas larvas se criam nas flores de *Aristolochia labiata* willd. (Aristolochiaceae). *Rev. Bras. Biol.* 45, 621–625.

Hipólito, J., Viana, B. F., Selbach-Schnadelbach, A., Galetto, L., and Kevan, P. G. (2012). Pollination biology and genetic variability of a giant perfumed flower (*Aristolochia gigantea* Mart. and Zucc., Aristolochiaceae) visited mainly by small Diptera. *Botany* 90, 815–829. doi: 10.1139/B2012-047

Hölldobler, B., Morgan, E. D., Oldham, N. J., and Liebig, J. (2001). Recruitment pheromone in the harvester ant genus *Pogonomyrmex*. *J. Insect Physiol.* 47, 369–374. doi: 10.1016/S0022-1910(00)00143-8

Jackson, B. D., Keegans, S. J., Morgan, E. D., Cammaerts, M. C., and Cammaerts, R. (1990). Trail pheromone of the ant *Tetramorium meridionale*. *Naturwissenschaften* 77, 294–296. doi: 10.1007/BF01131231

Johnson, S. D., and Jürgens, A. (2010). Convergent evolution of carrion and faecal scent mimicry in fly-pollinated angiosperm flowers and a stinkhorn fungus. *S. Afr. J. Bot.* 76, 796–807. doi: 10.1016/j.sajb.2010.07.012

Jürgens, A., Dötterl, S., and Meve, U. (2006). The chemical nature of fetid floral odours in stapeliads (Apocynaceae-Asclepiadoideae-Ceropegieae). *New Phytol.* 172, 452–468. doi: 10.1111/j.1469-8137.2006.01845.x

Jürgens, A., Wee, S.-L., Shuttleworth, A., and Johnson, S. D. (2013). Chemical mimicry of insect oviposition sites: a global analysis of convergence in angiosperms. *Ecol. Lett.* 16, 1157–1167. doi: 10.1111/ele.12152

Knoll, F. (1929). Die Gleitfalle als Blumentypus. *Verh. Zool. Ges. Wien.* 79, 9–12.

Knudsen, J. T., Eriksson, R., Gershenzon, J., and Ståhl, B. (2006). Diversity and distribution of floral scent. *Bot. Rev.* 72, 1–120.

Marshall, S. A. (2012). *Flies: The Natural History and Diversity of Diptera*. Richmond Hill, Ont: Firefly Books.

Martel, C., Cairampoma, L., Stauffer, F. W., and Ayasse, M. (2016). *Telipogon peruvianus* (Orchidaceae) flowers elicit pre-mating behaviour in *Eudejeania* (Tachinidae) males for pollination. *PLoS One* 11:e0165896. doi: 10.1371/journal.pone.0165896

Martin, K. R., Moré, M., Hipólito, J., Charlemagne, S., Schlumpberger, B. O., and Raguso, R. A. (2017). Spatial and temporal variation in volatile composition suggests olfactory division of labor within the trap flowers of *Aristolochia gigantea*. *Flora Morphol. Distrib. Funct. Ecol. Plants* 232, 153–168. doi: 10.1016/j.flora.2016.09.005

Menzel, F., and Mohrig, W. (2000). Revision der paläarktischen Trauermücken (Diptera: Sciaridae). *Stud. Dipterologica Suppl.* 6, 1–761.

Meve, U., and Liede, S. (1994). Floral biology and pollination in stapeliads – new results and a literature review. *Plant Syst. Evol.* 192, 99–116. doi: 10.1007/BF00985911

Morgan, E. D., Hölldobler, B., Vaisar, T., and Jackson, B. D. (1992). Contents of poison apparatus and their relation to trail-following in the ant *Daceton armigerum*. *J. Chem. Ecol.* 18, 2161–2168.

Nardi, E. (1984). The genus "*Aristolochia*" L. (Aristolochiaceae) in Italy. *Webbia* 38, 221–300.

Nardi, E. (1991). The genus *Aristolochia* (Aristolochiaceae) in Greece. *Webbia* 45, 31–69.

Neinhuis, C., Wanke, S., Hilu, K. W., Müller, K., and Borsch, T. (2005). Phylogeny of Aristolochiaceae based on parsimony, likelihood, and Bayesian analyses of trnL-trnF sequences. *Plant Syst. Evol.* 250, 7–26. doi: 10.1007/s00606-004-0217-0

Ngumbi, E., and Fadamiro, H. Y. (2014). Comparative responses of four *Pseudacteon* phorid fly species to host fire ant alarm pheromone and analogs. *Chemoecology* 25, 85–92. doi: 10.1007/s00049-014-0178-x

Novotny, M., Jemiolo, B., Harvey, S., Wiesler, D., and Marchlewska-Koj, A. (1986). Adrenal-mediated endogenous metabolites inhibit puberty in female mice. *Science* 231, 722–725. doi: 10.1126/science.3945805

Oelschlägel, B., Gorb, S., Wanke, S., and Neinhuis, C. (2009). Structure and biomechanics of trapping flower trichomes and their role in the pollination biology of *Aristolochia* plants (Aristolochiaceae). *New Phytol.* 184, 988–1002.

Oelschlägel, B., Nuss, M., von Tschirnhaus, M., Pätzold, C., Neinhuis, C., Dötterl, S., et al. (2015). The betrayed thief – the extraordinary strategy of *Aristolochia rotunda* to deceive its pollinators. *New Phytol.* 206, 324–351. doi: 10.1111/nph.13210

Oosterbroek, P. (2006). *The European Families of the Diptera*. Utrecht: KNNV Publishing. doi: 10.1163/9789004278066

Proctor, M., Yeo, P., and Lack, A. (1996). *The Natural History of Pollination*. London: Harper Collins Publishers.

Renner, S. S. (2006). "Rewardless flowers in the angiosperms and the role of insect cognition in their evolution," in *Plant-Pollinator Interactions: From Specialization to Generalization*, eds N. M. Waser, and J. Olerton (Chicago, IL: University of Chicago Press), 123–144.

Robacker, D. C., Aluja, M., Bartelt, R. J., and Patt, J. (2009). Identification of chemicals emitted by calling males of the Sapote fruit fly, *Anastrepha serpentina*. *J. Chem. Ecol.* 35, 601–609. doi: 10.1007/s10886-009-9631-7

Rulik, B., Wanke, S., Nuss, M., and Neinhuis, C. (2008). Pollination of *Aristolochia pallida* willd. (Aristolochiaceae) in the Mediterranean. *Flora* 203, 175–184. doi: 10.1016/j.flora.2007.02.006

Schiestl, F. P. (2005). On the success of a swindle: pollination by deception in orchids. *Naturwissenschaften* 92, 255–264. doi: 10.1007/s00114-005-0636-y

Schiestl, F. P., Peakall, R., Mant, J. G., Ibarra, F., Schulz, C., Franke, S., et al. (2003). The chemistry of sexual deception in an orchid-wasp pollination system. *Science* 302, 437–438. doi: 10.1126/science.1087835

Sharma, K. R., and Fadamiro, H. Y. (2013). Fire ant alarm pheromone and venom alkaloids act in concert to attract parasitic phorid flies, *Pseudacteon* spp. *J. Insect Physiol.* 59, 1119–1124. doi: 10.1016/j.jinsphys.2013.08.010

Sharma, K. R., Vander Meer, R. K., and Fadamiro, H. Y. (2011). Phorid fly, *Pseudacteon tricuspis*, response to alkylpyrazine analogs of a fire ant, *Solenopsis invicta*, alarm pheromone. *J. Insect Physiol.* 57, 939–944. doi: 10.1016/j.jinsphys.2011.04.007

Soini, H. A., Wiesler, D., Apfelbach, R., König, P., Vasilieva, N. Y., and Novotny, M. V. (2005). Comparative investigation of the volatile urinary profiles in different *Phodopus hamster* species. *J. Chem. Ecol.* 31, 1125–1143. doi: 10.1007/s10886-005-4252-2

Stashenko, E. E., Ordóñez, S. A., Marín, N. A., and Martínez, J. R. (2009). Determination of the volatile and semi-volatile secondary metabolites, and aristolochic acids in *Aristolochia ringens* Vahl. *J. Chromatogr. Sci.* 47, 817–821. doi: 10.1093/chromsci/47.9.817

Stensmyr, M. C., Urru, I., Collu, I., Celander, M., Hansson, B. S., and Angioy, A.-M. (2002). Rotting smell of dead-horse arum florets. *Nature* 420, 625–626. doi: 10.1038/420625a

Stökl, J., Strutz, A., Dafni, A., Svatos, A., Doubsky, J., Knaden, M., et al. (2010). A deceptive pollination system targeting drosophilids through olfactory mimicry of yeast. *Curr. Biol.* 20, 1846–1852. doi: 10.1016/j.cub.2010.09.033

Stutz, H. K., Silverman, G. J., Angelini, P., and Levin, R. E. (1991). Bacteria and volatile compounds associated with ground beef spoilage. *J. Food Sci.* 56, 1147–1153. doi: 10.1111/j.1365-2621.1991.tb04721.x

Trujillo, C. G., and Séric, A. N. (2006). Floral biology of *Aristolochia argentina* (Aristolochiaceae). *Flora* 201, 374–382. doi: 10.1016/j.flora.2005.07.013

Urru, I., Stensmyr, M. C., and Hansson, B. S. (2011). Pollination by brood-site deception. *Phytochemistry* 72, 1655–1666. doi: 10.1016/j.phytochem.2011.02.014

Vogel, S. (1978). Pilzmückenblumen als Pilzmimeten. Erster Teil. *Flora* 167, 329–366. doi: 10.1016/s0367-2530(17)31124-6

Wanke, S. (2006). *Evolution of the Genus Aristolochia – Systematics, Molecular Evolution and Ecology*. Ph.D. dissertation. Dresden: Universität Dresden.

Wanke, S., González, F., and Neinhuis, C. (2006). Systematics of Pipevines: combining morphological and fast-evolving molecular characters to investigate the relationships within subfamily Aristolochioideae (Aristolochiaceae). *Int. J. Plant Sci.* 167, 1215–1227. doi: 10.1086/508024

Wolda, H., and Sabrosky, C. W. (1986). Insect visitors to two forms of *Aristolochia pilosa* in Las Cumbres, Panama. *Biotropica* 18, 295–299.

Woodcock, T. S., Larson, B. M. H., Kevan, P. G., Inouye, D. W., and Lunau, K. (2014). Flies and flowers II: floral attractants and rewards. *J. Pollinat. Ecol.* 12, 63–94. doi: 10.2307/2992015

Zhang, Q. H., Jakuš, R., Schlyter, F., and Birgersson, G. (2003). Can *Ips typographus* (L.) (Col., Scolytidae) smell the carrion odours of the dead beetles in pheromone traps? Electrophysiological analysis. *J. Appl. Entomol.* 127, 185–188. doi: 10.1046/j.1439-0418.2003.00748.x

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 01 June 2021
doi: 10.3389/fevo.2021.639513

Colour Discrimination From Perceived Differences by Birds

Jair E. Garcia[1]*, *Detlef H. Rohr*[2] and *Adrian G. Dyer*[1,2]

[1] BIDS Lab, School of Media and Communication, RMIT University, Melbourne, VIC, Australia, [2] Evolution and Ecology Research Centre, University of New South Wales, Sydney, NSW, Australia

The ability of visual generalists to see and perceive displayed colour signals is essential to understanding decision making in natural environments. Whilst modelling approaches have typically considered relatively simple physiological explanations of how colour may be processed, data on key bee species reveals that colour is a complex multistage perception largely generated by opponent neural representations in a brain. Thus, a biologically meaningful unit of colour information must consider the psychophysics responses of an animal engaged in colour decision making. We extracted previously collected psychophysics data for a Violet-Sensitive (VS) bird, the pigeon (*Columba livia*), and used a non-linear function that reliably represents the behavioural choices of hymenopteran and dipteran pollinators to produce the first behaviourally validated and biologically meaningful representation of how VS birds use colour information in a probabilistic way. The function describes how similar or dis-similar spectral information can lead to different choice behaviours in birds, even though all such spectral information is above discrimination threshold. This new representation of bird vision will enable enhanced modelling representations of how bird vision can sense and use colour information in complex environments.

Keywords: colour, sigmoid, pigeon, power function, violet-sensitive, flower-signal, vision, just-noticeable-difference

Edited by:
Sara Diana Leonhardt, Technical University of Munich, Germany

Reviewed by:
Daniel Osorio, University of Sussex, United Kingdom
Thomas Pike, University of Lincoln, United Kingdom

***Correspondence:**
Jair E. Garcia
jair.garcia@rmit.edu.au

Specialty section:
This article was submitted to Coevolution, a section of the journal Frontiers in Ecology and Evolution

Received: *09 December 2020*
Accepted: *03 May 2021*
Published: *01 June 2021*

Citation:
Garcia JE, Rohr DH and Dyer AG (2021) Colour Discrimination From Perceived Differences by Birds. Front. Ecol. Evol. 9:639513. doi: 10.3389/fevo.2021.639513

1. INTRODUCTION

Many animals process visual information to inform decisions that result in fitness benefits to various species. Birds, for example, may first use their vision for locating a target of interest, and then for confirming correct identification (Troscianko et al., 2009). Whilst recognition of shape and texture is likely driven by achromatic processing in birds, discriminating the spectral component of a colour signal describing the quality of a stimulus is mainly driven by colour vision (Osorio et al., 1999).

Model bird species for studying evolution such as pigeons (Darwin, 1859, 1868) are known to be omnivorous, feeding on a wide variety of fruits, seeds, insects, flowers (Murton and Westwood, 1966; Crome, 1975; Innis, 1989; Baptista et al., 2009), and sometimes acting as pollinators when opportunistically feeding on nectar (Dalsgaard et al., 2016). In all these instances birds will likely use their colour vision to process information allowing for the initial detection of targets, and to subsequently discriminate a preferred option from sub-optimal alternatives simultaneously available.

When colour differences between stimuli are large, take for example preferred and opposing teams in a sport match dressed in yellow or blue, discrimination is both rapid and accurate. However, if respective teams were garbed in similar colours such as green and turquoise, both

accuracy and speed of discrimination would likely be impaired leading to a less accurate and slower response (Chittka and Osorio, 2007). Thus, well-beyond any theoretical discrimination limit to colour vision imposed by physiological aspects, colour similarity affects performance as it has been demonstrated on important pollinators like bees (Dyer and Chittka, 2004) and hoverflies (Hannah et al., 2019). Indeed, classic colour discrimination experiments with humans (MacAdam, 1942) show that accuracy diminishes with similarity up to a point where target and distracter become indistinguishable from each other and the observer chooses randomly between them; by the same token, increasing colour differences facilitate detection of targets concealed by background matching in real scenarios (Niu et al., 2020; Dyer and Garcia, 2021). Olsson et al. (2015) showed that accuracy of discrimination by chickens also increases with colour dissimilarity from chance level, to a range between 80 and 100% in a non-linear but continuous fashion. Such psychophysics evidence is likely to be mediated by the probabilistic way in which neurons can code and respond to the salience of different colour signals (Komatsu and Ideura, 1993).

The ability to predict the likely outcome of a colour discrimination event from physiological and physical properties of both observer and stimuli is fundamental for plant-animal interaction studies. For example, one could measure the reflectance spectra from flowers of different species and use a model for formulating testable hypothesis on the effect of colour signalling as means to establish relationships between plant and animal which are evolutionary meaningful (Pauw, 2019). Whilst such models are now available for hymenopteran pollinators such as honeybees and bumblebees (Garcia et al., 2017), and have been applied to mapping plant-pollinator interactions (Shrestha et al., 2019; Garcia et al., 2020), currently there is no model which allows for an accurate and unbiased prediction of animal responses considering (dis)similar colours as those likely to be encountered by a bird observer. The development of such models likely serves to improve our understanding of the processing for visual information produced by stimuli like fruits (Schaefer et al., 2008), egg shells (Hanley et al., 2019), or mating partners and nesting choices (Endler and Day, 2006).

Most models currently employed to understand colour discrimination by birds are based on purely physiological data at photoreceptor level, and specifically ignore the effects of neural processing by the brain of colour information (Avilés, 2020). This position of physiologically mediated colour discrimination does not fit evidence from primates showing that processing of such signals is a multistage process involving different brain regions with various degrees of specialisation (Solomon and Lennie, 2007). Furthermore there is strong evidence in invertebrates that colour processing is mediated a high levels of the insect brain (Paulk et al., 2009; Mota et al., 2013; Lin et al., 2016), strongly suggesting that colour information processing requires higher level structures in a wide range of animals.

Considering the current use of colour models for birds, the receptor noise (RN) model (Vorobyev and Osorio, 1998) has been proposed to be a solution for predicting the minimum colour difference of two stimuli required by a bird observer to discriminate between them, the so called *just noticeable difference* (JND). Interestingly, and in spite of its wide implementation for avian studies, the RN model assumptions are built on noise receptor data and behavioural responses from an invertebrate model: the European honeybee (*Apis mellifera*; Vorobyev et al., 2001). In the RN model, the difference between two coloured stimuli is not expressed in a colour space but is equated with a perceived difference expressed in JND (Pike, 2012). Whilst this model is proposed to be useful for both invertebrate and vertebrate observers, behavioural validation of the RN model predictions with avian observers has provided mixed results (Lind and Kelber, 2009).

Currently, it is accepted that validation of RN's predictions is subject to the appropriate choice of parameters, particularly, the noise level within photoreceptors (Olsson et al., 2015; Avilés, 2020). Regrettably, these measurements remain outstanding for any bird species, and approximations for the real values for these parameters based on purely theoretical assumptions are used instead (Olsson et al., 2018). Even for the two key model species for which photoreceptor noise values are available, the honeybee (Vorobyev et al., 2001) and the bumblebee (Skorupski et al., 2007), RN fails to predict discrimination accuracy observed in behavioural experiments when considering experimentally measured noise values (Vorobyev et al., 2001; Avarguès-Weber et al., 2010; Garcia et al., 2017, 2018).

An alternative formulation for predicting colour discrimination in animals was proposed by von Helversen (1972a). His approach aims to formulate a monotonic function linking a physical measure of colour dissimilarity, as for example the distance of two stimuli in a colour space, to a measure of the accuracy achieved by an observer when discriminating between these stimuli. This monotonic function approach proposed by von Helversen (1972a) contrasts starkly with the position of producing a single metric predicting perceptual difference proposed by the RN model.

A monotonic function describing the accuracy of colour discrimination task from a subject-independent measurement of (dis)similarity constitutes a representation of the psychophysical law (Norwich, 1987). More specifically, a colour discrimination function links an objective measurement of dissimilarity, distance in colour space (ΔC), with the *subjective perception* of colour dissimilarity experienced by an observer. Implementation of the psychophysical law for describing the relationship between objective and perceived differences in magnitude of stimuli such as heaviness, loudness, taste, and other stimuli (Stevens, 1957) suggests that this relationship is non-linear, and can described by either a logarithmic or power function depending on conditions (Norwich, 1987). Thus, a relationship informed by physchophysical data more fully represents the complete perceptual processing by the sensory system of the animal, which is the essential driver of observed behaviour.

Recently, such a psyschophysical approach has been proposed for predicting colour discrimination in hymenopteran pollinators based on non-linear, sigmoidal functions rather than JND magnitudes. These models predict changes in the probability of discrimination with colour distance based on the result of psychophysics experiments (Garcia et al., 2017), rather than just predicting if the considered colour distance is

either below or above a single theoretical JND discrimination threshold. In other words, models based on monotonic functions help assessing the uncertainty around an animal choice based on colour difference rather than just predicting if the animal can perceive, or not, the dissimilarity between two stimuli. The specific shape a colour discrimination function is given by the interaction between physiological aspects of colour vision for a given observer and the processing of such signals by the animal's brain as predicted by von Helversen (1972a).

In the present manuscript we develop a new framework for a colour discrimination function for a violet sensitive bird based on behavioural responses of the pigeon *Columba livia*. Importantly, pigeons are generalist foragers (Baptista et al., 2009; Dalsgaard et al., 2016) and a behaviourally accessible avian species for which it has been possible to collect precise psychophysics data on colour discrimination tasks (Wright, 1972). Our proposed model predicts the sensitivity index of colour discrimination, a more comprehensive measure of accuracy based on signal detection theory (see section 2 below), using a monotonic function described by a simple algebraic expression. We enable this solution without making assumptions about currently unknown photoreceptor noise parameters of a bird observer, thus overcoming one of the principal limitations of current modelling efforts. The function's accuracy to predict observed discrimination behaviour by the pigeon is then compared to that of the RN model for the same set of stimuli.

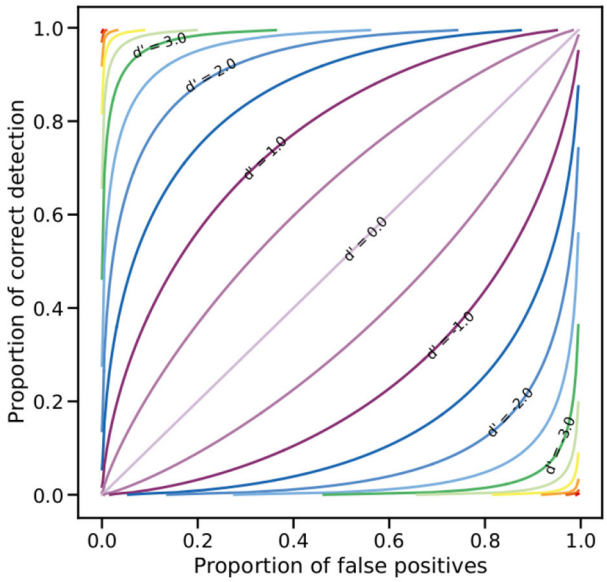

FIGURE 1 | Relative operating characteristic (ROC) on linear coordinates. Any point on a line represents the different combinations of hit rates and false positive rates leading to the same sensitivity index value (d'). The diagonal ($d' = 0$) represents the chance line where the hit and false positives rates are equal; as sensitivity increases, i.e., $d' > 0$, the slope of the lines also increase. Negative d' values are obtained when sensitivity decreases from chance level as a result of a higher proportion of false positive relative to the hit rate.

2. MATERIALS AND METHODS

2.1. Sensitivity Index of Colour Discrimination

In a dual choice experiment, for example a colour discrimination test, the response of a subject can be coded as the proportion of correct choices $p(c)$, expressed as the number of hits (n) obtained out of N trials ($p(c) = n/N$), and the proportion of incorrect choices $q(c) = 1 - p$. It is possible to obtain a deeper insight into the decision making process by coding two more variables representing the proportion of *false alarms*, mistakenly recognising the alternative choice as the reference; and, the proportion of *correct rejections* for the alternative choice (MacMillan and Creelman, 2005). These measurements can then be used to obtain a single measurement of sensitivity d' describing the ability of an observer to discriminate between stimuli of varying similarity (MacMillan and Creelman, 1990).

A subject with high sensitivity for a colour discrimination task is expected to have a relatively high hit rate relative to the false alarm rate even if its performance is not perfect. The magnitude of d' will generally increase with the proportion of correct choices or the decrease of false alarms; however, different hit/false ratios can be obtained for the same magnitude of d' (MacMillan and Creelman, 2005). This relationship is graphically depicted by a relative operating characteristic (ROC) curve (MacMillan and Creelman, 2005), as the one presented in **Figure 1**.

2.2. Colour Discrimination Experiment

In his discrimination experiment, Wright (1972) trained 4 pigeons (*C. livia*) to associate a set of 20 different quasi-monochromatic stimuli ranging from about 470 to 660 nm at 10nm intervals, with a food reward. Once the subjects had learned to associate a reference stimulus with the reward, pigeons were subsequently asked to discriminate the reference from a set of novel stimuli increasing in colour dissimilarity relative to the reference by having their peak transmission value shifted toward wavelengths shorter than that of the reference.

Experimental stimuli consisted of quasi monochromatic signals produced by passing a light source through a set of interference filters, each producing a quasi-monochromatic stimulus very similar to those presented in **Figure 2**. During the discrimination experiments, novel and reference stimuli were presented simultaneously in a bipartite screen thus ensuring a simultaneous viewing condition, which is essential for measuring colour discrimination for small differences (Wyszecki and Stiles, 1982; Kulikowski et al., 1991; Dyer and Neumeyer, 2005). The order of stimuli presentation was randomly determined for each one of the test subjects. Hit and false alarm rates produced by each subject where then used to calculate the sensitivity index (d') for the each tested colour. For any given subject, higher d' values are produced when the rate of hits is higher than false alarms, resulting from a lower number of errors.

We began constructing our discrimination model by recovering the d' values corresponding to each of the 20

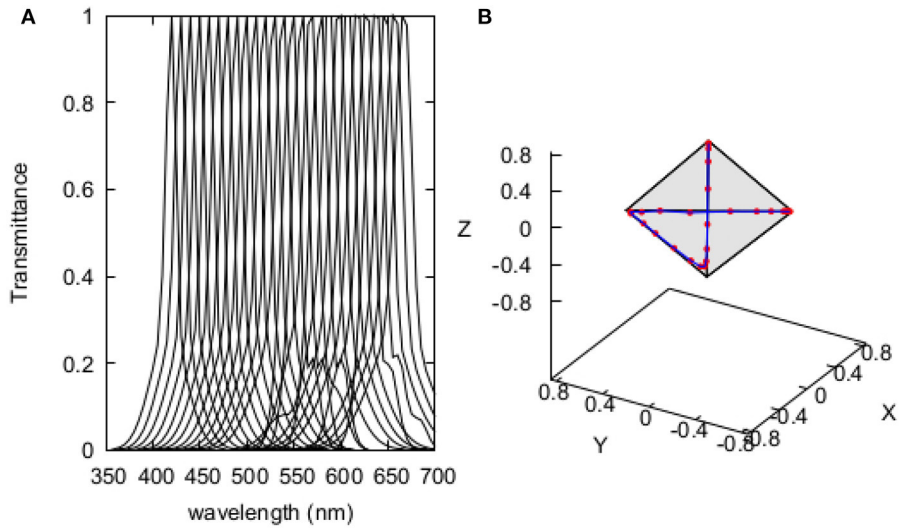

FIGURE 2 | Recovered spectral profiles of the 20 quasi-monochromatic stimuli used by Wright (1972) for determining the colour discrimination sensitivity index of the pigeon *Columba livia* **(A)**; and, their representation in the tetrahedron space **(B)**. Spectral profiles were obtained by implementing Equation (2) using coefficients in **Supplementary Table 1**.

reference stimuli tested by Wright (1972) using the expression:

$$d' = m \times \Delta\lambda, \quad (1)$$

where m is the slope of a simple, linear psychometric function describing change in sensitivity as a function of spectral difference between the reference and the stimuli ($\Delta\lambda$). We used the values of m reported in Table 1 of Wright (1972) for the calculations.

Similarly to models developed for hymenopteran pollinators (Garcia et al., 2017), our avian discrimination function uses colour difference between two stimuli ΔC as independent variable. We thus converted Wright's original $\Delta\lambda$ into ΔC values by calculating the Euclidean colour distance corresponding to each pair of the quasi monochromatic stimuli used in the experiment modelled in a tetrahedron colour space suitable for modelling bird vision (Endler and Mielke, 2005). Stimuli were characterised by means of the spectral transmittance profile of the interference filters used to produce them, modelled by a Gaussian function with three terms (Equation 2) fitted to the spectral transmittance chart of one of the Baush and Lomb interference filters originally used for the behavioural experiment.

$$T(\lambda) = a_1 * \exp\left(-\left(\frac{\lambda - b_1}{c_1}\right)^2\right) + a_2 * \exp\left(-\left(\frac{\lambda - b_2}{c_2}\right)^2\right) + a_3 * \exp\left(-\left(\frac{\lambda - b_3}{c_3}\right)^2\right). \quad (2)$$

Each transmittance function was evaluated from 300 to 700 nm at 5nm intervals. The λ coefficient in Equation (2) indicates the position of peak transmittance, so this term was systematically varied within the tested spectral range to recover the transmittance function of the different reference and test stimuli used in the behavioural experiment. Modelled spectra corresponding to the different stimuli are presented in **Figure 2** along with their representation in the tetrahedron colour space. Coefficients determining the various transmittance curves used as stimuli are provided in **Supplementary Table 1**.

2.3. Colour Modelling

There are currently three models suitable for modelling colour stimuli for avian vision (Goldsmith, 1991; Vorobyev and Osorio, 1998; Endler and Mielke, 2005). Of these, only models by Endler and Mielke (2005) and the RN model (Vorobyev and Osorio, 1998) account for light adaptation and colour constancy through the implementation of a von Kries-type scaling of photoreceptor sensitivity to match the spectral properties of the illumination and background (Vorobyev and Osorio, 1998; Endler and Mielke, 2005; Renoult et al., 2017). Only the model proposed by Endler and Mielke (2005) allows for a representation of colour stimuli independent from perceptual assumptions of the observer (Pike, 2012) by expressing colour samples as three-dimensional loci in the volume of a tetrahedron (Endler and Mielke, 2005). Even though the scaling of this colour space axis is arbitrary, and purely based on geometrical principles, its use permits current best practice for expressing colour dissimilarity by means of the Euclidean distance between loci.

We used the nomogram proposed by Stavenga et al. (1993) to model the spectral transmission of the photoreceptors present in the single cones of the pigeon (*C. livia*). For our modelling we used peak absorption of values of 404, 452, 506, 566 nm for the violet, short, medium, and long photoreceptors, respectively (Hart and Vorobyev, 2005). Spectral transmission profiles of the

oil droplets of the pigeon were modelled using the methods and parameters reported by Hart and Vorobyev (2005) with half maximum absorptance values of (λ_{mid}) of 470, 542, 613 nm for the clear, yellow and red types, respectively with a $\lambda_{T0.5} = 338$nm as suggested by Hart and Vorobyev (2005). Ocular transmittance was modelled using the average function for violet sensitive birds by Endler and Mielke (2005) by evaluating the function:

$$T_e(\lambda) = \ln(8.928 \times 10^{-13}\lambda^5 - 2.595 \times 10^{-9}\lambda^4 + 3.006 \times 10^{-6}\lambda^3 - 0.001736\lambda^2 - 55.56), \quad (3)$$

and subsequently shifting the resulting function along the x-axis by ($\lambda_{50} - 335.2$) with a $\lambda_{50} = 362$ as suggested for modelling violet-sensitive bird vision (Endler and Mielke, 2005). For our calculations we assumed a daylight illumination typical of an open sky during midday (CIE D6500) (Judd et al., 1964) expressed as quantum flux, and a background reflecting 25% of all incident radiation between 300 and 700 nm.

2.4. Curve Fitting and Statistical Analysis

Initial plots corresponding to the observed d' values for each of the tested reference stimuli showed a non-linear relationship between sensitivity index and the various colour differences tested. Pilot curve fitting trials suggested that a power function with general form:

$$p = a \times x^m, \quad (4)$$

fitted the data better than other functions involving logarithms or exponential terms.

Power functions, producing a monotonic curve, have a long tradition in psychophysical studies having been used to describe the relationship between perceived difference and magnitude of stimuli such as loudness, brightness, heaviness, and taste in experiments using human subjects (Stevens, 1957).

The function described by Equation (4) may take either a convex or concave shape depending on the magnitude of m (**Figure 3**): when the function takes a convex shape, initial small changes in the stimulus magnitude lead to large perceptual differences. On the other hand, when the function is concave large changes in the stimulus magnitude are required to drive small differences in perception. The parameter a defines the function slope: large magnitudes result in steeper functions whilst $a = 0$ produces a straight line where perceived magnitude would remain constant in spite of any changes to the stimulus magnitude (**Figure 3**).

We built an initial model based on Equation (4). The independent variable was the colour distance (ΔC) between each one of the 20 references, and their respective five test stimuli. The response variable was the observed d', corresponding to the ratio of hits and false alarm. The initial model included random terms for the a and m parameters to account for any potential differences in the shape or slope of the discrimination function with reference wavelength.

The initial non-linear model was fitted using the nlme package (Pinheiro and Bates, 2000) for R statistical language (R core). Significance of the random terms was tested by means of likelihood ratio tests (LRT) between the initial full model and reduced versions excluding the random terms for the a and m variables (Pinheiro and Bates, 2000). Once significant random terms were identified, we proceeded to evaluate the final model using Bayesian modelling techniques employing the package brms (Bürkner, 2017) v 2.13.5 for R. The Bayesian model was initialised assuming diffuse, normally distributed priors for the a and m parameters, and a half Cauchy distribution for the random terms (Zuur et al., 2013). The final model was fitted with 4 chains, each consisting of 100,000 iterations with a burn-in of 50,000 and thinning rate of 10. The data set consisted on 100 observations corresponding to five d' values for each of the 20 stimuli used as reference.

2.5. Receptor Noise Modelling and Model Comparison

We calculated colour difference (ΔS) between each of the 20 reference stimuli and their respective test signals. Differences were calculated implementing the receptor noise model for colour threshold (Vorobyev and Osorio, 1998; Vorobyev et al., 2001) given by:

$$\begin{aligned}(\Delta S)^2 = {} & ((e_1e_2)^2(\Delta_{q4} - \Delta_{q3})^2 + (e_1e_3)^2(\Delta_{q4} - \Delta_{q2})^2 + \\ & (e_1e_4)^2(\Delta_{q3} - \Delta_{q2})^2 + (e_2e_3)^2(\Delta_{q4} - \Delta_{q1})^2 + \\ & (e_2e_4)^2(\Delta_{q3} - \Delta_{q1})^2 + (e_3e_4)^2(\Delta_{q2} - \Delta_{q1})^2)/ \\ & ((e_1e_2e_3)^2 + (e_1e_2e_4)^2 + (e_1e_3e_4)^2 + (e_2e_3e_4)^2), \quad (5)\end{aligned}$$

where Δq denotes the difference in photon captured by q_i photoreceptors of spectral radiation reflected or emitted by two stimuli, after correcting for light adaptation using a von Kries transformation (Vorobyev and Osorio, 1998). Coefficients e denote the noise amount limiting colour discrimination in photoreceptor i. As noise values e_i have only been measured for two hymenopteran species (Vorobyev et al., 2001; Skorupski and Chittka, 2010), e_i values for the pigeon where estimated by means of $e_i = \nu_i/\sqrt{\eta_i}$ where ν_i denotes Weber fraction and η_i is the relative abundance of each of the i photoreceptor classes in the observer's retina (Vorobyev and Osorio, 1998). In our calculations we used $\nu = 0.05$, a value typically assumed for a wide range of vertebrate observers (Endler and Mielke, 2005; Santiago et al., 2020), and density ratios for the violet, short, medium, and long wavelength photoreceptors V:S:M:L of 1:1:1:2 as reported by Vorobyev and Osorio (1998). However here we explicitly state that the assumed value of $\nu = 0.05$ is used for convenience of modelling and currently no empirical data exists to support this value (Kemp et al., 2015).

Classically, the threshold for a colour discrimination experiment is set to $p(c) = 0.75$, or a 75% of correct choices (von Helversen, 1972b; Kelber et al., 2003). In its original formulation, the receptor noise model set ΔS to this value (Vorobyev and Osorio, 1998) which is approximately equivalent to a $d' = 1$ where 75% of correct choices correspond to a proportion of false positives of 0.25 (**Figure 1**). Endler and Mielke (2005) propose that $\Delta S = 2$ corresponds to a colour difference that can be distinguishable by a bird with an accuracy of 95%, equivalent to about $d' = 2$ with an increase in the proportion of false

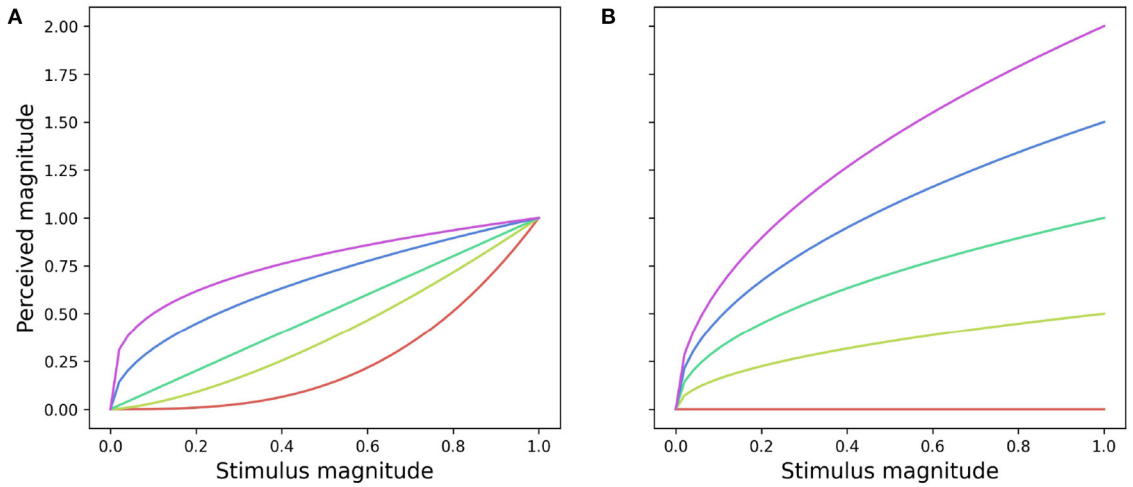

FIGURE 3 | Effect of coefficients *a* and *m* on the shape **(A)** and slope **(B)** of a power function (Equation 4) describing the magnitude of perceived change. **(A)** Effect of changing the value of *m* from 0.3 (red line) to 3 (purple line) for $a = 1.0$. **(B)** Changes in the slope of a power function resulting from increasing the value of *a* from 0 (red line) to 2 (purple line) for $m = 0.5$.

positives to a little more than 0.3, or to $d' = 2.5$ with a reduction in the proportion of false positives to about 0.25 (**Figure 1**). These relationships suggest that predictions from the RN model should be directly comparable with d' values thus allowing for a comparison of the goodness of fit of the RN model and the colour discrimination function to the described by Equation (4) to the behavioural data available.

Various Weber fraction values (ν) for the RN model and posterior distributions of the parameters *a* and *m* for the CDSF function, were used to measure effect of the respective parameter(s) value on the predictive power of each model. For the RN model, we sampled 100,000 pseudo-random ν-values from an uniform distribution ranging from $\nu = 0.05$ to $\nu = 0.10$, and used these to calculate ΔS corresponding to the outcome of Wright (1972) discrimination experiments. This range of the used ν-values encompasses the different magnitudes of Weber fraction reported by previous authors when validating the RN model as a method for predicting colour discrimination thresholds for bird vision (Olsson et al., 2018): $\nu = 0.05$ (Endler and Mielke, 2005), $\nu = 0.06$ (Olsson et al., 2015), and $\nu = 0.10$ (Vorobyev and Osorio, 1998; Lind et al., 2014). We calculated the root mean square error (RMSE) for predictions obtained from each ν-value to obtain an effect measurement of Weber fraction value on the predictive power of the RN model. Likewise, we evaluated the 100,000 *a* and *m* values making up the posterior distribution of these parameters to predict d' using the formulation of the CDSF function only including fixed-terms (Equation 4) and calculated respective RMSE values.

3. RESULTS

3.1. Colour Discrimination Sensitivity Function (CDSF)

Likelihood ratio tests evidence that the slope ($\chi^2_a = 80.8$, $P < 0.001$) of CDSF changes significantly with wavelength of the reference stimuli so a random term was included for the shape parameter (*a* in Equation 4). We found that the parameter defining the shape of the function changed significantly with reference wavelength ($\chi^2_m = 3.90$, $P = 0.0482$). Pilot modelling using a green adaptation background revealed a non significant variation of the function shape with wavelength of the reference stimuli ($\chi^2_m = 1.03$, $P = 0.309$); therefore we did not include a random term for shape in the final model.

The CDSF (**Figure 4**) describes changes in sensitivity of a VS bird when discriminating coloured stimuli from 470 to 660nm. The model allows predicting discrimination accuracy in terms changes of sensitivity index (d') for colour differences ranging from $\Delta C = 0$ to $\Delta C = 1$ between 470 and 660 nm, and is mathematically described by:

$$d' = (a + \alpha_i) \times \Delta C^m, \tag{6}$$

where the fixed terms *a* and *m* describe the slope and shape of the function, respectively, whilst the α coefficient describes random variation in the function slope at each one of the $i = 20$ reference wavelengths tested. Median and 95% credibility intervals for the posterior distributions of the fixed parameters of the model are provided in **Table 1**. Details on the posterior distributions of random terms are available as **Supplementary Material 2**.

The shape of the CDSF suggests that small changes in colour distance rapidly increment the sensitivity index thus increasing the probability of accurate discrimination whilst diminishing the likelihood of false positives (**Figure 4**). Indeed, the function predicts that a change from about $\Delta C = 0.1$ between similar colours lead to a change in sensitivity values of about one d' unit.

The colour discrimination sensitivity model for a VS bird predicts that differences of about $\Delta C = 0.204$ tetrahedron units (THu) can be discriminated with a sensitivity of $d' = 2.0$, which is approximately equivalent to a correct discrimination of about 80% with a false alarm rate of <20% (**Figure 1**). Likewise,

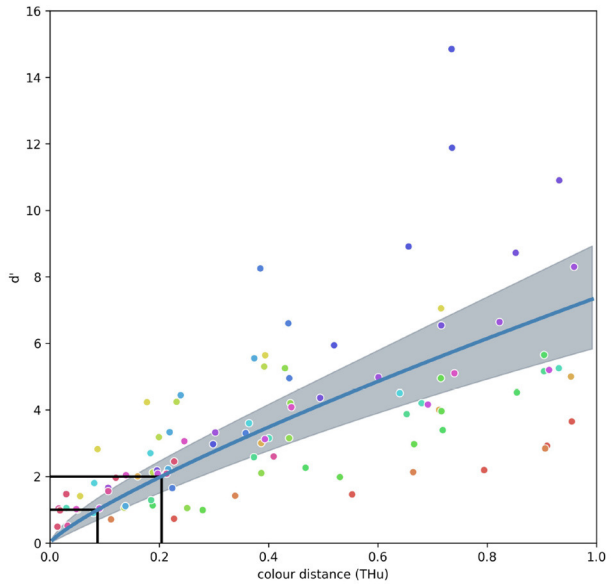

FIGURE 4 | Fixed terms model representing the colour discrimination sensitivity function (CDSF) and associated 95 % credibility intervals for a violet-sensitive bird observer predicting the sensitivity index (d') for a discrimination task between stimuli of increasing colour dissimilarity (ΔC). Sensitivity index is related to the ratio of hits to false positives with $d' \geq 1$ representing a proportion of hits above 75% with ratio of false positives <30% as illustrated by the ROC curve in **Figure 1**. Colour circles represent the 20 different reference wavelengths tested by Wright (1972), colour code is the same as in **Figure 5**. Black lines indicate the ΔC values of 0.088 and 0.204 THu required to attain $d' = 1$ and $d' = 2$, respectively.

TABLE 1 | Median and 95% credibility intervals of the *a* and *m* parameters describing the colour discrimination function of a violet sensitive bird to a set of 20 different quasi monochromatic stimuli from 470 to 670 nm.

Coefficients	Median	95% Credibility interval
a	7.37	5.87, 8.99
m	0.821	0.684, 0.970

colour differences of about $\Delta C = 0.144$ THu yield a $d' = 1.5$ equivalent to about the same proportion of correct choices, but with a higher false alarm rate. Finally, colour differences $\Delta C = 0.088$ THu are predicted to be correctly discriminated about 75% of the time with a false alarm rate of about 25% ($d' = 1.0$), whilst colour differences $\Delta C \approx 0$ THu will fall very close to chance level ($d' = 0$). Values of ΔC corresponding to any d' of interest can be obtained by inverting Equation (4) and using values reported in **Table 1** for the fixed terms a and m. Random terms α_i corresponding to each of the $i = 20$ reference stimuli are presented in **Supplementary Table 2**. These calculations can be easily performed using any spreadsheet program, or coded into functions for programming languages such as R or Python.

When considering the random effects for each tested wavelength (**Figure 5**), the CDSF predicts the greatest sensitivity index for spectral radiation of 600 and 500 nm, indicating that birds can best discriminate colour signals rich in long wavelength radiation.

3.2. Model Evaluation

Mean RMSE value for the RN model was of 11.6 (95% confidence intervals (CI) 7.48–17.8). The minimum RMSE value of 7.33 was obtained when $\nu = 0.10$, whilst the maximum RMSE of 18.31 corresponds to $\nu = 0.05$, the value often recommended as the best approximation for the unknown Weber fraction for birds. On the other hand, the mean RMSE value for the fixed-term only CDSF function (Equation 4) was of 2.04 (95% CI 1.95–2.27). Minimum RMSE for the CDSF function was of 1.92 with a maximum value of 4.40. The RMSE value for the CDSF function is further reduced to 0.927 when considering the random terms corresponding to each one of the stimuli tested by Wright (Equation 6).

When only considering predictions of the RN model within a discrimination threshold $\Delta S = 1 - 2$, corresponding to a probability of correct choices between 75 and 95%, we obtained a mean RMSE of 6.02 (95% CI 4.07–8.97). For this subset, the minimum RMSE was also obtained when $\nu = 0.10$. On the other hand, mean RMSE for the CDSF function for d' values between 1 and 2 was of 1.12 (95% CI 0.882–1.40). A graphical summary of these results are provided in **Figure 6**.

DISCUSSION

In the current study we consider if psychometric functions may offer a holistic modelling solution for understanding VS-sensitive, avian decision making considering (dis)similar colours when compared to the noise-limited colour discrimination model (RN).

Predictions from RN depend on the precise value of its various parameters, and in particular, on the magnitude of noise coefficients (e_i) assigned to the different photoreceptors (Lind and Kelber, 2009). In spite of the wide use of the RN model for answering questions regarding avian visual ecology such as: discrimination of parasitic eggs by hosts (Hanley et al., 2019), camouflage by female birds (Cain et al., 2019), and perceived flower colour variation by pollinating species at population level (Whitney et al., 2020); to cite just a few recent examples, no noise measurement data currently exist for any avian species. In most cases, the unknown but essential noise values are derived from applying theoretical assumptions of signal detection theory to photoreceptor density data (Vorobyev and Osorio, 1998), although it still remains unclear how neural processing by the brain may affect these assumptions. This is important as birds and other animals are known to use various strategies to counteract limitations imposed to vision by the physiological properties of their visual system. For example, spatial pooling for increasing signal-to-noise ratio under dim illumination (Warrant, 1999) is likely to occur in some species of owls (Orlowski et al., 2012) and nocturnal parrots (Corfield et al., 2011; Iwaniuk et al., 2020). Likewise, it remains largely unknown how external factors such as temperature and light intensity may affect photoreceptor noise values, and how neural processing may

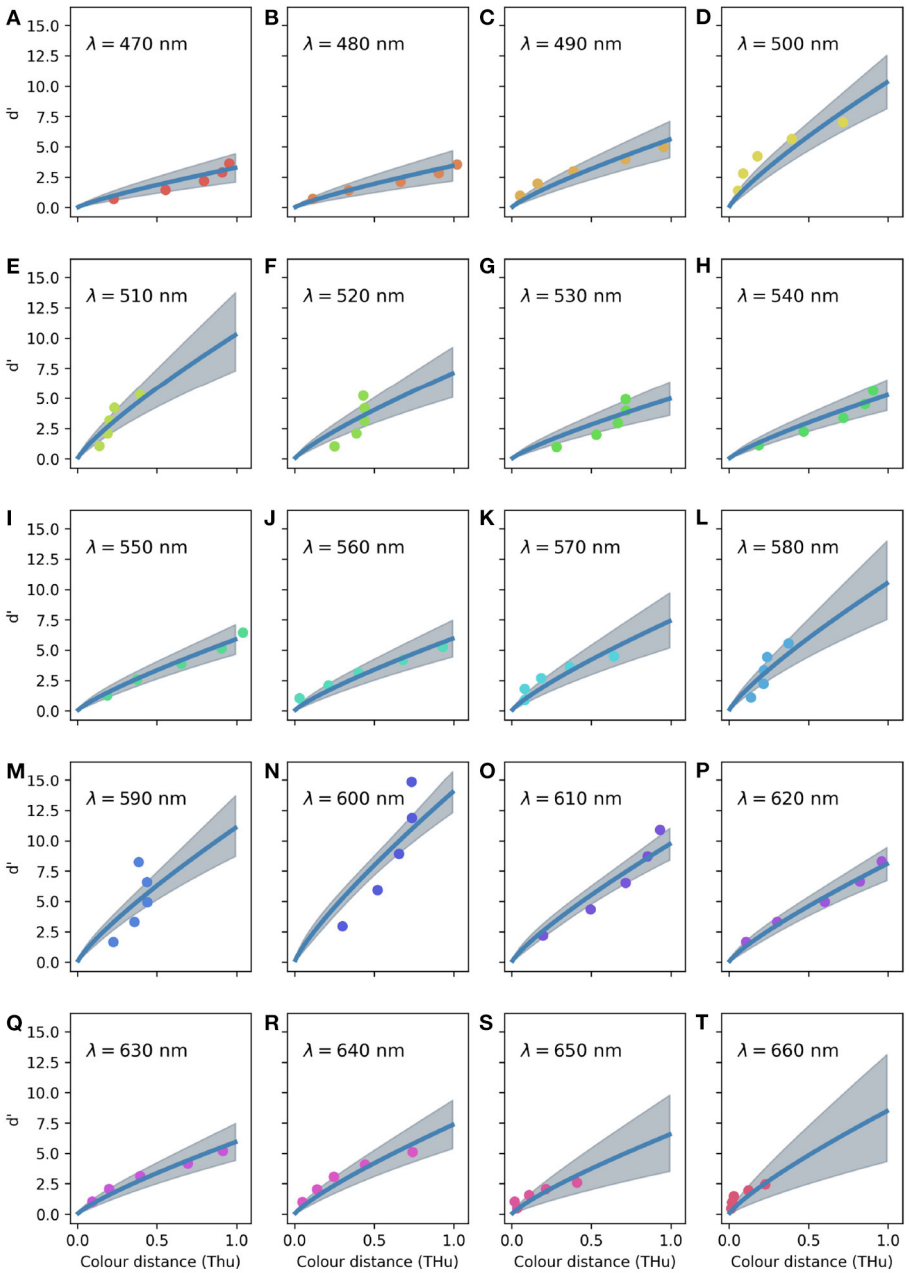

FIGURE 5 | Colour discrimination sensitivity model for a violet sensitive bird accounting for fixed and random terms for of the 20 reference wavelengths tested by Wright (1972) as indicated on the legend for **(A–T)**. Circles indicate observations corresponding to the five different stimuli tested for each reference wavelength. Marker colours have no relationship with the visual appearance of the stimuli, hues were selected to ease visual interpretation.

compensate for such fluctuations. Indeed, experiments on frogs under laboratory controlled conditions (Aho et al., 1988) suggest that visual performance could be affected by temperature if receptor noise were the only or primary factor mediating colour discrimination. Such basic physiological limitations would likely apply to many animals unless neural corrections resolve how colour can be reliably perceived in naturally occurring visual conditions.

The colour discrimination sensitivity function (CDSF) for a violet-sensitive bird observer (**Figure 4**), takes as input a measurement of colour dissimilarity and returns the *likely* outcome of the discrimination process expressed as a sensitivity index (d'). Through this approach CDSF accounts for the "inner-conditions" driving animal behaviour as originally hypothesised by von Helversen (1972a). Our function thus represents an application of the psychophysics law where the relationship

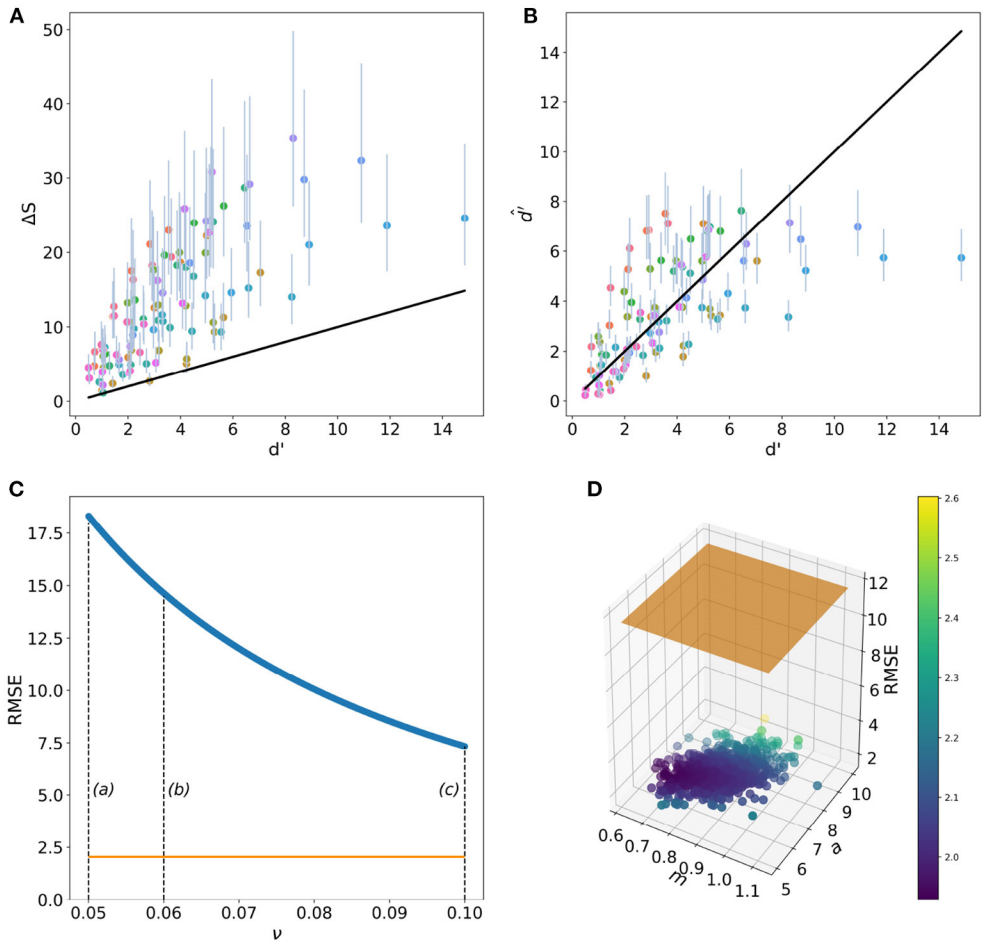

FIGURE 6 | Performance of the receptor-noise (RN) limited model **(A)** and the CDSF model **(B)** when predicting the outcome of Wright's (1972) colour discrimination experiment (solid lines). Circular markers and vertical lines in panel A represent the mean and 95 % confidence intervals (CI) of ΔS values from 100,000 predictions of the colour discrimination experiments by Wright (1972) using Weber fraction values (ν) ranging from 0.05 to 0.1. Markers and vertical lines in **(B)** represent mean and 95 % CI d' values predicted by the CDSF model ($\hat{d}'$) after evaluating the 100,000 coefficients present in the posterior distributions for the fixed terms a and m in Equation (4) for the same stimuli. Root mean square error (RMSE) for the predictions of the RN **(C)** and CDSF **(D)** discrimination functions. **(C)** Shows how the RMSE for RN diminishes as the noise parameter departs from $\nu = 0.05$. Dashed lines in **(C)** indicate the magnitude of Weber fraction values typically reported in the literature: (a) $\nu = 0.05$ (Endler and Mielke, 2005), (b) $\nu = 0.06$ (Olsson et al., 2015), and (c) $\nu = 0.1$ (Vorobyev and Osorio, 1998; Lind et al., 2014). The orange line indicates the mean RMSE value corresponding to the CDSF function for the same colour differences. **(D)** Shows a 1,000 RMSE values sub sample out of the total 100,000 RMSE values obtained after evaluating the CDSF function with all the *a* and *m* parameters making up the posterior distributions recovered from the Bayesian fitting procedure. Marker colour represents RMSE value as indicated by the colour bar. Orange plane represents the mean RMSE value for the RN model for the same set of stimuli.

between objective and perceived colour difference is modelled by a power function (Equation 6) as experimentally obtained for modelling perceived changes in the magnitude of stimuli such as heaviness and loudness (Stevens, 1957).

An additional advantage of the CDSF function is that it allows for a precise definition of the just noticeable difference for colour discrimination by means of the Weber fraction. The Weber fraction describes the difference in stimulus magnitude that is just noticeable by an observer (Debats et al., 2012), quantitatively expressed as a derivative of the psychophysics law (Norwich, 1987). By differentiating Equation (6) (**Supplementary Figure 1**), and evaluating the resulting function for a range of colour differences, we can see how for bird vision large Weber fraction values are initially obtained for small colour differences and subsequently falling with increasing stimuli dissimilarity into a plateau region, as observed for other perceptual tasks such as taste and brightness (Norwich, 1987). This approach allows for a more precise definition of the just noticeable colour differences perceivable by a bird observer, representing an improvement over the extrapolation of the behavioural and physiological results obtained from observations on the insect model species used for validating the RN assumptions (Vorobyev et al., 2001). Indeed, previous behavioural validation of RN assumptions for bird colour discrimination have provided inconclusive results on the predictive accuracy of this model in the absence

of measured noise data. For example, in their experiment with domestic chicks, Olsson et al. (2015) found that a Weber fraction $\nu = 0.06$ provided a good fit for observed behavioural data on a two option colour discrimination task under bright light conditions. This value is 40% smaller than the $\nu = 0.1$ sometimes suggested as noise parameter for modelling bird vision (Vorobyev and Osorio, 1998; Lind et al., 2014), evidencing the susceptibility of RN predictions to errors when assumptions of their parameters are made (Lind and Kelber, 2009; Bitton et al., 2017).

In spite of its algebraic simplicity (Equation 1), the CDSF is flexible enough as to accommodate for potential changes in sensitivity produced by stimuli of different hue (**Figure 5**). Indeed, the statistical significance of the random term α_i in Equation (6) suggests that the magnitude of colour dissimilarity required to perceive two loci as being different changes with the spectral position of the transmittance peak of the stimuli (**Figure 5**); in other words, birds likely discriminate some colours better than others. Asymmetries in the ability to discriminate colour stimuli depending on hue have also been reported for human observers (Wyszecki and Stiles, 1982), macaque monkeys (Komatsu and Ideura, 1993), honeybees (von Helversen, 1972b; Dyer and Neumeyer, 2005), bumblebees (Dyer et al., 2008), stingless bees (Spaethe et al., 2014), *Drosophila* flies (de Salomon and Spatz, 1983), hoverflies (Hannah et al., 2019), and domestic chicks (*Gallus gallus*) following associative training (Olsson et al., 2015). Moreover, by using two coefficients the colour discrimination sensitivity function can take different shapes (**Figure 3**) potentially describing the effect of other cognitive-like processes affecting colour discrimination such as memory and conditioning as reported for some hymenopteran species (Dyer and Chittka, 2004; Giurfa, 2004; Avarguès-Weber et al., 2010; Dyer et al., 2011; Garcia et al., 2020).

Our data shows that modelling colour discrimination by means of the purely physiologically informed RN model (**Figure 6A**) leads to a greater RMSE, and thus poorer predictive power, than the CDSF either including or excluding the wavelength-specific random terms (**Figure 6**). For the empirical colour discrimination data for pigeons, RN predictions for the typically assumed $\nu = 0.05$ value for modelling photoereceptor noise in birds, we obtained an RMSE error 95 % higher than the one resulting from implementing the discrimination function only including fixed terms (**Figure 6C**). This result is consistent with previous studies reporting that RN is not well suited for modelling perceptual colour distances of 1–3 jnd which are beyond the discrimination threshold (Bitton et al., 2017; Marshall, 2018; Olsson et al., 2018). Whilst an improvement of the RN model predictions was obtained when using a $\nu = 0.1$, RMSE for this value was still 28% higher than the mean RMSE value for the CDSF. The CFSD model still provides a better estimate of colour discrimination by birds than the RN alternative when considering the 75–95% discrimination threshold range for which the RN was originally calibrated using an insect model (Vorobyev et al., 2001).

The CDFS function accurately predicts bird discrimination for colour differences between $0.25 < \Delta C \leq 0.5$ THu for which pigeons show a sensitivity $d' > 2$ (**Figures 4**, **5**). Colour differences of this magnitude are large enough as to be discriminable with an accuracy of about 90 % with a false positive proportion of <10% (**Figure 1**) indicating that they are easily distinguishable by a violet sensitive bird. Such salient and robust colour signals are likely to be produced during plant pollinator interactions to attract animal pollination vectors like birds or bees (Lunau et al., 2011) as a solution to overcome the "colour noise" produced by natural variability of flower pigmentation that might confuse decision making (Dyer et al., 2012; Garcia et al., 2018; van der Kooi et al., 2019; Garcia et al., 2020).

Perceptual effects of colour differences $\Delta C > 6.0$ are poorly predicted by the CDSF model. This result may be interpreted as the effect of processes such as categorisation when judging large colour differences as suggested by early experiments on this species (Wright and Cumming, 1971). Furthermore, a large colour difference between target and background increases salience of the signal attracting gaze and attention (Siuda-Krzywicka et al., 2019), which may have an effect on colour discrimination accuracy as suggested by experiments on honeybees (Giurfa, 2004; Avarguès-Weber et al., 2010). Another limitation of the CDSF model is that its predictions are currently based on pigeon responses to monochromatic stimuli. The intensity and purity of these signals may have an effect on the photoreceptor adaptation process potentially affecting discrimination accuracy when compared to responses obtainable from broad-band, colour stimuli as those typically produced by organic pigments. Nevertheless, the methodology we present can be used to re-calibrate the CDSF function to account for animal responses, which may even exist within subjects (Giurfa, 2004), to these type of stimuli once they become available. Indeed, such functions have already been derived for predicting the accuracy of colour discrimination by four hymenopteran species when observing broad-band stimuli (Garcia et al., 2017).

Pigeons have many feral and domestic breeds around the world and a demonstrated capacity to forage on a wide variety of foods including: flowers, insects, fruits, and seeds (Darwin, 1859, 1868; Murton and Westwood, 1966; Baptista et al., 2009). On the island of Cuba pigeons have been observed to feed from nectar rich flowers (Dalsgaard et al., 2016), and in Queensland, Australia, fruit pigeons were observed in a 5-year study to feed from 89 different species of plants and have diets including a variety of fruits and flowers (Innis, 1989). This suggests that pigeons require a good capacity to generalise information. Our findings that pigeons have a colour visual system that is more fully explained by a continuous discrimination function (**Figures 4**, **6**) suggests that for other animals that have a requirement for foraging on a variety of colour stimuli it will be of value to consider this model of colour processing.

Psychometric functions linking objective measurements of dissimilarity, expressed as distance in a colour space, with perceived difference, measured as discrimination accuracy can easily accommodate a more holistic understanding of cognitive aspects of colour vision including memory, individual experience (Skorupski and Chittka, 2011), and effects of colour variability which cannot be accurately modelled by purely physiologically informed colour models (Garcia et al., 2020). This new generation of analytical tools open the door for testing interesting

hypothesis about the perceptual effect of colour signalling such as flower display and plant—animal interactions with new, fresh eyes.

DATA AVAILABILITY STATEMENT

The datasets corresponding to posterior distributions of the CDSF coefficients, ΔS values for the various Weber fractions simulated and their specific values presented in this study can be found in Figshare data depository (doi: 10.6084/m9.figshare.13350926).

AUTHOR CONTRIBUTIONS

JG and AD designed the experiments. JG analysed the data and performed the statistical analyses. All authors wrote and edited the manuscript.

FUNDING

AD was supported by the Australian Research Council Discovery Project DP160100161.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.639513/full#supplementary-material

Supplemental data for this submission includes **Supplementary Tables 1**, **2** containing coefficients used for producing the Gaussian functions modelling transmittance of the interference filters used for the behavioural experiment, and median and 95% credibility intervals for the α_i parameters corresponding to the random term in Equation (4), respectively.

REFERENCES

Aho, A. C., Donner, K., Hyden, C., Larsen, L. O., and Reuter, T. (1988). Low retinal noise in animals with low body temperature allows high visual sensitivity. *Nature* 334, 348–350. doi: 10.1038/334348a0

Avargués-Weber, A., de Brito Sanchez, M. G., Giurfa, M., and Dyer, A. G. (2010). Aversive reinforcement improves visual discrimination learning in free-flying honeybees. *PLoS ONE* 5:e15370. doi: 10.1371/journal.pone.0015370

Avilés, J. M. (2020). Avian egg and nestling detection in the wild: should we rely on visual models or behavioural experiments? *Philos. Trans. R. Soc. B Biol. Sci.* 375:20190485. doi: 10.1098/rstb.2019.0485

Baptista, L. F., Martínez Gómez, J. E., and Horblit, H. M. (2009). Darwin's pigeons and the evolution of the columbiforms: recapitulation of ancient genes. *Acta Zool. Mex.* 25, 719–741. doi: 10.21829/azm.2009.253674

Bitton, P. P., Janisse, K., and Doucet, S. M. (2017). Assessing sexual dicromatism: The importance of proper parameterization in tetrachromatic visual models. *PLoS ONE* 12:e169810. doi: 10.1371/journal.pone.0169810

Bürkner, P.-C. (2017). BRMS: an R package for Bayesian multilevel models using stan. *J. Stat. Softw.* 80:32916. doi: 10.18637/jss.v080.i01

Cain, K. E., Hall, M. L., Medina, I., Leitao, A. V., Delhey, K., Brouwer, L., et al. (2019). Conspicuous plumage does not increase predation risk: a continent-wide test using model songbirds. *Am. Nat.* 193, 359–372. doi: 10.1086/701632

Chittka, L., and Osorio, D. (2007). Cognitive dimensions of predator responses to imperfect mimicry. *PLoS Biol.* 5:e339. doi: 10.1371/journal.pbio.0050339

Corfield, J. R., Gsell, A. C., Brunton, D., Heesy, C. P., Hall, M. I., Acosta, M. L., et al. (2011). Anatomical specializations for nocturnality in a critically endangered parrot, the kakapo (*Strigops habroptilus*). *PLoS ONE* 6:e22945. doi: 10.1371/journal.pone.0022945

Crome, F. H. (1975). The ecology of fruit pigeons in tropical Northern Queensland. *Wildl. Res.* 2, 93–101. doi: 10.1071/WR9750155

Dalsgaard, B., Baquero, A. C., Rahbek, C., Olesen, J. M., and Wiley, J. W. (2016). Speciose opportunistic nectar-feeding avifauna in Cuba and its association to hummingbird island biogeography. *J. Ornithol.* 157, 627–634. doi: 10.1007/s10336-016-1326-6

Darwin, C. (1859). *On the Origin of Species by Means of Natural Selection or the Preservation of Favored Races in the Struggle for Life*. London: John Murray. doi: 10.5962/bhl.title.68064

Darwin, C. (1868). *The Variation of Animals and Plants Under Domestication*. London: John Murray.

de Salomon, H. C., and Spatz, H.-C. (1983). Colour vision in *Drosophila melanogaster*: wavelength discrimination. *J. Comp. Physiol. A* 150, 31–37. doi: 10.1007/BF00605285

Debats, N., Kingma, I., Beek, P. J., and Smeets, J. B. J. (2012). Moving the weber fraction: the perceptual precision for moment of inertia increases with exploration force. *PLoS ONE* 7:e42941. doi: 10.1371/journal.pone.0042941

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G. P., Simonov, V., and Wong, B. B. M. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. B Biol. Sci.* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Dyer, A. G., and Chittka, L. (2004). Biological significance of distinguishing between similar colours in spectrally variable illumination: bumblebees (*Bombus terrestris*) as a case study. *J. Comp. Physiol.* 190, 105–114. doi: 10.1007/s00359-003-0475-2

Dyer, A. G., and Garcia, J. E. (2021). Plant camouflage: fade to grey. *Curr. Biol.* 31, R78–R80. doi: 10.1016/j.cub.2020.11.061

Dyer, A. G., and Neumeyer, C. (2005). Simultaneous and successive colour discrimination in the honeybee (*Apis mellifera*). *J. Comp. Physiol. A* 191, 547–557. doi: 10.1007/s00359-005-0622-z

Dyer, A. G., Paulk, A. C., and Reser, D. H. (2011). Colour processing in complex environments: insights from the visual system of bees. *Proc. R. Soc. B Biol. Sci.* 278, 952–959. doi: 10.1098/rspb.2010.2412

Dyer, A. G., Spaethe, J., and Prack, S. (2008). Comparative psychophysics of bumblebee and honeybee colour discrimination and object detection. *J. Comp. Physiol. A* 194, 617–627. doi: 10.1007/s00359-008-0335-1

Endler, J. A., and Day, L. B. (2006). Ornament colour selection, visual contrast and the shape of colour preference functions in great bowerbirds, *Chlamydera nuchalis*. *Anim. Behav.* 72, 1405–1416. doi: 10.1016/j.anbehav.2006.05.005

Endler, J. A., and Mielke, P. W. (2005). Comparing entire colour patterns as birds see them. *Biol. J. Linn. Soc.* 86, 405–431. doi: 10.1111/j.1095-8312.2005.00540.x

Garcia, J. E., Phillips, R., Peter, C. I., and Dyer, A. G. (2020). Changing how biologists view flowers - color as a perception not a trait. *Front. Plant Sci.* 11:1775. doi: 10.3389/fpls.2020.601700

Garcia, J. E., Shrestha, M., and Dyer, A. G. (2018). Flower signal variability overwhelms receptor-noise and requires plastic color learning in bees. *Behav. Ecol.* 29, 1286–1297. doi: 10.1093/beheco/ary127

Garcia, J. E., Spaethe, J., and Dyer, A. G. (2017). The path to colour discrimination is S-shaped: behaviour determines the interpretation of colour models. *J. Comp. Physiol. A* 203, 983–997. doi: 10.1007/s00359-017-1208-2

Giurfa, M. (2004). Conditioning procedure and color discrimination in the honeybee *Apis mellifera*. *Naturwissenschaften* 91, 228–231. doi: 10.1007/s00114-004-0530-z

Goldsmith, T. H. (1991). "Chapter 5: The evolution of visual pigments and colour vision," in *The Perception of Color*, ed P. Gouras (London: MaxMillan), 62–89.

Hanley, D., López, A. V., Fiorini, V. D., Reboreda, J. C., Grim, T., and Hauber, M. E. (2019). Variation in multicomponent recognition cues alters egg rejection decisions: a test of the optimal acceptance threshold hypothesis. *Philos. Trans. R. Soc. B Biol. Sci.* 374:1769. doi: 10.1098/rstb.2018.0195

Hannah, L., Dyer, A. G., Garcia, J. E., Dorin, A., and Burd, M. (2019). Psychophysics of the hoverfly: categorical or continuous color discrimination? *Curr. Zool.* 65, 483–492. doi: 10.1093/cz/zoz008

Hart, N. S., and Vorobyev, M. (2005). Modelling oil droplet absorption spectra and spectral sensitivities of bird cone photoreceptors. *J. Comp. Physiol.* 191, 381–392. doi: 10.1007/s00359-004-0595-3

Innis, G. J. (1989). Feeding ecology of fruit pigeons in subtropical rainforests of South-Eastern Queensland. *Wildl. Res.* 16, 365–394. doi: 10.1071/WR9890365

Iwaniuk, A. N., Keirnan, A. R., Janetzki, H., Mardon, K., Murphy, S., Leseberg, N. P., et al. (2020). The endocast of the Night Parrot (*Pezoporus occidentalis*) reveals insights into its sensory ecology and the evolution of nocturnality in birds. *Sci. Rep.* 10, 1–9. doi: 10.1038/s41598-020-70122-x

Judd, D. B., MacAdam, D. L., Wyszecki, G., Budde, H. W., Condit, H. R., Henderson, S. T., et al. (1964). Spectral distribution of typical daylight as a function of correlated color temperature. *J. Opt. Soc. Am.* 54:1031. doi: 10.1364/JOSA.54.001031

Kelber, A., Vorobyev, M., and Osorio, D. (2003). Animal colour vision - behavioural test and physiological concepts. *Biol. Rev.* 78, 81–118. doi: 10.1017/S1464793102005985

Kemp, D. J., Herberstein, M. E., Fleishman, L. J., Endler, J. A., Bennett, A. T. D., Dyer, A. G., et al. (2015). An integrative framework for the appraisal of coloration in nature. *Am. Nat.* 185, 705–724. doi: 10.1086/681021

Komatsu, H., and Ideura, Y. (1993). Relationships between color, shape, and pattern selectivities of neurons in the inferior temporal cortex of the monkey. *J. Neurophysiol.* 70, 677–694. doi: 10.1152/jn.1993.70.2.677

Kulikowski, J. J., Walsh, V., and Murray, I. J. (1991). "Chapter 5: On the limits of colour detection and discrimination," in *Limits of Vision*, eds J. J. Kulikowski, V. Walsh, I. J. Murray, and J. Cronly-Dillon (London: MacMillan Press), 202–220.

Lin, T.-Y., Luo, J., Shinomiya, K., Ting, C.-Y., Lu, Z., Meinertzhagen, I. A., et al. (2016). Mapping chromatic pathways in the drosophila visual system. *J. Comp. Neurol.* 524, 213–227. doi: 10.1002/cne.23857

Lind, O., Chavez, J., and Kelber, A. (2014). The contribution of single and double cones to spectral sensitivity in budgerigars during changing light conditions. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 200, 197–207. doi: 10.1007/s00359-013-0878-7

Lind, O., and Kelber, A. (2009). Avian colour vision: effects of variation in receptor sensitivity and noise data on model predictions as compared to behavioural results. *Vis. Res.* 49, 1939–1947. doi: 10.1016/j.visres.2009.05.003

Lunau, K., Papiorek, S., Eltz, T., and Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *J. Exp. Biol.* 214, 1607–1612. doi: 10.1242/jeb.052688

MacAdam, D. L. (1942). Visual sensitivities to color differences in daylight. *J. Opt. Soc. Am.* 32:247. doi: 10.1364/JOSA.32.000247

MacMillan, N. A., and Creelman, C. D. (1990). Response bias: characteristics of detection theory, threshold theory, and 'nonparametric' indexes. *Psychol. Bull.* 107, 401–413. doi: 10.1037/0033-2909.107.3.401

MacMillan, N. A., and Creelman, C. D. (2005). *Detection Theory, 2nd Edn*. New York, NY: Psychology Press. doi: 10.4324/9781410611147

Marshall, J. (2018). Do not be distracted by pretty colors: a comment on Olsson et al. *Behav. Ecol.* 29, 286–287. doi: 10.1093/beheco/arx164

Mota, T., Gronenberg, W., Giurfa, M., and Sandoz, J.-C. (2013). Chromatic processing in the anterior optic tubercle of the honey bee brain. *J. Neurosci.* 33, 4–16. doi: 10.1523/JNEUROSCI.1412-12.2013

Murton, R. K., and Westwood, N. J. (1966). The foods of the Rock Dove and Feral Pigeon. *Bird Study* 13, 130–146. doi: 10.1080/00063656609476116

Niu, Y., Stevens, M., and Sun, H. (2020). Commercial harvesting has driven the evolution of camouflage in an alpine plant. *Curr. Biol.* 31, 446–449.e4. doi: 10.1016/j.cub.2020.10.078

Norwich, K. H. (1987). On the theory of weber fractions. *Percept. Psychophys.* 42, 286–298. doi: 10.3758/BF03203081

Olsson, P., Lind, O., and Kelber, A. (2015). Bird colour vision: behavioural thresholds reveal receptor noise. *J. Exp. Biol.* 218, 184–193. doi: 10.1242/jeb.111187

Olsson, P., Lind, O., and Kelber, A. (2018). Chromatic and achromatic vision: parameter choice and limitations for reliable model predictions. *Behav. Ecol.* 29, 273–282. doi: 10.1093/beheco/arx133

Orlowski, J., Harmening, W., and Wagner, H. (2012). Night vision in barn owls: visual acuity and contrast sensitivity under dark adaptation. *J. Vis.* 12, 1–8. doi: 10.1167/12.13.4

Osorio, D., Mikló, A., and Gonda, Z. (1999). Visual ecology and perception of coloration patterns by domestic chicks. *Evol. Ecol.* 13, 673–689. doi: 10.1023/A:1011059715610

Paulk, A. C., Dacks, A. M., and Gronenberg, W. (2009). Color processing in the medulla of the bumblebee (Apidae: Bombus impatiens). *J. Comp. Neurol.* 513, 441–456. doi: 10.1002/cne.21993

Pauw, A. (2019). A bird's-eye view of pollination: biotic interactions as drivers of adaptation and community change. *Annu. Rev. Ecol. Evol. Syst.* 50, 477–502. doi: 10.1146/annurev-ecolsys-110218-024845

Pike, T. (2012). Preserving perceptual distances in chromaticity diagrams. *Behav. Ecol.* 23, 723–728. doi: 10.1093/beheco/ars018

Pinheiro, J. C., and Bates, D. M. (2000). *Mixed-Effects Models in S and S-PLUS*. New York, NY: Springer New York. doi: 10.1007/978-1-4419-0318-1

Renoult, J., Kelber, A., and Schaefer, M. H. (2017). Colour spaces in ecology and evolutionary biology. *Biol. Rev.* 92, 292–315. doi: 10.1111/brv.12230

Santiago, C., Green, N. F., Hamilton, N., Endler, J. A., Osorio, D. C., Marshall, N. J., et al. (2020). Does conspicuousness scale linearly with colour distance? A test using reef fish. *Proc. R. Soc. B Biol. Sci.* 287:20201456. doi: 10.1098/rspb.2020.1456

Schaefer, H. M., McGraw, K., and Catoni, C. (2008). Birds use fruit colour as honest signal of dietary antioxidant rewards. *Funct. Ecol.* 22, 303–310. doi: 10.1111/j.1365-2435.2007.01363.x

Shrestha, M., Dyer, A. G., Garcia, J. E., and Burd, M. (2019). Floral colour structure in two Australian herbaceous communities: it depends on who is looking. *Ann. Bot.* 124, 221–232. doi: 10.1093/aob/mcz043

Siuda-Krzywicka, K., Boros, M., Bartolomeo, P., and Witzel, C. (2019). The biological bases of colour categorisation: from goldfish to the human brain. *Cortex* 118, 82–106. doi: 10.1016/j.cortex.2019.04.010

Skorupski, P., and Chittka, L. (2010). Differences in photoreceptor processing speed for chromatic and achromatic vision in the bumblebee, *Bombus terrestris*. *J. Neurosci.* 30, 3896–3903. doi: 10.1523/JNEUROSCI.5700-09.2010

Skorupski, P., and Chittka, L. (2011). Is colour cognitive? *Opt. Laser Technol.* 43, 251–260. doi: 10.1016/j.optlastec.2008.12.015

Skorupski, P., Döring, T. F., and Chittka, L. (2007). Photoreceptor spectral sensitivity in island and mainland populations of the bumblebee, *Bombus terrestris*. *J. Comp. Physiol. A* 193, 485–494. doi: 10.1007/s00359-006-0206-6

Solomon, S. G., and Lennie, P. (2007). The machinery of colour vision. *Nat. Rev. Neurosci.* 8, 276–286. doi: 10.1038/nrn2094

Spaethe, J., Streinzer, M., Eckert, J., May, S., and Dyer, A. G. (2014). Behavioural evidence of colour vision in free flying stingless bees. *J. Comp. Physiol. A* 200, 485–486. doi: 10.1007/s00359-014-0886-2

Stavenga, D., Smits, R., and Hoenders, B. (1993). Simple exponential functions describing the absorbance bands of visual pigment spectra. *Vis. Res.* 33, 1011–1017. doi: 10.1016/0042-6989(93)90237-Q

Stevens, S. S. (1957). On the psychophysical law. *Psychol. Rev.* 64, 153–181. doi: 10.1037/h0046162

Troscianko, T., Benton, C. P., Lovell, P. G., Tolhurst, D. J., and Pizlo, Z. (2009). Camouflage and visual perception. *Philos. Trans. R. Soc. London Ser. B- Biol. Sci.* 364, 449–461. doi: 10.1098/rstb.2008.0218

van der Kooi, C. J., Dyer, A. G., Kevan, P. G., and Lunau, K. (2019). Functional significance of the optical properties of flowers for visual signalling. *Ann. Bot.* 123, 263–276. doi: 10.1093/aob/mcy119

von Helversen, O. (1972a). "Chapter 9: The relationship between difference in stimuli and choice frequency in training experiments with the honeybee," in *Inf. Process. Vis. Syst. Anthr*, ed R. Wehner (Berlin; Heidelberg: Springer), 323–334. doi: 10.1007/978-3-642-65477-0_46

von Helversen, O. (1972b). Zur spektralen unterschiedsempfindlichkeit der honigbiene. *J. Comp. Physiol.* 80, 439–472. doi: 10.1007/BF00696438

Vorobyev, M., Brandt, R., Peitsch, D., Laughlin, S. B., and Menzel, R. (2001). Colour thresholds and receptor noise: behaviour and physiology compared. *Vis. Res.* 41, 639–653. doi: 10.1016/S0042-6989(00)00288-1

Vorobyev, M., and Osorio, D. (1998). Receptor noise as a determinant of colour thresholds. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* 265, 351–358. doi: 10.1098/rspb.1998.0302

Warrant, E. J. (1999). Seeing better at night: life style, eye design and the optimum strategy of spatial and temporal summation. *Vis. Res.* 39, 1611–1630. doi: 10.1016/S0042-6989(98)00262-4

Whitney, K. D., Smith, A. K., Williams, C., and White, T. E. (2020). Birds perceive more intraspecific color variation in bird-pollinated than bee-pollinated flowers. *Front. Plant Sci.* 11:1806. doi: 10.3389/fpls.2020.590347

Wright, A. A. (1972). Psychometric and psychophysical hue discrimination functions for the pigeon. *Vis. Res.* 12, 1447–1464. doi: 10.1016/0042-6989(72)90171-X

Wright, A. A., and Cumming, W. W. (1971). Color-naming functions for the pigeon. *J. Exp. Anal. Behav.* 15, 7–17. doi: 10.1901/jeab.1971.15-7

Wyszecki, G., and Stiles, W. S. (1982). *Color Science Concepts and Methods, Quantitative Data and Formulae, 2nd Edn*. New York, NY: John Wiley & Sons, Inc.

Zuur, A. F., Hilbe, J. M., and Ieno, E. N. (2013). *A beginner's Guide to GLM and GLMM With R*. Newburgh: Highland Statistics.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

BRIEF RESEARCH REPORT
published: 14 June 2021
doi: 10.3389/fevo.2021.676369

Opsin Evolution in Flower-Visiting Beetles

Camilla R. Sharkey[1], Gareth S. Powell[2] and Seth M. Bybee[2]*

[1] Department of Ecology, Evolution and Behavior, University of Minnesota, Saint Paul, MN, United States, [2] Department of Biology & Monte L. Bean Museum, Brigham Young University, Provo, UT, United States

Flowers have evolved signals that exploit the sensory systems of insect visitors. In the case of visual cues, color signals are thought to have been shaped in large part by the spectral sensitivity of key pollinators, such as hymenopterans. Beetles were some of the first plant pollinators, pre-dating the angiosperm radiation but with the exception of a few well-studied species, the evolution of flower-visiting beetle visual systems is poorly understood. Thus, the ability of beetles to detect and distinguish flower color signals and perhaps their potential role in shaping flower coloration is not well understood. Traditional models of pollinator visual systems often assume a putative tri- or tetrachromatic flower-visitor, as is found in bees, flies and butterflies. Beetles are unique among modern pollinators as ancestrally they did not possess the machinery for trichromatic vision, lacking the blue-sensitive photoreceptor class. Research on the evolution of visual genes responsible for wavelength sensitivity (opsins) has revealed that beetles with putative tri- and tetrachromatic visual systems have evolved independently, along multiple lineages. We explore the evolution of beetle visual genes using newly generated and publicly available RNA-seq data from 25 species with flower associations, including previously unexplored key flower-visitor groups and 20 non-flower visiting relatives. Our findings serve as a resource to inform and guide future studies on beetle-flower interactions, where insight from both signal and receiver is needed to better understand these poorly explored systems.

Keywords: opsin, insect vision, pollinators, gene duplication, coleoptera (beetles)

OPEN ACCESS

Edited by:
Casper J. Van Der Kooi,
University of Groningen, Netherlands

Reviewed by:
Markus Friedrich,
Wayne State University, United States
Jair E. Garcia,
RMIT University, Australia

****Correspondence:***
Camilla R. Sharkey
camilla.r.sharkey@gmail.com

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *05 March 2021*
Accepted: *14 May 2021*
Published: *14 June 2021*

Citation:
Sharkey CR, Powell GS and Bybee SM (2021) Opsin Evolution in Flower-Visiting Beetles. Front. Ecol. Evol. 9:676369. doi: 10.3389/fevo.2021.676369

INTRODUCTION

Beetles were some of the earliest pollinators and remain the primary pollinators of ancient plant group, gymnosperms (Toon et al., 2020), while also functioning as common pollinators of more recent angiosperm (flowering plant) radiations. Angiosperm radiations during the Cretaceous co-occurred with accelerated diversification among the holometabolous insects that comprise the dominant insect pollinators: hymenopterans, lepidopterans, dipterans and coleopterans (Doyle, 2012; Misof et al., 2014). This co-radiation is thought to be in part due to the establishment of close associations or mutualisms between pollinator and plant, which likely contributed to the diversification of floral pollinator signals seen in modern flowers (Bronstein et al., 2006; Cardinal and Danforth, 2013). It is estimated that there are over 77,000 extant beetle species with flower associations (Wardhaugh, 2015) and flower visitation in these species has arisen *via* various evolutionary routes. While some species have retained existing ancestral gymnosperm associations (e.g., Boganiidae) (Cai et al., 2018), pollination behavior likely transitioned in some beetle lineages

from gymnosperms to angiosperms (e.g., Oedemeridae and Kateretidae) (Peris et al., 2017, 2020). In other beetle lineages, flower associations evolved without preexisting pollination behavior (e.g., Glaphyridae) (Sabatinelli et al., 2020), as in Anthophila (bees) (Cardinal and Danforth, 2013; Peters et al., 2017).

Traditionally, flowers visited by beetles have been described as dull in coloration and highly scented, suggesting that beetles do not use or are not reliant on spectral cues to detect flowers (Faegri and Van der Pijl, 1979). Perhaps unsurprisingly, considering the diversity of beetles, as more beetle-flower interactions have been described it has become clear that many beetle species do use spectral information for flower detection. Pollinators in the families Meloidae (blister beetles), Glaphyridae (bumble-bee scarabs), and Scarabaeidae (monkey beetles), use color alone as a cue for flower detection (Dafni et al., 1990; Steiner, 1998; Van Kleunen et al., 2007; Paudel et al., 2017). In carabids and fireflies, heavier investment in vision (larger eyes) rather than olfaction (reduced antennae) has been shown to be driven by visually mediated tasks (Bauer and Kredler, 1993; Stanger-Hall et al., 2018). It is not yet known whether there are similar trade-offs between flower visiting beetles that utilize predominantly visual or olfactory cues. Angiosperms that are pollinated primarily by beetles span at least 34 families (**Figure 1**; Bernhardt, 2000) thus there are likely many beetle-flower associations still to be described and the role of visual cues to be determined.

The role of insect pollinator visual systems in shaping floral color cues has been well studied in the context of hymenopteran pollination systems (e.g., Chittka, 1996; Dyer et al., 2012). The spectral sensitivities of hymenopteran UV-, blue- and green-sensitive photoreceptors are positioned for optimum wavelength discrimination of floral spectral cues (Chittka and Menzel, 1992). Rather than tuning of insect spectral sensitivity, this relationship is thought to have arisen from spectral tuning of floral reflectance, to existing photoreceptor sensitivities (Chittka, 1996; Dyer et al., 2012). The underlying sensitivity of a photoreceptor is determined by the structure of a GPCR protein (opsin), which is coupled to a light-absorbing chromophore pigment. Unlike the vast majority of insects studied, including bees and butterflies, beetles lack a key opsin (SW) that typically confers sensitivity to blue wavelengths of light (Jackowska et al., 2007), lost prior to the radiation of beetles (Sharkey et al., 2017). Based on spectral sensitivity measurements of beetles with this ancestral condition, it is assumed that the ancestor of all beetles had a dichromatic UV-green color visual system (Gribakin, 1981; Warrant and McIntyre, 1990).

The lack of a dedicated blue-sensitive photoreceptor channel impacts the discriminatory capabilities of certain wavelengths, particularly within the violet-blue region of the light spectrum where photon catch is low. The ancestral beetle visual system is less complex than bees, for example, which have retained all three insect opsin classes UV, SW and LW (long wavelength), resulting in UV- blue- and green-sensitivity. Opsin duplication events are the major route for acquiring additional spectral channels. Duplications alone do not lead to novel spectral sensitivities; selection for changes in function is required via mutations that lead to changes in protein (subfunctionalization). In a number of coleopteran lineages, duplications of the UV opsin and subsequent subfunctionalization has led to the ability of these taxa to perceive blue wavelengths (Sharkey et al., 2017) by essentially "re-evolving" a dedicated blue-sensitive photoreceptor, for example in coccinellids (Lin, 1993) and chrysomelids (Döring and Skorupski, 2007). There is also evidence that flower-visiting lineages of beetles in the families Scarabaeidae, Nitidulidae, and Curculionidae have expanded their opsin repertoire, likely expanding their wavelength sensitivities (Sharkey et al., 2017). It is not known if this is true across the diversity of beetle pollinators.

We explore opsin expansions across the diversity of beetle pollinators using publicly available RNA-seq data, opsin sequences and an additional nine transcriptomes generated in this study. We aimed to investigate the evolution of the visual genes (opsins) that underpin sensitivity to spectral (color) information. Transcriptome data allow us to examine the diversity of opsin genes and hence putative wavelength sensitivities, from these flower-visiting species and their close relatives. In addition, we examine relative eye size in these species as an indicator of visual system investment.

MATERIALS AND METHODS

RNA Extraction and Assembly

Seven flower-visiting beetle species (*Trirhabda eriodictyonis*, *Sibinia setosa*, *Tychius meliloti*, *Anthrenus lepidus*, *Mordella albosuturalis*, *Nitops pallipennis,* and *Anaspis rufa*) were collected from flowers in southern Utah into RNAlater and later frozen (–80°C) until processed. Multiple individuals were pooled for RNA extraction with the exception of *T. eriodictyonis* where one male was used. Total RNA was extracted from adult whole bodies using Nucleospin spin columns (Clontech) and reverse transcribed into cDNAs using the Illumina TruSeq RNA v2 kit. Sequencing was done on an Illumina HiSeq 2,500 generating 100 bp paired-end reads (BYU DNA sequencing center). RNA from two additional nitidulid species was extracted using the RNeasy Mini kit (Qiagen) and transcribed with the KAPA Stranded mRNA-Seq kit (Roche). Sequencing was completed on an Illumina HiSeq 2,500 generating 250 bp paired-end reads (BYU DNA sequencing center). Additionally, paired-end RNA-seq data from 36 species were downloaded from the Sequence Read Archive (SRA). Data were trimmed using Trimmomatic (Bolger et al., 2014), removing adapter sequences and poor-quality bases using the parameters: SLIDINGWINDOW:4:5 LEADING:5 TRAILING:5 MINLEN:25. Trinity (v2.11.0) (Grabherr et al., 2011; Haas et al., 2013) was used to assemble the remaining reads. Completeness of assemblies was estimated by searching each for the presence of 1367 insect Benchmarking Universal Single-Copy Orthologs using BUSCO v5 (Simão et al., 2015; Seppey et al., 2019). See **Supplementary Table 1** for all species information. Opsin sequences have been deposited in GenBank with accession numbers MW885977—MW886096 and RNA-seq data have been uploaded to the Sequence Read Archive (PRJNA718629).

FIGURE 1 | Flower-visiting beetle families included in this study. From left to right: Cetoniinae (*Trichaulax philipsii*), Buprestidae (*Acmaeodera* sp.), Lycidae (*Lycus* sp.), Curculionidae and Nitidulidae (*Aethina concolor*). Images by: Chris Moeseneder, GSP, Russ Anderson, Steven Marshall, GSP.

Opsin Search

Coding regions were predicted using TransDecoder v5.5.0,[1] retaining the longest open reading frame (ORF). All predicted ORFs were also BLASTed[2] against a database of known arthropod opsins (orthodb EOG8NKF98), with the addition of Lampyridae and *Thermonectus marmoratus* full-length opsin copies with an *e*-value of 0.001. All remaining ORFs were searched against our insect opsin database using hmmscan in HMMER (v3.3) (Eddy, 2011). Cross-contamination and pseudogenes were removed using phylogenies of DNA and protein opsin sequences and by examining alignments. Opsins with > 99% similarity in protein sequence, likely structurally and functionally identical, were removed (CD-hit v4.8.1; Li and Godzik, 2006; Fu et al., 2012). Duplicates with 100% sequence identity using local alignment (BLASTp) were considered to be one opsin gene (*Anthocomus equestris* and *Pharaxonotha floridana*; see asterisks **Figure 2** and **Supplementary Table 2**).

Final DNA sequences and additional insect opsins were subject to codon alignment (MAFFT v7.453; Katoh et al., 2002; Katoh and Standley, 2013) with automatic alignment strategy detection. Maximum likelihood opsin DNA gene trees were generated using IQ-tree (v1.6.12) (Minh et al., 2013; Nguyen et al., 2015). The substitution model GTR + F + I + G4 was selected automatically using ModelFinder (Kalyaanamoorthy et al., 2017). A species topology was generated in Mesquite 3.2 (Maddison and Maddison, 2018) based on McKenna et al. (2019). For statistical analyses, beetle species were categorized as obligate flower-visitors that require floral resources for food or reproduction (A), facultative flower visitors with known floral associations (e.g., facultative pollinators) but no reliance on floral resources for food or reproduction (B) and non-flower visitors with no known association with flowers (C).

Eye Size Measurements and Statistics

Relative eye size was generated using the measurement tools in Adobe Photoshop v.19.1.6 from high resolution habitus photos (available upon request due to copyright) with an unimpeded dorsal view of the head. Two measurements were taken for each photo: total head width including eyes, and interocular distance (see **Supplementary Figure 1** for example image). These two measurements were used to generate the total width of the eyes in the dorsal view and used to calculate a percentage of lateral head space dedicated to eye tissue as viewed dorsally. We tested for a relationship between UV and LW opsin copy number (Pearson's correlation test) and if flower visitation behavior predicted relative eye-size (one-way ANOVA). Both were included as predictors in the final MANOVA. Because LW and UV were not correlated, we chose to test the effects of relative eye size and flower visitation on both LW and UV opsin copy number (MANOVA). For the purpose of this analysis, species without UV opsin copies were omitted. All data met required assumptions, and no data transformations were performed. All eye size statistical tests were executed in SPSS 27 (IBMCorp.).

RESULTS

Opsin Duplications

A transcriptomics approach was taken to explore the putative wavelength sensitivities of flower visiting and non-flower visiting beetle species. To determine possible wavelength sensitivity expansions, RNA-seq data from 45 species spanning 26 families were mined for opsins and data were analyzed for the presence of duplication events. Nine transcriptomes from flower-visiting species were generated for this study to expand the diversity of sampled lineages. We recovered 120 opsin copies, 92 of which were full length copies with a minimum length of 101 amino acids. We classified 18 beetle species as obligate flower visitors (category A), seven as facultative flower visitors (category B), and 20 with no known floral associations (category C). Additionally, we included 18 previously published beetle opsins from five category A species, 11 opsins from four category B species and 14 opsins from five category C species (Sharkey et al., 2017; **Supplementary Table 1**).

In our dataset both obligate (A) and facultative (B) flower visitors had 2-fold higher proportions of UV duplications present, 39.1 and 45.5%, respectively, than non-flower visitors (C) with only 20%. LW duplications were more common in obligate flower visitors (52.2%) than either facultative (18.2%) or non-flower visitors (12%). The prevalence of either opsin duplication was highest in obligate flower visitors (73.9%), lower in flower-associated species (54.5%) and lowest in non-flower visitors sampled (28%). We report 33 independent opsin duplication events (12 UV and 21 LW), which have occurred across the diversity of Coleoptera (**Figure 2**). Duplication events occurred

[1]http://transdecoder.github.io

[2]https://blast.ncbi.nlm.nih.gov/

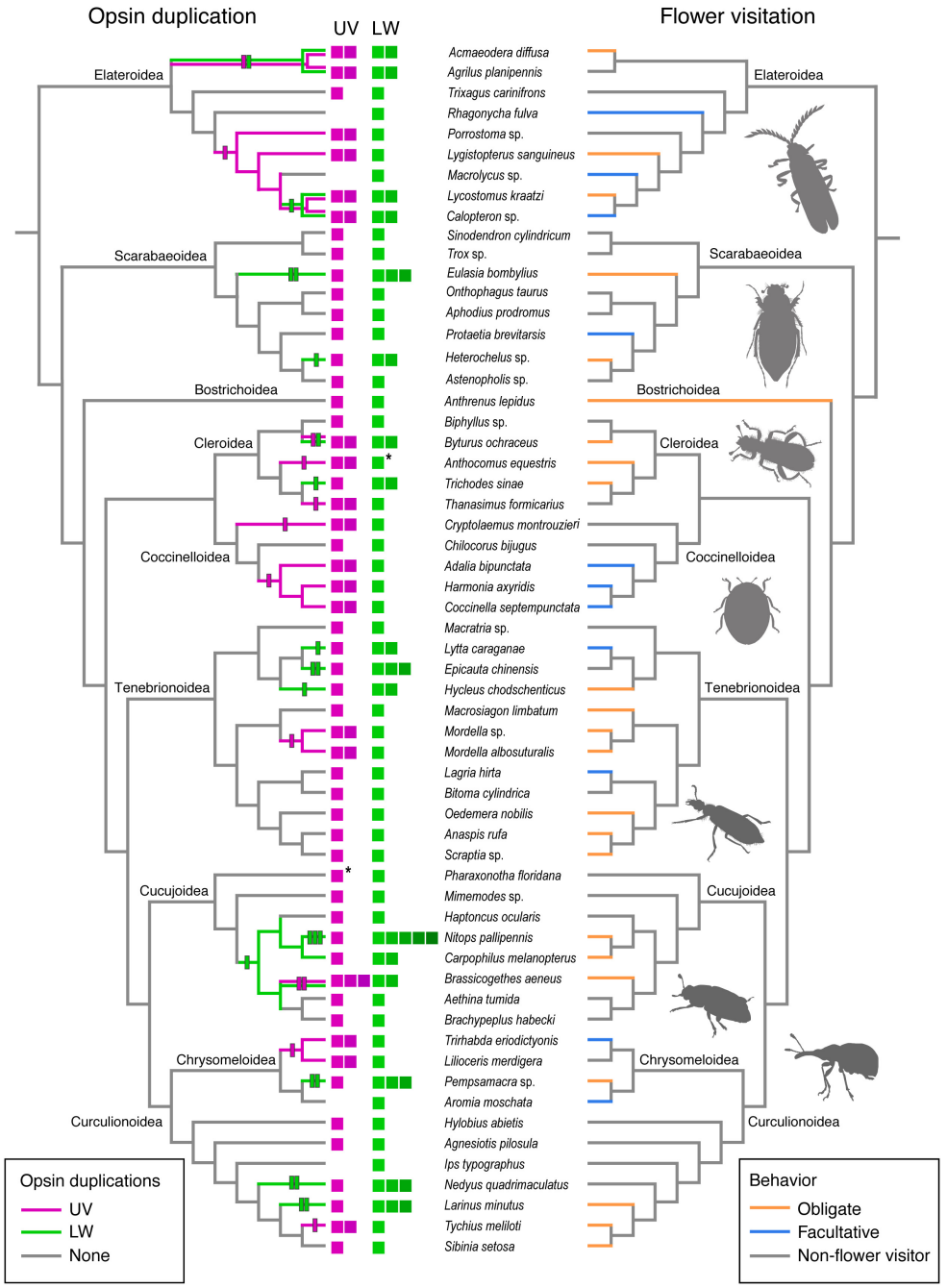

FIGURE 2 | Patterns of opsin duplication and flower-visitation behavior for 59 beetle species. The cladograms represent a simplified version of beetle relationships after McKenna et al. (2019) with the position of superfamilies indicated. The left-hand cladogram shows opsin duplication events with opsin copy number at the terminals and duplication events indicated on the branches. Duplication events were estimated using the opsin tree (see **Supplementary Figure 2**). Asterisks (*) denote cases where additional duplication copies were found but determined not to be functionally distinct (see Opsin Search Methods text). The right-hand cladogram reflects known behavioral categories for the level of flower visitation. No attempt at ancestral reconstruction was made due to the available taxon sampling.

within all but one superfamily, Bostrichoidea, which has only a single flower-visiting lineage (the Dermestidae) represented by *Anthrenus lepidus*. Percent identity (sequence similarity) of aligned duplicate opsins (BLASTp, see text footnote 2) ranged from 64 to 98% sequence identity and a mean of 80% (see **Supplementary Table 2**).

Within Scarabaeoidea only flower visiting scarabs exhibit opsin duplications. These duplications were independent and

solely among LW opsins in *Heterochelus* sp. and *Eulasia bombylius* with one and two duplications, respectively (**Figure 2**). Notably, *Protaetia brevitarsis*, which can commonly be found on flowers (B) but is not considered an obligate flower visitor, did not have opsin duplicates. Among the five Lycidae (Elateroidea) species sampled, the UV opsin duplicates present were orthologous (**Figure 2** and **Supplementary Figure 2**) and therefore represent an early duplication event in this group. Two LW opsin copies were present in two lycid species *Lycostomus kraatzi* and *Calopteron* sp. However, this was not linked to flower visiting behavior with the former likely an obligate (A) and the latter only facultatively associated with flowers (B). Rather, these species are among the most derived members of this group and therefore highlight a potential ancestral LW opsin duplication event that occurred during the lycid radiation. All flower visiting members (A) of Cleroidea have either UV or LW duplicates. Each duplication event was independent, including within the subfamily Clerinae where a UV duplication occurred in *Thanasimus formicarius* and a LW duplication in the flower visitor *Trichodes sinae*.

Coccinellid opsin UV duplicates have been previously examined (Sharkey et al., 2017). We add the species *Chilocorus bijugus* (tribe: Chilocorini), which has no known associations with flowers. No UV duplicate was recovered for this species making this species unique among the coccinellids sampled thus far. Our opsin phylogeny (**Supplementary Figure 2**) suggest that two separate UV duplication events occurred, one prior to the diversification of the tribe Coccinellini and the other along lineage sister to the rest of the coccinellids, *Cryptolaemus montrouzieri* (tribe: Coccidulini). We note only two opsin duplication events within the Tenebrionoidea, within the blister beetles Meloidae (*Hycleus chodschenticus*) (LW duplication) and tumbling flower beetles Mordellidae (*Mordella* sp. and *Mordella albosuturalis*) (UV duplication). Opsin orthologs are present in both *Mordella* species, suggesting that this duplicate may be shared among other members of this genus.

A close relationship between flower visitation and opsin copy number can be clearly seen among the cucujoids (**Figure 2**). Single UV and LW opsin copies are present in all non-flower visiting species but UV and/or LW opsin duplications are present among all flower-visiting nitidulids sampled (**Figure 2**). The LW opsin duplicates form two clades shared among these species suggesting an early LW duplication event in Nitidulidae that has potentially been lost in the three non-flower visiting lineages. Two of the three obligate flower visiting weevils (Curculionidae) species possess additional opsin copies.

While duplications more commonly occurred amongst flower visiting species (categories A and B), than in non-flower visitors (C), 26% of obligate flower visitors have retained the ancestral UV-LW opsin condition. Among the obligate flower visitors, four of the seven tenebrionid species sampled have single opsin copies, including both members of Scraptiidae (*Scraptia* sp. and *Anaspis rufa*), *Oedemera nobilis* (Oedemeridae), and *Macrosiagon limbatum* (Ripiphoridae) (**Figure 2**). Carpet beetle *Anthrenus lepidus* (Dermestidae: Bostrichoidea), *Biphyllus* sp. (Biphyllidae: Cleroidea) and two weevil species, *Tychius meliloti* and *Sibinia setosa* (Curculionidae: Curculionoidea) also did not have opsin duplications. We did not recover UV opsins from four non-obligate flower visiting species, *Rhagonycha fulva*, *Macrolycus* sp., *Aromia moschata* or *Ips typographus* but LW opsins were recovered from all of these species.

Eye Size, Flower Visitation and Opsin Copy Number

Relative eye size was used as an approximate measure of visual investment across the species in this study. We found no significant correlation between UV and LW opsin copy number ($p = 0.893$). Flower visitation category (A, B or C) did significantly predict relative eye size ($F = 8.52$, $df = 2$, $p < 0.001$). *Post hoc* tests revealed obligate flower visiting species (A) have significantly larger eyes than those without any floral association (C) ($p < 0.001$, mean difference = 11.15%). Flower visitation category in conjunction with relative eye size was a significant predictor of LW copy number ($F = 3.895$, $df = 32$, $p = 0.015$), but not UV copy number ($F = 2.06.11$, $df = 32$, $p = 0.122$). While the overall model showed evidence for the combination of both flower visitation and eye size influencing LW copy number, neither was significant individually.

DISCUSSION

A previous study (Sharkey et al., 2017) found opsin duplications in four of five flower-visiting beetle species. While LW opsin duplications were not common generally, in the coleopterans sampled, they were ubiquitous among these flower-visitors. These findings suggested that there may be a selective advantage for duplications, such as an expansion of wavelength sensitivity, among species that may use floral cues. Our opsin analysis across numerous flower-visiting species of coleopterans suggests that associations with flowers has often led to or precedes the expansion of wavelength sensitivity through opsin duplication. We cannot attribute an opsin duplication event strictly to the evolution of flower visitation behavior due to associated factors, such as diurnality (Sondhi et al., 2021) and other visually guided behaviors (e.g., host-plant seeking), for which expanded wavelength sensitivity may be beneficial. However, by including closely related non-flower visiting relatives in this study, we show that duplication events and particularly LW opsin duplications, do commonly occur along lineages associated with flowers, more so than those that have no floral associations. The frequency of duplications varies among other pollinator groups. Hymenopterans exhibit relatively stable opsin copy numbers (Spaethe and Briscoe, 2004; Oeyen et al., 2020) but in contrast the opsin repertoire of lepidopterans is highly variable, particularly within the butterflies, Papilionoidea (Sondhi et al., 2021). Our finding that there have been many independent duplication events in flower-visiting coleopterans may be attributed to the ancestral loss of the SW opsin in this group, or as in butterflies, may suggest increased spectral richness of the visual system.

Opsin duplications do not in all cases lead to new photoreceptor sensitivities, as is the extreme case in odonates, where there is a large excess of opsin copies (Futahashi et al.,

2015; Suvorov et al., 2016) compared to measured photoreceptor sensitivities (Laughlin, 1976; Yang and Osorio, 1991). In contrast, it has been shown that beetles are relatively conservative in opsin copy number (Sharkey et al., 2017). Therefore, opsin duplicates can be used as a guide for photoreceptor sensitivity diversity. For example, all sampled jewel beetles (Buprestidae) and *Carabus* sp. (Carabidae) have 2 UV and 2 LW opsin copies (Lord et al., 2016; Sharkey et al., 2017) that underpin UV-, blue-, green-, and red-sensitive photoreceptors (Hasselmann, 1962; Meglič et al., 2020), demonstrating increased function of photoreceptor sensitivity for these groups as a result of opsin duplication. Further, the presence of blue-sensitive photoreceptors also aligns well with the presence of a UV opsin duplication, in coccinellids (Lin and Wu, 1992) and chrysomelids (Döring and Skorupski, 2007). Additionally, species with ancestral UV-green sensitivity and complementary opsin data only possess a single UV and a single LW opsin copy (e.g., Lampyridae and *Dendroctonus*) (Groberman and Borden, 1982; Lall et al., 2010; Martin et al., 2015; Sander and Hall, 2015).

UV opsins were absent in four species. The UV opsin typically has lower expression levels as often UV-sensitive photoreceptors are less numerous than long wavelength-sensitive receptors in insects. Thus, we cannot be sure whether this finding reflects inadequate sequencing depth in these species or a true opsin loss. The use of head or eye tissue rather than whole body specimens, or deeper sequencing, may be necessary for future studies. Additionally, opsin expressed in photoreceptors with low abundance, such as those used in highly specialized regions (e.g., the polarization-sensitive dorsal rim area) may also have low signal. Further study to determine opsin abundance and expression patterns is required to better understand how well opsin copy number is estimated from whole body specimens, in particular those with reduced eyes.

Flowers commonly frequented by beetles have been traditionally described as dull in coloration (white, green or yellow) but highly scented, thought to exploit existing attraction to certain volatile compounds, such as skatole, attractive to coprophagous beetles (Schiestl and Dötterl, 2012). Despite compelling evidence that many beetles use floral visual cues for flower visitation, the study of associated visual adaptations is lacking. Scarab pollinators provide compelling evidence for vision as the primary cue for flower detection. Bumble-bee scarabs (Glaphyridae) and monkey beetles (Hopliini) use the dark center of a flower as a visual cue, termed "beetle marks" (Dafni et al., 1990; Johnson and Midgley, 2001). Other non-scarab beetle pollinators are also attracted to dark spots on flowers, e.g., nitidulids (Free and Williams, 1978) and mordellids (Westmoreland and Muntan, 1996).

In scarab beetles, anthophagy (flower feeding) has evolved at least seven times (Ahrens et al., 2014). Three of these lineages have been sampled in this study: Glaphyridae, Hopliini and Cetoniinae. We found LW opsin duplications among the two lineages that are known to have preferences for red and orange flowers, Glaphyridae (Sabatinelli et al., 2020) and Hopliini (Johnson and Midgley, 2001). The bumble-bee scarab *Pygopleurus israelitus* (Glaphyridae) has both green- and red-sensitive photoreceptors (λ_{max}: 631 nm) the latter of which increase the conspicuousness of red bowl-shaped flowers they visit (Martínez-Harms et al., 2012). This suggests a role for LW opsin duplication and subfunctionalization to expand long-wavelength sensitivity for floral detection. Electrophysiological measurements of *Protaetia brevitarsis* (Cetoniinae), a facultative flower visitor, are in agreement with our finding that this species only has UV and green sensitivity (Lin and Wu, 1992) but curiously this species exhibits attraction to red over green stimuli (Cai et al., 2021). We have yet to find any opsin duplications in the eight additional scarabs examined thus far (Sharkey et al., 2017), highlighting flower-visiting scarabs as an interesting group to study visual systems and signals in the context of anthophily.

Flower visitation is not always a predictor of an opsin duplication event; opsin duplications were absent from six flower-visiting lineages. It is possible that these species rely more heavily on olfaction than vision, and we may expect to see greater investment in these sensory structures (e.g., larger antennae), rather than vision (Bauer and Kredler, 1993; Stanger-Hall et al., 2018). Such sensory adaptations can be seen in the flabellate (fan-shaped) antennae of *Macrosiagon limbatum* (Ripiphoridae). Additionally, if odor was a primary cue in ancient cycads, as in modern species (Toon et al., 2020), lineages such as the false oil beetles (Oedemeridae) may have initially established olfactory rather than visual specializations that persisted after they transitioned to angiosperm hosts (Peris et al., 2017). A UV-green color channel system may be adequate to detect floral cues commonly attractive to beetles, e.g., white and yellow (Reverté et al., 2016), but wavelength sensitivity expansion may be advantageous to detect floral cues that fall outside this spectral range (e.g., pink or violet).

In this study we measured eye size as a proportion of the head (i.e., relative eye size), which has been used in prior studies of beetles to test relationships between visual investment and behavior (Bauer and Kredler, 1993; Stanger-Hall et al., 2018). This does not give a perfect estimation of eye volume due to the variation in eye shape across coleopterans with extreme morphological diversity. However, using relative eye size to examine the potential link between flower visitation, opsin copy number and investment in vision reveals some interesting findings. For the species examined in this study, eye size was found to be predicted by flower visitation behavior with obligate flower visitors having larger eyes as a proportion of the head. This suggests that flower-visitation or associated visual ecology may have driven selection for greater investment in vision. Similarly, flower visitors were also more likely to have a greater LW opsin copy number, suggesting that an expansion of long-wavelength sensitivity is advantageous for behaviors associated with flower visitation in the species we have examined. This is certainly true among beetles that prefer orange and red flowers and have dedicated red-sensitive photoreceptors, but red flower visitation is not commonplace among other species with LW duplications examined herein. Interestingly, among all species, LW opsin copy number was not correlated with eye size suggesting that it is not eye size alone that predicts the number of opsin duplicates. This may point to greater visual specialization in flower visiting beetles.

Our aim was to demonstrate that there is much more exciting work that needs to be done to better understand the evolution of beetle visual systems. This is particularly true for flower visitors, which have multiple origins within the majority of coleopteran superfamilies. Equally exciting is the large visual system diversity, morphological, molecular and certainly functional, among the anthophilous Coleoptera. In short, beetles represent a largely untapped area to study insect plant interactions at both the fine and coarse scales of evolution.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA718629.

AUTHOR CONTRIBUTIONS

CS conceived the study, ran transcriptomic and phylogenetic analyses, and wrote the manuscript. GP advised on beetle taxa, collected and analyzed eye size measurements. SB advised on beetle taxa and generated the species topology. All authors edited the manuscript and approved the submitted version.

FUNDING

This project was made possible by funding from the National Science Foundation DEB-1655981 to SB.

ACKNOWLEDGMENTS

We thank all the researchers who generated RNA-seq data that was used in this study. We are grateful to Nathan Lord and Gavin Martin for help with sample collection and processing. We also thank Alexandra Duffy for statistics advice.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.676369/full#supplementary-material

REFERENCES

Ahrens, D., Schwarzer, J., and Vogler, A. P. (2014). The evolution of scarab beetles tracks the sequential rise of angiosperms and mammals. *Proc. R. Soc. B Biol. Sci.* 281:20141470. doi: 10.1098/rspb.2014.1470

Bauer, T., and Kredler, M. (1993). Morphology of the compound eyes as an indicator of life-style in carabid beetles. *Can. J. Zool.* 71, 799–810. doi: 10.1139/z93-105

Bernhardt, P. (2000). Convergent evolution and adaptive radiation of beetle-pollinated angiosperms. *Plant Syst. Evol.* 222, 293–320. doi: 10.1007/BF00984108

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Bronstein, J. L., Alarcón, R., and Geber, M. (2006). The evolution of plant – insect mutualisms. *New Phytol.* 172, 412–428. doi: 10.1111/j.1469-8137.2006.01864.x

Cai, C., Escalona, H. E., Li, L., Yin, Z., Huang, D., and Engel, M. S. (2018). Beetle pollination of cycads in the mesozoic. *Curr. Biol.* 28, 2806–2812. doi: 10.1016/j.cub.2018.06.036

Cai, H., Zhang, T., Su, Y., Wang, Z., Zhang, X., Wang, S., et al. (2021). Influence of trap color, type, and placement on capture efficacy for *Protaetia brevitarsis* (Coleoptera: Scarabaeidae). *J. Econ. Entomol.* 114, 225–230. doi: 10.1093/jee/toaa259

Cardinal, S., and Danforth, B. N. (2013). Bees diversified in the age of eudicots. *Proc. R. Soc. B Biol. Sci.* 280:20122686. doi: 10.1098/rspb.2012.2686

Chittka, L. (1996). Does bee color vision predate the evolution of flower color? *Naturwissenschaften* 83, 136–138. doi: 10.1007/s001140050263

Chittka, L., and Menzel, R. (1992). The evolutionary adaptation of flower colours and the insect pollinators' colour vision. *J. Comp. Physiol. A* 171, 171–181. doi: 10.1007/BF00188925

Dafni, A., Bernhardt, P., Shmida, A., Ivri, Y., Greenbaum, S., O'Toole, C. H., et al. (1990). Red bowl-shaped flowers: convergence for beetle pollination in the Mediterranean region. *Isr. J. Bot.* 39, 81–92.

Döring, T. F., and Skorupski, P. (2007). Host and non-host leaves in the colour space of the Colorado potato beetle (Coleoptera: Chrysomelidae). *Entomol. Gen.* 29, 81–95. doi: 10.1127/entom.gen/29/2007/81

Doyle, J. A. (2012). Molecular and fossil evidence on the origin of angiosperms. *Annu. Rev. Earth Planet. Sci.* 40, 301–326. doi: 10.1146/annurev-earth-042711-105313

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G. P., Simonov, V., and Wong, B. B. M. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. Biol. Sci.* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Eddy, S. R. (2011). Accelerated profile HMM searches. *PLoS Comput. Biol.* 7:e1002195. doi: 10.1371/journal.pcbi.1002195

Faegri, K., and Van der Pijl, L. (1979). *The principles of pollination ecology*. Oxford, UK: Pergamon Press.

Free, J. B., and Williams, I. H. (1978). The responses of the pollen beetle, *Meligethes aeneus*, and the seed weevil, *Ceuthorhynchus assimilis*, to oil-seed rape, *Brassica napus*, and other plants. *J. Appl. Ecol.* 15, 761–774. doi: 10.2307/2402773

Fu, L., Niu, B., Zhu, Z., Wu, S., and Li, W. (2012). CD-HIT: Accelerated for clustering the next-generation sequencing data. *Bioinformatics* 28, 3150–3152. doi: 10.1093/bioinformatics/bts565

Futahashi, R., Kawahara-Miki, R., Kinoshita, M., Yoshitake, K., Yajima, S., Arikawa, K., et al. (2015). Extraordinary diversity of visual opsin genes in dragonflies. *Proc. Natl. Acad. Sci.* 112, E1247–E1256. doi: 10.1073/pnas.1424670112

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* 29, 644–652. doi: 10.1038/nbt.1883

Gribakin, F. G. (1981). Automatic spectrosensitometry of photoreceptors in *Lethrus* (Coleoptera, Scarabaeidae). *J. Comp. Physiol. A* 142, 95–102. doi: 10.1007/BF00605481

Groberman, L. J., and Borden, J. H. (1982). Electrophysiological response of *Dendroctonus pseudotsugae* and *Ips paraconfusus* (Coleoptera: Scolytidae) to selected wavelength regions of the visible spectrum. *Can. J. Zool.* 60, 2180–2189. doi: 10.1139/z82-279

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., et al. (2013). *De novo* transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat. Protoc.* 8, 1494–1512. doi: 10.1038/nprot.2013.084

Hasselmann, E. M. (1962). Über die relative spektrale Empfindlichkeit von Käfer- und Schmetterlingsaugen bei verschiedenen Helligkeiten. *Zool. Jahrbucher* 69, 537–576.

Jackowska, M., Bao, R., Liu, Z., McDonald, E. C., Cook, T. A., and Friedrich, M. (2007). Genomic and gene regulatory signatures of cryptozoic adaptation: Loss of blue sensitive photoreceptors through expansion of long wavelength-opsin expression in the red flour beetle *Tribolium castaneum*. *Front. Zool.* 4:24. doi: 10.1186/1742-9994-4-24

Johnson, S. D., and Midgley, J. J. (2001). Pollination by monkey beetles (Scarabaeidae: Hopliini): Do color and dark centers of flowers influence alighting behavior? *Environ. Entomol.* 30, 861–868. doi: 10.1603/0046-225X-30.5.861

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., Von Haeseler, A., and Jermiin, L. S. (2017). ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nat. Methods* 14, 587–589. doi: 10.1038/nmeth.4285

Katoh, K., Misawa, K., Kuma, K., and Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 30, 3059–3066. doi: 10.1093/nar/gkf436

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Lall, A. B., Cronin, T. W., Carvalho, A. A., de Souza, J. M., Barros, M. P., Stevani, C. V., et al. (2010). Vision in click beetles (Coleoptera: Elateridae): pigments and spectral correspondence between visual sensitivity and species bioluminescence emission. *J. Comp. Physiol. A* 196, 629–638. doi: 10.1007/s00359-010-0549-x

Laughlin, S. B. (1976). The sensitivities of dragonfly photoreceptors and the voltage gain of transduction. *J. Comp. Physiol. A* 111, 221–247. doi: 10.1007/BF00606466

Li, W., and Godzik, A. (2006). Cd-hit: A fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* 22, 1658–1659. doi: 10.1093/bioinformatics/btl158

Lin, J. T. (1993). Identification of photoreceptor locations in the compound eye of *Coccinella septempunctata* Linnaeus (Coleoptera, Coccinellidae). *J. Insect Physiol.* 39, 555–562. doi: 10.1016/0022-1910(93)90037-R

Lin, J. T., and Wu, C. Y. (1992). A comparative study on the color vision of four coleopterous insects. *Bull. Inst. Zool. Acad. Sin.* 31, 81–88.

Lord, N. P., Plimpton, R. L., Sharkey, C. R., Suvorov, A., Lelito, J. P., Willardson, B. M., et al. (2016). A cure for the blues: opsin duplication and subfunctionalization for short-wavelength sensitivity in jewel beetles (Coleoptera: Buprestidae). *BMC Evol. Biol.* 16:107. doi: 10.1186/s12862-016-0674-4

Maddison, W., and Maddison, D. (2018). *Mesquite: a modular system for evolutionary analysis. Version 3.2*. Available Online at: http://www.mesquiteproject.org

Martin, G. J., Lord, N. P., Branham, M. A., and Bybee, S. M. (2015). Review of the firefly visual system (Coleoptera: Lampyridae) and evolution of the opsin genes underlying color vision. *Org. Divers. Evol.* 15, 513–526. doi: 10.1007/s13127-015-0212-z

Martínez-Harms, J., Vorobyev, M., Schorn, J., Shmida, A., Keasar, T., Homberg, U., et al. (2012). Evidence of red sensitive photoreceptors in *Pygopleurus israelitus* (Glaphyridae: Coleoptera) and its implications for beetle pollination in the southeast Mediterranean. *J. Comp. Physiol. A* 198, 451–463. doi: 10.1007/s00359-012-0722-5

McKenna, D. D., Shin, S., Ahrens, D., Balke, M., Beza-Beza, C., Clarke, D. J., et al. (2019). The evolution and genomic basis of beetle diversity. *Proc. Natl. Acad. Sci. U. S. A.* 116, 24729–24737. doi: 10.1073/pnas.1909655116

Meglič, A., Ilić, M., Quero, C., Arikawa, K., and Belušič, G. (2020). Two chiral types of randomly rotated ommatidia are distributed across the retina of the flathead oak borer *Coraebus undatus* (Coleoptera: Buprestidae). *J. Exp. Biol.* 223:225920. doi: 10.1242/jeb.225920

Minh, B. Q., Nguyen, M. A. T., and Von Haeseler, A. (2013). Ultrafast approximation for phylogenetic bootstrap. *Mol. Biol. Evol.* 30, 1188–1195. doi: 10.1093/molbev/mst024

Misof, B., Liu, S., Meusemann, K., Peters, R. S., Donath, A., Mayer, C., et al. (2014). Phylogenomics resolves the timing and pattern of insect evolution. *Science* 346, 763–767. doi: 10.1126/science.1257570

Nguyen, L. T., Schmidt, H. A., Von Haeseler, A., and Minh, B. Q. (2015). IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* 32, 268–274. doi: 10.1093/molbev/msu300

Oeyen, J. P., Baa-Puyoulet, P., Benoit, J. B., Beukeboom, L. W., Bornberg-Bauer, E., Buttstedt, A., et al. (2020). Sawfly genomes reveal evolutionary acquisitions that fostered the mega-radiation of parasitoid and eusocial Hymenoptera. *Genome Biol. Evol.* 12, 1099–1188. doi: 10.1093/GBE/EVAA106

Paudel, B. R., Shrestha, M., Dyer, A. G., and Li, Q.-J. (2017). Ginger and the beetle: Evidence of primitive pollination system in a Himalayan endemic alpine ginger (*Roscoea alpina*, Zingiberaceae). *PLoS One* 12:e0180460. doi: 10.1371/journal.pone.0180460

Peris, D., Labandeira, C. C., Barrón, E., Delclòs, X., Rust, J., and Wang, B. (2020). Generalist pollen-feeding beetles during the Mid-Cretaceous. *iScience* 23:100913. doi: 10.1016/j.isci.2020.100913

Peris, D., Pérez-de la Fuente, R., Peñalver, E., Delclòs, X., Barrón, E., and Labandeira, C. C. (2017). False blister beetles and the expansion of gymnosperm-insect pollination modes before angiosperm dominance. *Curr. Biol.* 27, 897–904. doi: 10.1016/j.cub.2017.02.009

Peters, R. S., Krogmann, L., Mayer, C., Donath, A., Gunkel, S., Meusemann, K., et al. (2017). Evolutionary history of the Hymenoptera. *Curr. Biol.* 27, 1013–1018. doi: 10.1016/j.cub.2017.01.027

Reverté, S., Retana, J., Gómez, J. M., and Bosch, J. (2016). Pollinators show flower colour preferences but flowers with similar colours do not attract similar pollinators. *Ann. Bot.* 118, 249–257. doi: 10.1093/aob/mcw103

Sabatinelli, G., Eberle, J., Fabrizi, S., and Ahrens, D. (2020). A molecular phylogeny of Glaphyridae (Coleoptera: Scarabaeoidea): evolution of pollination and association with 'Poppy guild' flowers. *Syst. Entomol.* 45, 838–848. doi: 10.1111/syen.12429

Sander, S. E., and Hall, D. W. (2015). Variation in opsin genes correlates with signalling ecology in North American fireflies. *Mol. Ecol.* 24, 4679–4696. doi: 10.1111/mec.13346

Schiestl, F. P., and Dötterl, S. (2012). The evolution of floral scent and olfactory preferences in pollinators: Coevolution or pre-existing bias? *Evolution* 66, 2042–2055. doi: 10.1111/j.1558-5646.2012.01593.x

Seppey, M., Manni, M., and Zdobnov, E. M. (2019). BUSCO: assessing genome assembly and annotation completeness. *Methods Mol. Biol.* 1962, 227–245. doi: 10.1007/978-1-4939-9173-0_14

Sharkey, C. R., Fujimoto, M. S., Lord, N. P., Shin, S., McKenna, D. D., Suvorov, A., et al. (2017). Overcoming the loss of blue sensitivity through opsin duplication in the largest animal group, beetles. *Sci. Rep.* 7:8. doi: 10.1038/s41598-017-00061-7

Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., and Zdobnov, E. M. (2015). BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 31, 3210–3212. doi: 10.1093/bioinformatics/btv351

Sondhi, Y., Ellis, E. A., Bybee, S. M., Theobald, J., and Kawahara, A. (2021). Light environment drives evolution of color vision genes in butterflies and moths. *Commun. Biol.* 4:177. doi: 10.1038/s42003-021-01688-z

Spaethe, J., and Briscoe, A. D. (2004). Early duplication and functional diversification of the opsin gene family in insects. *Mol. Biol. Evol.* 21, 1583–1594. doi: 10.1093/molbev/msh162

Stanger-Hall, K. F., Lower, S. E. S., Lindberg, L., Hopkins, A., Pallansch, J., and Hall, D. W. (2018). The evolution of sexual signal modes and associated sensor morphology in fireflies (Lampyridae, Coleoptera). *Proc. R. Soc. B Biol. Sci.* 285:20172384. doi: 10.1098/rspb.2017.2384

Steiner, K. E. (1998). The evolution of beetle pollination in a South African orchid. *Am. J. Bot.* 85, 1180–1193. doi: 10.2307/2446351

Suvorov, A., Jensen, N. O., Sharkey, C. R., Fujimoto, M. S., Bodily, P., Wightman, H. M. C., et al. (2016). Opsins have evolved under the permanent heterozygote model: insights from phylotranscriptomics of Odonata. *Mol. Ecol.* 26, 1306–1322. doi: 10.1111/mec.13884

Toon, A., Terry, L. I., Tang, W., Walter, G. H., and Cook, L. G. (2020). Insect pollination of cycads. *Austral. Ecol.* 45, 1033–1058. doi: 10.1111/aec.12925

Van Kleunen, M., Nänni, I., Donaldson, J. S., and Manning, J. C. (2007). The role of beetle marks and flower colour on visitation by monkey beetles (Hopliini) in the greater cape floral region, South Africa. *Ann. Bot.* 100, 1483–1489. doi: 10.1093/aob/mcm256

Wardhaugh, C. W. (2015). How many species of arthropods visit flowers? *Arthropod. Plant. Interact.* 9, 547–565. doi: 10.1007/s11829-015-9398-4

Warrant, E. J., and McIntyre, P. D. (1990). Limitations to resolution in superposition eyes. *J. Comp. Physiol. A* 167, 785–803. doi: 10.1007/BF00189768

Westmoreland, D., and Muntan, C. (1996). The influence of dark central florets on insect attraction and fruit production in Queen Anne's Lace (*Daucus carota* L.). *Am. Midl. Nat.* 135, 122–129. doi: 10.2307/2426878

Yang, E.-C., and Osorio, D. (1991). Spectral sensitivities of photoreceptors and lamina monopolar cells in the dragonfly, *Hemicordulia tau*. *J. Comp. Physiol. A* 169, 663–669. doi: 10.1007/BF00194895

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 16 June 2021
doi: 10.3389/fevo.2021.659176

Specialization for Tachinid Fly Pollination in the Phenologically Divergent Varieties of the Orchid *Neotinea ustulata*

Carlos Martel[1†], Demetra Rakosy[2,3,4†], Stefan Dötterl[5], Steven D. Johnson[6], Manfred Ayasse[7], Hannes F. Paulus[2], L. Anders Nilsson[8], Hans Mejlon[8] and Jana Jersáková[9]*

[1] Institute of Omic Sciences and Applied Biotechnology, Pontifical Catholic University of Peru, Lima, Peru, [2] Department of Evolutionary Biology, Faculty of Life Sciences, University of Vienna, Vienna, Austria, [3] Department of Community Ecology, Helmholtz Centre for Environmental Research - UFZ, Halle, Germany, [4] German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Leipzig, Germany, [5] Department of Biosciences, Paris-Lodron University of Salzburg, Salzburg, Austria, [6] Centre for Functional Biodiversity, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa, [7] Institute of Evolutionary Ecology and Conservation Genomics, University of Ulm, Ulm, Germany, [8] Museum of Evolution, Uppsala University, Uppsala, Sweden, [9] Faculty of Science, University of South Bohemia, České Budějovice, Czechia

OPEN ACCESS

Edited by:
Johannes Spaethe, Julius Maximilian University of Würzburg, Germany

Reviewed by:
Judith Trunschke, Kunming Institute of Botany, Chinese Academy of Sciences, China
Giovanni Scopece, University of Naples Federico II, Italy

****Correspondence:***
Carlos Martel
carlos.martel@pucp.pe

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *27 January 2021*
Accepted: *14 May 2021*
Published: *16 June 2021*

Citation:
Martel C, Rakosy D, Dötterl S, Johnson SD, Ayasse M, Paulus HF, Nilsson LA, Mejlon H and Jersáková J (2021) Specialization for Tachinid Fly Pollination in the Phenologically Divergent Varieties of the Orchid Neotinea ustulata. Front. Ecol. Evol. 9:659176. doi: 10.3389/fevo.2021.659176

Despite increased focus on elucidating the various reproductive strategies employed by orchids, we still have only a rather limited understanding of deceptive pollination systems that are not bee- or wasp-mediated. In Europe, the orchid *Neotinea ustulata* has been known to consist of two phenologically divergent varieties, neither of which provide rewards to its pollinators. However, detailed studies of their reproductive biology have been lacking. Our study aimed to characterize and understand the floral traits (i.e., morphology, color, and scent chemistry) and reproductive biology of *N. ustulata*. We found that the two varieties differ in all their floral traits; furthermore, while *Neotinea ustulata* var. *ustulata* appears to be pollinated by both bees (e.g., *Anthophora*, *Bombus*) and flies (e.g., *Dilophus, Tachina*), var. *aestivalis* is pollinated almost entirely by flies (i.e., *Nowickia, Tachina*). Tachinids were also found to be much more effective than bees in removing pollinaria, and we show experimentally that they use the characteristic dark inflorescence top as a cue for approaching inflorescences. Our results thus suggest that while both *N. ustulata* varieties rely on tachinids for pollination, they differ in their degree of specialization. Further studies are, however, needed to fully understand the reproductive strategy of *N. ustulata* varieties.

Keywords: food deception, flower color, flower morphology, floral scent, pollinator efficiency, pollinator specialization

INTRODUCTION

The orchid family is one of the largest plant families in the world, accounting for around 28,000 described species (Willis, 2017; Fay, 2018). At least one third of these species are deceptive, not providing their pollinators with a reward (Renner, 2005; but see Shrestha et al., 2020). Deceptive orchids employ various strategies to lure in their pollinators, which range from the advertisement

of false nectar or pollen sources to the imitation of brood sites or mating partners (Jersáková et al., 2006; Johnson and Schiestl, 2016). Each type of deception is thought to be associated with a particular combination of floral traits, often influenced by their reliance on particular groups of pollinators (Johnson and Schiestl, 2016; Valenta et al., 2017). Conspicuous visual displays coupled with reduced floral scent emission and the attraction of male and female pollinators are traditionally considered an indication of food-deceptive pollination (Galizia et al., 2004; Jersáková et al., 2012, 2016; Johnson and Schiestl, 2016). Less conspicuous visual displays, the exclusive attraction of either male or female insects and the emission of particular scent bouquets are, in contrast, associated with pollination by sexual deception or brood-site mimicry (Vereecken and Schiestl, 2009; Ayasse et al., 2011; Bohman et al., 2016; Martel et al., 2019; but see Streinzer et al., 2010). For many deceptive orchids the investigation of the floral traits employed can provide an accurate estimate of their pollination strategy; however, some orchid species may have complex strategies that are much more difficult to categorize (e.g., Vogel, 1972; Bino et al., 1982; Vöth, 1989; Valterová et al., 2007; Scopece et al., 2009). This seems also to be the case for the European orchid species, *Neotinea ustulata* (L.) R. M. Bateman, Pridgeon and M. W. Chase.

Neotinea ustulata, like up to 81% of orchid taxa occurring in Europe, Asia Minor and North Africa (Paulus, 2005; Delforge, 2006), is deceptive, i.e., does not offer any readily identifiable reward to potential floral visitors (Claessens and Kleynen, 2011). While most of these deceptive orchids rely on either bumblebees or solitary bees for reproduction, *N. ustulata* has been considered to be potentially fly- and beetle-pollinated (Vöth, 1984; van der Cingel, 1995). The evidence for *N. ustulata*'s pollination system has, however, been mostly anecdotal. The identification of its reproductive strategy has been further complicated by the occurrence of two phenologically distinct varieties, the early flowering (from April to June, or even until July) *Neotinea ustulata* var. *ustulata* and the late flowering (from June to August) *N. ustulata* var. *aestivalis* (Kümpel) Tali, M. F. Fay and R. M. Bateman. The first recorded pollination event in *N. ustulata* var. *ustulata* probably dates back to Godfery (1933), who observed two *Tachina* (*Echinomyia*) aff. *magnicornis* (Zett.) flies, one of them with pollinaria attached to its proboscis, visiting flowers in a population from Eastern France. Further records stem from Vöth (1984), who observed several individuals of *T.* aff. *magnicornis* carrying pollinaria in Austria. While tachinid flies seem to be common visitors, other records include bees (Borsos, 1962), bumblebees (Paulus, 2005; Claessens and Kleynen, 2016), and empidid flies (Paulus, 2005; Claessens and Kleynen, 2016). Pollinator records are even more scarce for *N. ustulata* var. *aestivalis*, for which there have been only observations of beetles, namely *Chrysanthia* sp. (Oedemeridae) (Danesch and Danesch, 1962) and *Vadonia* (*Leptura*) *livida* (F.) (Cerambycidae) (Mrkvicka, 1991), carrying pollinaria on various parts of their head. These records are, however, insufficient to determine the reproductive system of *N. ustulata* as they only account for sporadic and isolated visitation events.

Throughout their range of distribution, the two *N. ustulata* varieties do not usually occur in sympatry (Tali et al., 2004); var. *ustulata* being mostly found in dry meadows, whereas var. *aestivalis* in both dry and wet meadows (e.g., Molinetum vegetation) (Paulus, pers. obs.). Plants of var. *aestivalis* are also usually taller than those of var. *ustulata* (Haraštová-Sobotková et al., 2005). Despite these differences, the two varieties are genetically undifferentiated (Tali et al., 2006; but see Haraštová-Sobotková et al., 2005) and have the same chromosome number (Tali, 2004), which indicates no association of autopolyploidy with the phenological differences as reported in others plants (e.g., Simón-Porcar et al., 2017). The mechanisms driving their divergence, thus remains unknown. It is possible that both pollinator- and non-pollinator-mediated selection could have played a role in driving the phenological divergence among populations, leading to the evolution of these two varieties. However, testing the role of pollinator as selection mediators in *N. ustulata* requires firstly a better assessment of its pollinators and of the traits involved in pollinator attraction. To this date there are only very few assessments of floral traits of *N. ustulata* varieties (i.e., Haraštová-Sobotková et al., 2005; Schiestl and Cozzolino, 2008; D'auria et al., 2021). Inflorescences of both varieties have a similar color pattern (Paulus, 2005), both having a very dark brown-purple top (hereafter referred as "dark top"). This provides a strong contrast with the remaining part of the inflorescence (Godfery, 1933; Stace, 2010), and may serve as a signal for attracting pollinators; however, this hypothesis so far has not been tested. Perhaps the most unusual trait of *N. ustulata* is its floral scent. Food-deceptive orchids rarely rely upon flower scent for pollinator attraction, this floral trait is, however, essential in sexually deceptive species (Ayasse et al., 2011; Bohman et al., 2016). Schiestl and Cozzolino (2008) were the first to show that the floral scent of *N. ustulata* contains alkenes, which, although uncommon in most flowers, are frequently associated with sexually deceptive ones (Ayasse et al., 2011; Bohman et al., 2016); in particular, *N. ustulata* flowers produce high relative quantities of 11-tricosene. The role of this specific compound in pollinator attraction is so far unknown, but other tricosene isomers are known to act as sex pheromones in the house fly *Musca domestica* (Carlson et al., 1971; Rogoff et al., 1973) or aggregation pheromones in *Drosophila* (Hedlund et al., 1996). At the moment, we do not know if alkenes could be found in both varieties or these are restricted to just one of them. The particular combination of visual signals associated with the chemical composition of the flower scent raises the interesting possibility that *N. ustulata* may rely on intricate signals to attract its pollinators.

In order to understand the reproductive strategy and, eventually, the evolution of *N. ustulata*, we need to accurately quantify the differences in floral traits between its two varieties as well as identify their reproductive biology. Therefore, our study aims are to compare *N. ustulata* varieties in terms of (i) morphological, visual and chemical floral traits, (ii) pollination success and patterns of fruit set, and (iii) pollinator spectra. Furthermore, we aimed to (iv) assess efficiency in pollinaria removal among the principal pollinator guilds, and (v) identify the role of the inflorescence dark top in the attraction of predominant pollinators.

MATERIALS AND METHODS

Study Species

Neotinea ustulata is a perennial tuberous herb, which is typically found on extensively used meadows (Tali et al., 2004). Each plant of this species bears a single inflorescence, which has a burnt appearance when its flowers are in bud; the species is therefore known as the burnt-tip orchid. *Neotinea ustulata* flowers have three semi-fused sepals, which form a hood; the outer side of the sepals (especially in buds) displays a dark reddish to blackish color (**Figure 1**). The lip is white with dark red spots and is deeply divided into three parts (**Figure 1**). The flowers have a nectarless spur, which is blunt. *Neotinea ustulata* occurs in central and southern Europe with populations reaching Spain and Greece in the south, England and southern Sweden in the north, and as far east as the Caucasus and Ural Mountains (Tali et al., 2004; Delforge, 2006). Despite its broad geographical range, the species is vulnerable to extinction and the number of populations have been greatly reduced during the last century (Neiland, 2001; Tali et al., 2004; Baumann et al., 2005; Jacquemyn et al., 2005).

The species consists of two varieties, which, while phenologically distinct, do not differ strongly in their vegetative traits (Kümpel and Mrkvicka, 1990; Wucherpfennig, 1992; Haraštová-Sobotková et al., 2005): the early flowering *N. ustulata* var. *ustulata* and the late flowering var. *aestivalis*. *Neotinea ustulata* var. *ustulata* is 10–35 cm tall, rarely larger, and its flowering period ranges from April till June in most parts of Europe (Tali et al., 2004; Baumann et al., 2005), but from June to July at high altitudes in the Alps (Paulus, pers. obs.). The dense, narrow, up to 8 cm long inflorescence is composed on an average of 30 flowers (Tali et al., 2004; Baumann et al., 2005; Haraštová-Sobotková et al., 2005; **Figure 1**). *Neotinea ustulata* var. *aestivalis* has in turn a somewhat taller stem, of 33 cm long in average, (rarely up to 80 cm; Haraštová-Sobotková et al., 2005) with longer and narrower leaves, and sharply pointed inflorescences (especially in young stage) (**Figure 1**). The inflorescences have on average around 40 flowers (Jensen and Pedersen, 1999). Plants of this variety flower from the end of June or July till mid-August (Tali et al., 2004; Baumann et al., 2005; Paulus, pers. obs.). In order to confirm the identity of the two varieties, detailed digital photographs of individuals from our sampling localities were compared against material deposited in diverse herbaria (e.g., CB, BRNM, OHN, PR, PRC, UPS, VN, and W).

Study Sites

Field observations and experiments were carried out in Austria, the Czech Republic, the Slovak Republic and Sweden (**Figure 2**), during May–June (for details see **Supplementary Table 1**). In total, thirteen populations of var. *ustulata* and ten populations of var. *aestivalis* were sampled to analyze different aspects of their reproductive biology (**Figure 2**; for population details see **Supplementary Table 1**).

Morphological, Visual, and Chemical Floral Traits

Morphology

We sampled inflorescences of *N. ustulata* var. *ustulata* and var. *aestivalis* from two populations each (i.e., 30, 30, 23, and 15 inflorescences from U8, U9, A2, and A4, respectively, see **Supplementary Table 1** for population codes). From the middle of each inflorescence, we picked a single, fully opened and yet unpollinated flower. Flowers were placed in 70% ethanol (Sigma Aldrich, Munich, Germany) and stored until further usage. Individual flowers were mounted on a styrofoam base and then photographed from three different angles with the aid of a Dino-Lite digital microscope (AnMo Electronics Corporation, Taiwan). Size calibration and distance measurements were performed with the in-house software DinoCapture (AnMo Electronics Corporation, Taiwan). Eight floral traits (i.e., hood opening width, hood opening height, spur opening width, spur width at 3/4 spur length, width at spur bottom, spur length, distance from column to the spur opening, and lip length) were measured.

FIGURE 1 | The burnt tip orchid *Neotinea ustulata*. **(A)** Habitus of *N. ustulata*. **(B)** Inflorescence from a var. *ustulata* plant. **(C)** Inflorescence from a var. *aestivalis* plant. Pictures by J. Jersáková.

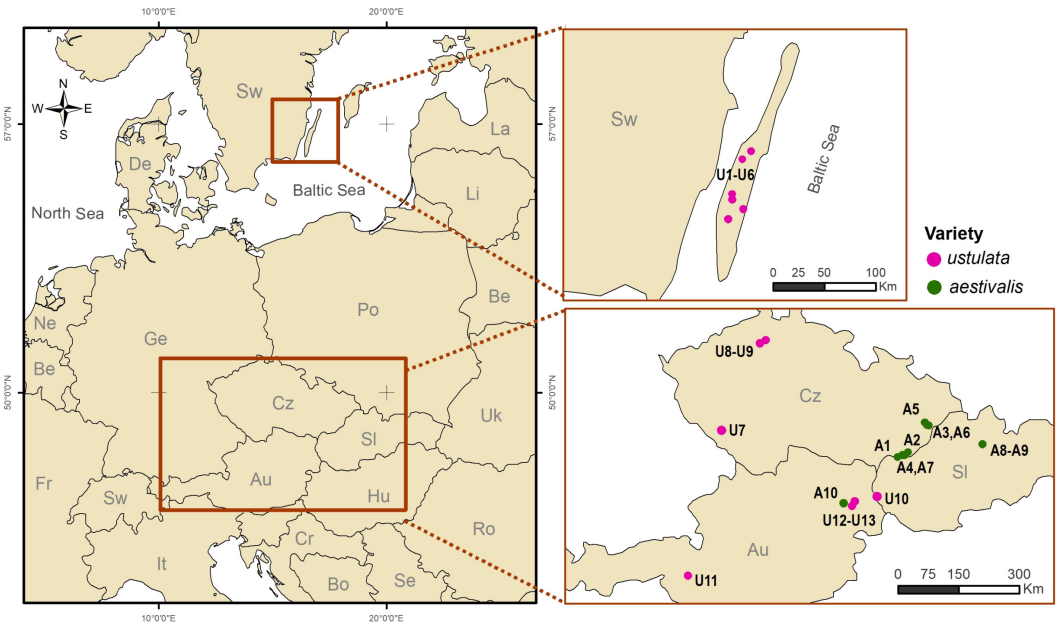

FIGURE 2 | Maps showing the localities where field work was carried out in diverse European countries. The variety identity of *Neotinea ustulata* populations is indicated by circle colors (violet: var. *aestivalis*; green: var. *ustulata*). Populations codes follow **Supplementary Table 1**.

To identify which morphological traits, if any, are responsible for differences among varieties of *N. ustulata*, we performed a principal component analysis (PCA) using the *factoextra* package (Kassambara and Mundt, 2020) of the R software (R version 3.6.1; R Core Team., 2017). Morphological data were scaled and normalized prior to conducting the PCA. To explore differences in morphology between the two varieties, we performed a permutational multivariate analysis of variance (PERMANOVA) with the raw morphological data. We constructed a matrix based on Euclidean distances to run the PERMANOVA with 10,000 permutations, considering "variety" as fixed factor and "population" as nested factor in "variety." PERMANOVA was performed using the *vegan* package (Oksanen et al., 2019) of R.

Color

The spectral reflectance was measured for 44 flowers (one flower per plant) from two populations per *N. ustulata* variety (i.e., 14, 10, 10, and 10 flowers from U8, U9, A2, and A4, respectively). We specifically measured the reflectance of the dark top, outer sepals and lips. All floral reflectance measurements were performed using an Ocean optics UV-VIS Spectrometer JAZ and an Ocean Optics DT-mini light source (200–1,100 nm; Dunedin, FL, United States), calibrated with a white reflection standard (WS-1-SL, Ocean Optics). Readings were taken through a fiber-optic reflection probe (UV/VIS 400 μm) held at 45°ca. 5 mm from the surface of the object. The visual similarity between varieties, as perceived by their visitors, was determined by plotting their reflectance spectra as loci in bee- and fly-specific color spaces, assuming adaptation to foliage background (Chittka and Kevan, 2005). Selection of these two insect models was based on our pollinator list (see section "Results"). To model bee color vision, we used the color hexagon (Chittka, 1992), based on the spectral sensitivities of *Apis mellifera* (L.). To model fly color vision, we used Vorobyev and Osorio, 1998) receptor noise model. This method uses spectral sensitivities and the relative numbers of each fly photoreceptor type to calculate the perceived color contrast between objects. We did not use Troje's (1993) fly categorical color vision model because it was already shown to be unreliable when contrasted with experimental data (e.g., Jersáková et al., 2012; Renoult et al., 2014; An et al., 2018). Based on color loci, we also run a PERMANOVA, considering "variety" and "inflorescence part" as fixed factors and "population" as a nested factor in variety, to test for differences in spectral reflectance between varieties while considering population origin. The visual modeling and PERMANOVA were performed using the *pavo* (Maia et al., 2021) and *vegan* packages of R, respectively.

Floral Scent

Highly volatile compounds of the floral scents of both *N. ustulata* varieties were collected using dynamic headspace techniques (i.e., 10, 9, and 5 flowers from U8, U9, and A2, respectively), whereas semi- and low-volatile compounds were collected through solvent extracts (i.e., 10, 10, 11, and 10 flowers from U8, U9, A2, and A4, respectively). Samples were analyzed by a gas-chromatograph and gas chromatograph/mass spectrometer using a DB-5 non-polar column (for detailed description of the analytical techniques see **Supplementary Methods 1**). Chemical identification was carried out by comparing the mass spectra of pure compounds with the ones of commercial libraries (NIST, ADAMS) or of our own libraries, and the retention indexes of detected compounds with those from the literature (e.g., Adams, 2017). Mass spectra were processed using the

Automated Mass Spectral Deconvolution and Identification System—AMDIS—software (National Institute of Standards and Technology, Gaithersburg, United States). Differences in floral scent bouquets of both varieties were analyzed by comparing the relative proportions of compounds identified in our two type of samples. We calculated the Bray-Curtis similarity index to determine semi-quantitative differences in odor bouquets. Then, we performed a PERMANOVA with 10,000 permutations to test for differences in scent bouquets between the two varieties while considering population origin. A non-metric multidimensional scaling (NMDS) was performed to graphically display the (dis)similarities in floral scent patterns among samples. PERMANOVA and NMDS were performed using the *vegan* package and displayed graphically using the *ggplot2* package (Wickham et al., 2020) of R.

Reproductive Success Patterns

During 6 years (i.e., 1978, 1981, 1983, 1984, 2000, and 2001), we examined seven populations of *N. ustulata* var. *ustulata* and four populations of var. *aestivalis* for fruit production (see **Supplementary Table 1** for population details). We recorded the total number of flowers and position of fruits within inflorescences for each inflorescence sampled. The differences in the number of flowers, the number of fruits produced and the proportion of flowers that set fruits between the *N. ustulata* varieties were tested by means of generalized linear mixed-models (GLMMs) with Poisson model distribution with log link function for number of flowers and fruits, and binomial model distribution with logit link function for the proportion of flowers that set fruits; "population" was set as a random factor.

In deceptive systems, it is expected to find position effects on fruit production, as first open flowers are more likely to be visited by yet unexperienced insects than late flowers; however, it is unknown if this pattern generalized and also happens in *N. ustulata*. Therefore, we checked whether the development of fruits is affected by the relative position of the flower in the inflorescence by dividing each inflorescence into three sections (i.e., bottom, middle, and top), calculated mean reproductive success for each section, and then performed a GLMM with a binomial model distribution and log link functions, and conducted Tukey *post hoc* tests to compare pairs of means; "plant individual" was set as a random factor. GLMMs and *post hoc* tests were carried out using the *lme4* (Bates et al., 2019) and *emmeans* packages (Lenth et al., 2020), respectively, in R.

Diversity of Pollinator Guilds and Their Efficiency

Flower Visitors, Pollinators, and Their Overall Efficiency

Insect visitors on *N. ustulata* flowers were recorded by video footages (73 and 37 h of footage on *N. ustulata* var. *ustulata* and var. *aestivalis*, respectively) and complemented by direct observations in our study sites (see **Supplementary Table 1** for population details). Floral visitor identity and identification of visitors as pollinators were assessed based on both direct observations and video recordings; whereas frequency of visits and floral visitor behavior on the inflorescence were assessed based on the video footage only. Visitors were identified to the order and family level, and when possible up to genus/species level. To test for differences in the flower visitor composition, a G-test of independence comparing the frequency of species within orders between varieties was performed using the *DescTools* package (Signorell, 2020) of R. Representatives of each floral visiting species were collected using entomological nets in parallel to the video recordings in search of attached pollinaria and for insect identification. We also checked for presence and placement of pollinaria in insects feeding on rewarding flowers of plants co-occurring with *N. ustulata*, such as species of *Aegopodium* L. (Apiaceae), *Crataegus* L. (Rosaceae), *Dorycnium* Mill. (Fabaceae) and *Euonymus* L. (Celastraceae). To corroborate the pollinaria identity, they were compared with pollinaria directly collected from *N. ustulata* individuals. Insects were considered pollinators based on both the presence of attached pollinaria and evidence of pollinaria removal (assessed from the video footage or direct observations).

Furthermore, we also calculate male (i.e., pollinaria removed/total flowers × 100) and female (i.e., pollinia deposited/total flowers × 100) reproductive success, and pollen transfer efficiency (i.e., ratio of pollinia deposited to pollinaria removed × 100; Nunes et al., 2016), which can be linked to specialization. We performed a GLMM with Poisson model distribution and log link function to estimate the effects of variety on pollen transfer efficiency and with "population" set as a random factor. GLMM was carried out using the *lme4* package of R.

Efficiency in Pollinaria Removal Between Pollinator Guilds

We compared the pollinaria removal efficiency between the tachinid fly *Tachina fera* (L.) and the bee *Anthophora plumipes* (Pallas), in order to identify whether pollinators of different guilds are equally efficient. Our set up was as follows: one inflorescence of *N. ustulata* var. *ustulata* with pollinaria present in all its open flowers was placed in a plastic container (100 × 50 mm i.d., Greiner Bio-One GmbH., Frickenhausen, Germany) in which a single individual of either *T. fera* (n = 6) or *A. plumipes* (n = 12) was introduced. We recorded the removal of pollinaria in up to three attempts (i.e., insertion of the proboscis in the flower) of each *T. fera* and *A. plumipes*. We performed a GLMM with binomial model distribution and logit link function to test for removal efficiency between pollinators. We also conducted Tukey *post hoc* tests to compare pairs of means. Our GLMM for repeated measures, compound symmetry was used as covariance pattern model, included "pollinator guild" and "attempt number" as fixed factors and "insect individual" as a random factor. GLMM and *post hoc* tests were performed using the *lme4* and *emmeans* packages in R.

Role of Visual Traits in Pollination Success

In 1999 we sampled inflorescences from eight populations from Sweden and Czech Republic belonging to both varieties (see **Supplementary Table 1** for population details). They

were examined for inflorescence display and dark top display. *Neotinea ustulata* inflorescences have a cylindrical arrangement and, therefore, the inflorescence display was calculated as its two-dimensional projection (i.e., the area of a rectangle: length × width of inflorescence); while the dark top has a circular shape and, therefore, dark top display was approximated as the area of a circle (i.e., $\pi \times \{\text{width of dark top}/2\}^2$). We also assessed the number of flowers with removed pollinaria, deposited pollinia and flowers where pollination events occur; they were then transformed to proportions by dividing them by the number of open flowers. We performed GLMMs with binomial model distribution and logit link function to estimate the effects of visual display on the probability of a flower to be pollinated. Our models included "inflorescence display," "dark top display" and "variety" as fixed effects, and "population" as random factor. GLMMs were performed using the *lme4* package in R.

Role of Dark Top in Fly Pollinator Attraction

We tested the importance of the dark top in the attraction of tachinids since it might be an attractant to flies (Godfery, 1933; van der Cingel, 1995). *Tachina* flies have been previously considered the main pollinators of *N. ustulata* (Vöth, 1984; Trunschke et al., 2017), and their importance as pollinators is supported by our own observations (see section "Results"). Experiments were carried out with both varieties. For var. *ustulata*, four inflorescence pairs were selected for experiments. For each trial, two inflorescences of equal height and similar phenological state were placed less than 10 cm apart. Each inflorescence pair was first observed for about 30 min, during which the number of tachinids attracted by each inflorescence was recorded. After the initial observations the dark top of one of the two inflorescences was arbitrarily cut off. The inflorescence pairs (i.e., one intact, one without the dark top) were then observed again for the same amount of time and the number of tachinids which landed and/or probed the flowers was recorded. In case of var. *aestivalis*, only two inflorescence pairs were selected, but the experimental setup was similar to that described for var. *ustulata*, with two exceptions: (i) For the second trial, the cut off dark top of var. *aestivalis* was placed below the selected plant, in such a way that it was hidden from the view to approaching flies. This setup ensured that olfactory cues released by the dark top were still present when the dark top was cut off. (ii) An additional trial was carried out, in which the dark top was reattached to the plant. Then, the inflorescence pairs were offered again to tachinids. This trial controlled for any effect of the damage (e.g., green leaf volatiles after plant tissue damage) to the inflorescence on their attractiveness to *Tachina* flies. We performed GLMMs with negative binomial model distribution and log link function to test for differences in the attractiveness of manipulated and non-manipulated inflorescences within each variety. Our GLMMs for repeated measures incorporated a compound symmetry covariance matrix included "treatment stage" and "manipulation condition" as fixed factors, "plant individual" as the subject for repeated measures before and after the manipulation, and "number of visits" as the response variable. GLMMs were performed using the *MASS* package (Ripley et al., 2020) in R.

RESULTS

Comparison of Morphological, Visual, and Chemical Floral Traits

Morphology

The flowers of *N. ustulata* var. *ustulata* are larger in all measured traits in comparison to var. *aestivalis* (**Table 1**). In our PCA, the first two PCs accounted for 62.1% of the total variance (PC1: 50.5%, PC2: 11.6%). All the morphological floral traits loaded negatively on the PC1 (**Supplementary Table 2**), which separates var. *ustulata* (negative scores) from var. *aestivalis* (positive scores) (**Figure 3**). Indeed, the PERMANOVA analysis revealed that the floral morphology differed significantly between var. *ustulata* and var. *aestivalis* [Pseudo-$F_{(1,\ 97)} = 81.12$, $p < 0.0001$], and between populations [Pseudo-$F_{(2,\ 97)} = 5.32$, $p < 0.001$].

Color

The dark top and the lightly colored lip plotted apart in both the fly and bee color models, indicating that these two parts of the flower appear highly contrasting, and are therefore easily discriminated by bees as well as flies. Loci of the individual color measurements of the dark top ($n = 44$) plotted, except by one case, in the UV-blue color category within the bee hexagon and also grouped together within the fly tetrahedron (**Figure 4**). Similarly, color loci of the lip ($n = 44$) clustered together within both the bee hexagon and the fly tetragon (**Figure 4**). The color loci of the hood ($n = 44$) were more broadly distributed, spanning the space from the dark top loci to the lip loci (**Figure 4**). In terms of color information, the dark top and hood may often be perceived as uncolored against the leaf background by bees based on color hexagon distances. The color loci of different inflorescence parts were different, independently of variety, as our PERMANOVA analysis confirmed [Pseudo-$F_{(7,\ 131)} = 52.64$, $p < 0.0001$]. Furthermore, PERMANOVA analyses also indicated that there are statistical differences in the spectral reflectance between *N. ustulata* varieties [Pseudo-$F_{(1,\ 131)} = 9.28$, $p < 0.01$] and within varieties among populations [Pseudo-$F_{(3,\ 131)} = 2.81$, $p < 0.05$].

TABLE 1 | Measurements (in mm) of the floral morphology traits in *Neotinea ustulata*.

Morphological floral trait	var. *ustulata* (mean ± SD)	var. *aestivalis* (mean ± SD)
Hood opening width	4.20 ± 0.79	2.28 ± 0.57
Hood opening height	2.23 ± 0.67	1.90 ± 0.44
Spur opening width	0.66 ± 0.09	0.42 ± 0.13
Spur width at 3/4 spur length	1.11 ± 0.19	0.88 ± 0.18
Width at the spur bottom	0.64 ± 0.08	0.52 ± 0.09
Spur length	2.40 ± 0.32	1.83 ± 0.22
Distance from column to spur opening	1.67 ± 0.24	1.48 ± 0.19
Lip length	3.19 ± 0.58	2.53 ± 0.56

Mean and standard deviation values for each trait and variety are shown.

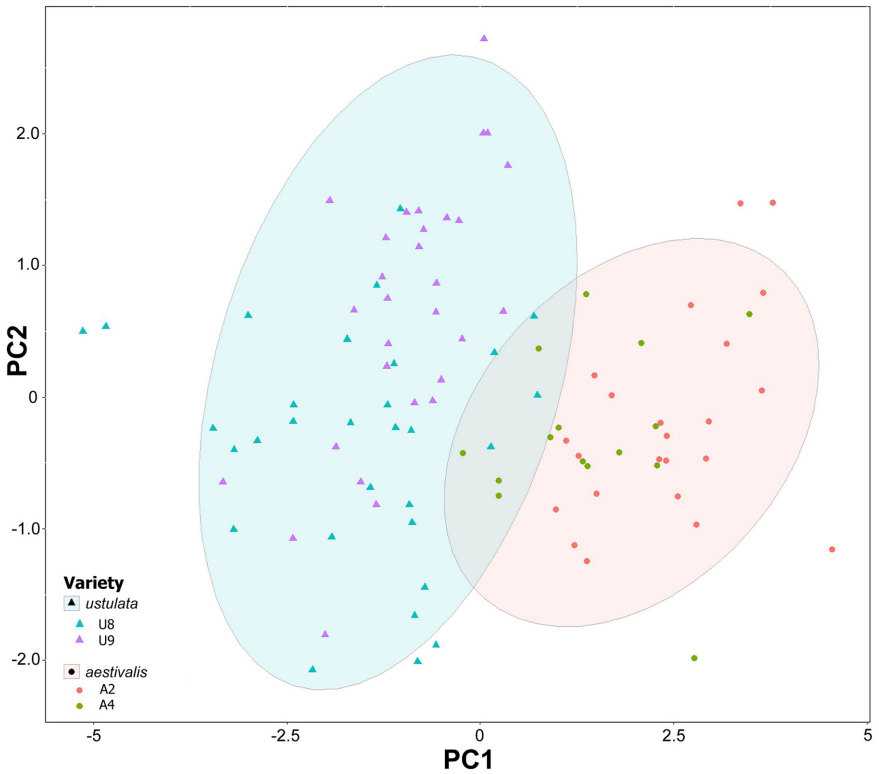

FIGURE 3 | Plotting of *Neotinea ustulata* samples based on floral morphology traits in PC1 and PC2. Different symbols represent different varieties and ellipses display 95% confidence area of each variety. Different symbol colors represent different populations. Population codes as in **Supplementary Table 1**.

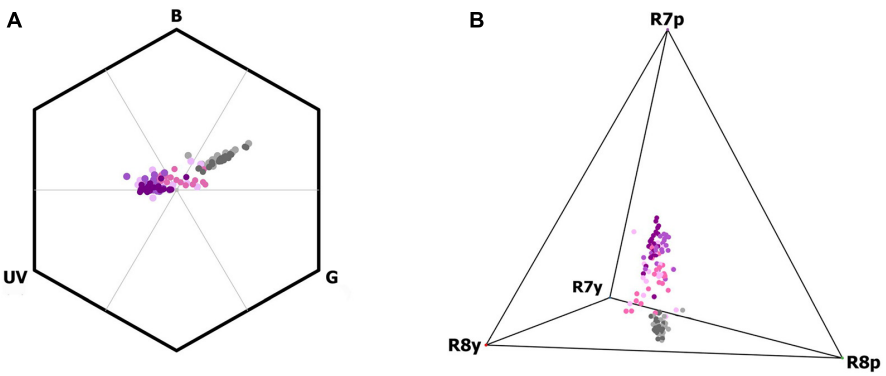

FIGURE 4 | Color loci (circles) of different inflorescence parts of *Neotinea ustulata* varieties (purple: dark top; pink: hood; gray: lip) plotted within the vision color models of **(A)** bees (bee-hexagon) and **(B)** flies (fly-tetrahedron). Var. *ustulata*: dark-colored tones (i.e., light purple, light pink, light gray); var. *aestivalis*: light-colored tones (i.e., dark purple, dark pink, dark gray). Bee (UV, B, G) and fly (R7p, R7y, R8p, R8y) receptors are shown.

Floral Scent

We detected 44 floral volatiles in the headspace samples and 54 chemical compounds in the floral extracts of *N. ustulata* with the majority of the compounds present in both varieties (see **Supplementary Tables 3, 4**). Headspace samples were dominated by terpenes (e.g., eucalyptol, limonene, linalool, β-myrcene, (*E*)-β-ocimene), although benzenoids (e.g., 2-phenylethyl alcohol) were also identified (see **Supplementary Table 3**); whereas extracts were dominated by alkanes (e.g., pentacosane, heptacosane) and alkenes (e.g., tricosene and heptacosene isomers), but also aldehydes (see **Supplementary Table 4**). The (dis)similarity of floral scent composition between *N. ustulata* var. *ustulata* and var. *aestivalis* is shown in **Figure 5**. Although there were differences in floral scent patterns among populations of the same varieties [PERMANOVA headspace samples: Pseudo-$F_{(1,\ 23)} = 4.72$, $p < 0.01$; PERMANOVA extract samples: Pseudo-$F_{(2,\ 40)} = 12.65$, $p < 0.0001$], we did not

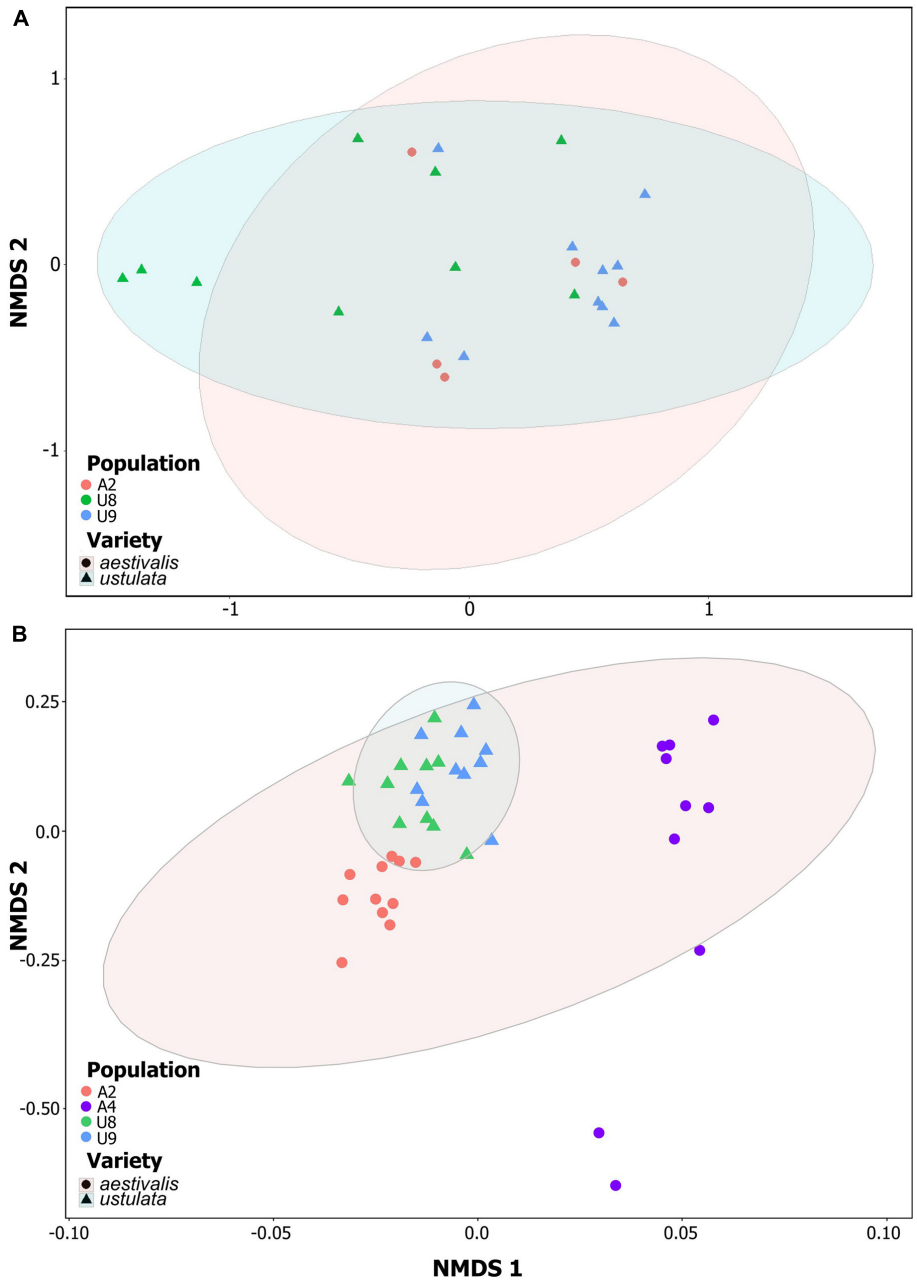

FIGURE 5 | Comparison of floral scent composition between *Neotinea ustulata* varieties in a non-metric multidimensional scaling (NMDS) based on semi-quantitative Bray-Curtis similarities: **(A)** headspace (stress = 0.15), and **(B)** solvent extracts (stress = 0.09). Different symbols represent different varieties and different symbol colors represent different populations (see **Supplementary Table 1** for population codes). Ellipses display 95% confidence area of each variety.

find any significant differences between the two varieties when comparing headspace samples [Pseudo-$F_{(1,\ 23)}$ = 1.48, p = 0.171]. Significant differences were only found when comparing the composition of the floral extracts of the two varieties [Pseudo-$F_{(1,\ 40)}$ = 37.33, p < 0.0001]. *Neotinea ustulata* var. *ustulata* could thus be distinguished from var. *aestivalis* through the low- or semi-volatile compounds emitted, but not by the more volatile fraction of the flower scent.

Pollination Success and Patterns of Fruit Set

Differences in the number of flowers between varieties were significant (var. *aestivalis*: mean ± SD: 40.89 ± 13.57; var. *ustulata*: mean ± SD: 33.34 ± 11.37; χ^2 = 4.83, *df* = 1, p < 0.05; **Figure 6**), while differences in the number of fruits approached statistical significance (var. *aestivalis*: mean ± SD: 9.82 ± 9.32; var. *ustulata*: mean ± SD: 3.93 ± 5.01; χ^2 = 3.59,

df = 1, $p = 0.058$; **Figure 6**); however, no differences were found comparing the proportion of flowers that set fruits (var. *aestivalis*: mean ± SD: 0.22 ± 0.19; var. *ustulata*: mean ± SD: 0.13 ± 0.17; $\chi^2 = 1.41$, *df* = 1, $p = 0.24$; **Figure 6**). The number of flowers per inflorescence had a positive impact on the probability to set at least one fruit ($\chi^2 = 6.23$, *df* = 2, $p < 0.05$), and the estimated probability to set a fruit increased 4% for each 10 flowers.

The probability to set a fruit varied significantly with the position of a flower within the inflorescence ($\chi^2 = 30.88$, *df* = 2, $p < 0.0001$; **Supplementary Figure 1**), but not the variety ($\chi^2 = 1.54$, *df* = 1, $p = 0.21$) or the interaction of variety and position ($\chi^2 = 1.71$, *df* = 2, $p = 0.42$; **Supplementary Figure 1**). *Post hoc* tests comparing the fruit set among the sections within each variety showed that compared to the top section, probability to set fruit was higher in the bottom (*aestivalis*: $z = 4.040$, $p < 0.001$; *ustulata*: $z = 3.995$, $p < 0.001$) and middle sections (*aestivalis*: $z = 2.882$, $p < 0.05$; *ustulata*: $z = 2.915$, $p < 0.05$); while no differences were found when comparing bottom and middle sections (*aestivalis*: $z = 1.556$, $p = 0.63$; *ustulata*: $z = 1.210$, $p = 0.83$; **Supplementary Figure 1**).

Pollinator Guilds and Their Efficiency in Pollinaria Removal

Flower Visitors, Pollinators, and Their Overall Efficiency

Inflorescences of both varieties were visited by a broad range of insect taxa within the Coleoptera, Diptera, Hymenoptera and Lepidoptera. Diptera and Hymenoptera were more frequently observed in our video recordings and together accounted for around 90% of the total insect visits (i.e., 415 insect visitors) to both *N. ustulata* varieties (**Figure 7** and see **Supplementary Table 5** for details of insect identity and frequency). Based on the same video recordings, the frequencies of the attracted insect orders significantly differed between var. *aestivalis* and var. *ustulata* (G = 71.68, *df* = 3, $p < 0.0001$; **Figure 7**). *Anthophora plumipes* and *Lasioglossum* sp. bees were particularly frequent on *N. ustulata* var. *ustulata,* and *T.* aff. *magnicornis* flies were most abundant on var. *aestivalis* (**Supplementary Table 5**). Despite the high number of insect visits, only three *Anthophora* bees carrying pollinaria of *N. ustulata* var. *ustulata* and one *T.* aff. *magnicornis* removing pollinaria of var. *aestivalis* were observed in our videos. By direct observations, we recorded some *Tachina* individuals visiting the flowers and carrying pollinaria (**Supplementary Table 6**), thereby acting as pollinators of *N. ustulata* var. *ustulata* and var. *aestivalis*. While bees almost exclusively landed on the white part of the inflorescence moving upwards in their search for a reward, *Tachina* flies always settled briefly (for 1–4 s.) on the dark top of the inflorescence; then they searched for potential nectar in the uppermost freshly open flowers and introduced their head to reach the base of the flower spur with their body in downright position (**Figure 8**). The pollinaria then became attached on the ventral side of the lower part of the proboscis (**Figure 8**). Identification of tachinid specimens revealed that they belong to three morphologically similar species, *Tachina fera, T. magnicornis* and *Nowickia ferox*. Both taxa pollinate flowers of *N. ustulata* var. *ustulata,* while var. *aestivalis* was only observed to be pollinated by *T. magnicornis* and *N. ferox* (**Supplementary Table 6**). Beside tachinids, solitary bees and bumblebees acted as regular pollinators, whereas beetles and other flies were highly occasional (see **Supplementary Table 6** and **Supplementary Figure 2**).

Differences in female success between both varieties (var. *aestivalis*: mean ± SD: 6.73 ± 15.57, *n* = 86; var. *ustulata*: 20.80 ± 22.74, *n* = 172) were marginally significant ($\chi^2 = 3.787$, *df* = 1, $p = 0.052$). However, we did not find differences in male success ($\chi^2 = 0.444$, *df* = 1, $p = 0.51$) and pollen transfer efficiency ($\chi^2 = 0.477$, *df* = 1, $p = 0.49$) between var. *aestivalis* (male success:

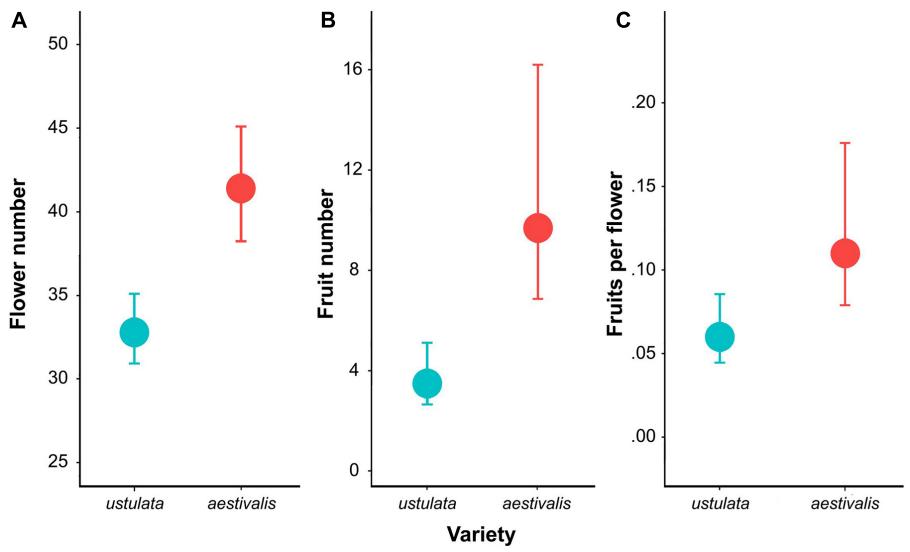

FIGURE 6 | Floral traits related to reproductive success: **(A)** the number of flowers, **(B)** the number of fruits, and **(C)** proportion of flowers that set fruit between *Neotinea ustulata* var. *ustulata* and var. *aestivalis*. Back-transformed means and standard errors are shown.

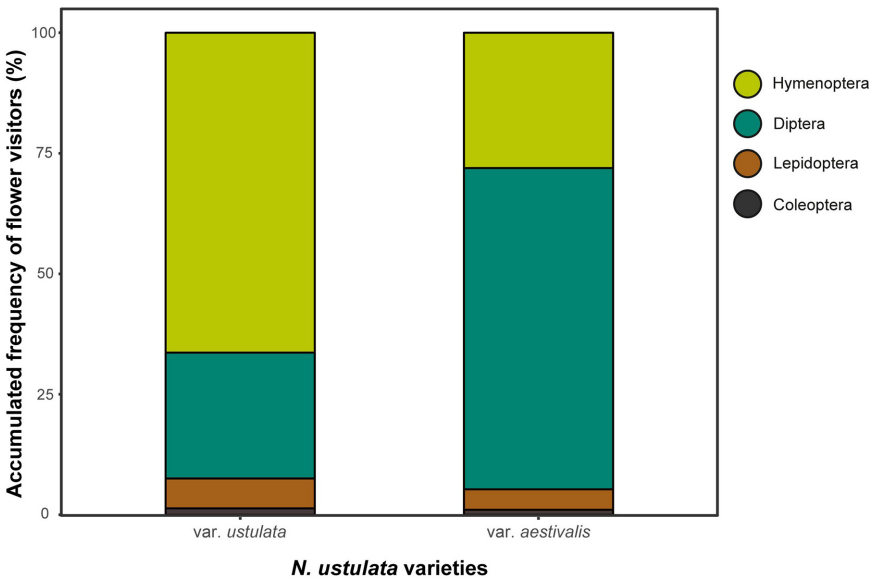

FIGURE 7 | Percentage of floral visits by insect groups to *Neotinea ustulata* var. *ustulata* and var. *aestivalis*. Insects were classified at the taxonomic order rank. Note that the graphs are based solely on video recordings. For records of insect diversity and insect responses see **Supplementary Table 5**.

mean ± SD: 23.03 ± 20.28, $n = 86$; pollen transfer efficiency: mean ± SD: 55.14 ± 237.41, $n = 70$) and var. *ustulata* (male success: mean ± SD: 24.29 ± 19.81, $n = 172$; pollen transfer efficiency: mean ± SD: 83.07 ± 81.82, $n = 151$).

Efficiency in Pollinaria Removal Between Pollinator Guilds

While all tachinids but one, which probed the flower with the body in an upright position, removed pollinaria at their first attempt, some bees did not remove pollinaria even after three attempts. When testing the efficiency of pollinaria removal, we found that pollinator type had significant effects on pollinaria removal ($\chi^2 = 7.01$, $df = 1$, $p < 0.01$; **Figure 9**) but no effects of number of attempts on pollinaria removal success ($\chi^2 = 2.34$, $df = 2$, $p = 0.31$; **Figure 9**). *Post hoc* tests indicated that the efficiency of pollinaria removal was significantly higher within each attempt for *T. fera* compared to *A. plumipes* ($z = -2.850$, $p \leq 0.05$; **Figure 9**).

The Role of Visual Traits in Pollination Success

Inflorescence display was significantly larger in *N. ustulata* var. *aestivalis* (mean ± SD: 758.1 ± 320.1 mm^2) than in var. *ustulata* (mean ± SD: 543.9 ± 325.4 mm^2; $\chi^2 = 62.71$, $df = 1$, $p < 0.0001$); whereas dark top display did not differ between *N. ustulata* var. *ustulata* (mean ± SD: 121.0 ± 35.8 mm^2) and var. *aestivalis* (mean ± SD: 123.0 ± 31.5 mm^2; $\chi^2 = 0.20$, $df = 1$, $p = 0.655$). We also did not find an effect of the dark top display on the probability of pollinaria removal ($\chi^2 = 0.54$, $df = 1$, $p = 0.464$), pollinia deposition ($\chi^2 = 2.30$, $df = 1$, $p = 0.130$) and pollination event ($\chi^2 = 1.29$, $df = 1$, $p = 0.255$). Although we also did not detect an effect of the total inflorescence display on the probability of pollinaria removal ($\chi^2 = 2.11$, $df = 1$, $p = 0.15$), effect of inflorescence display on pollinia deposition approached statistical significance ($\chi^2 = 3.27$, $df = 1$, $p = 0.070$) and was marginally non-significant for pollination events ($\chi^2 = 3.74$, $df = 1$, $p = 0.053$).

Role of Dark Top in Fly Pollinator Attraction

In our experiment with manipulation of the inflorescence dark top, there were significant effects of the treatment stage ($\chi^2 = 5.68$, $df = 1$, $p < 0.05$), the inflorescence type (i.e., selected for dark top excision or not; $\chi^2 = 10.29$, $df = 1$, $p < 0.01$) and the interaction treatment stage × inflorescence type ($\chi^2 = 15.97$, $df = 1$, $p < 0.001$) on the number of *Tachina* aff. *fera* visits to var. *ustulata* (**Figure 10**). In the case of var. *aestivalis*, we did not find significant effects of the treatment stage on the number of *Nowickia ferox* visits ($\chi^2 = 0.017$, $df = 2$, $p = 0.99$), however the inflorescence type ($\chi^2 = 10.27$, $df = 1$, $p < 0.01$) and the interaction treatment stage × inflorescence type ($\chi^2 = 6.98$, $df = 1$, $p < 0.05$) had significant effects on pollinator visits (**Figure 10**). *Post hoc* tests indicated that inflorescences with the dark top had significantly more visits than inflorescences without it ($z = 3.507$, $p < 0.01$; **Figure 9**), but no other pair-wise differences were found.

DISCUSSION

Despite being widely distributed, *Neotinea ustulata* is one of the most enigmatic European orchids in terms of its reproductive biology. Our study reveals that morphological (e.g., larger flowers in var. *ustulata*), visual (e.g., spectral reflectance pattern) and chemical (e.g., pattern of semi- and low- volatile compounds) floral traits distinguish *N. ustulata* var. *ustulata* from var. *aestivalis*. Our observations across different locations in Europe also support some early records that *N. ustulata* is mainly

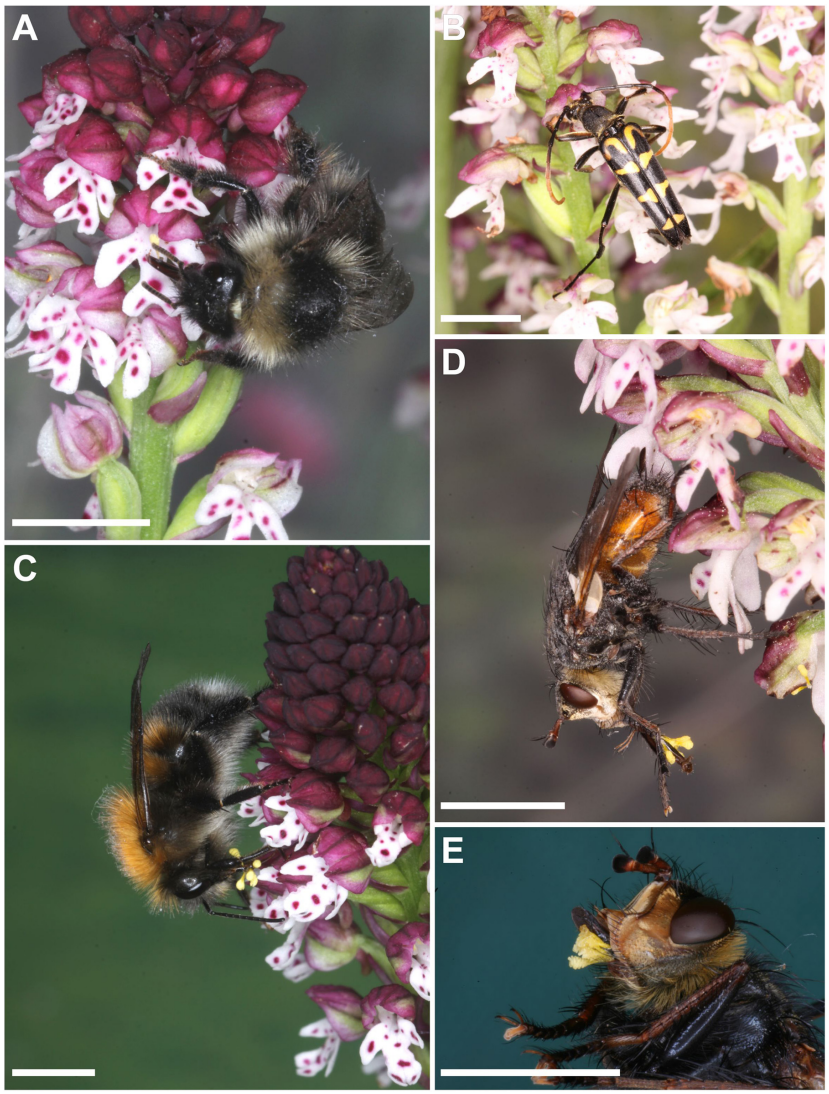

FIGURE 8 | Pollinators of *Neotinea ustulata*: **(A)** *Bombus mucidus*, **(B)** *Leptura annularis*, **(C)** *Bombus hypnorum*, **(D)** *Tachina fera*, **(E)** *Tachina fera* close-up. They were recorded visiting flowers of *N. ustulata* var. *ustulata* **(A,B,D,E)** and var. *aestivalis* **(C)**. White bars represent scale of 5 mm. Photographs by H. Paulus.

pollinated by tachinid flies (Godfery, 1933; Vöth, 1984). However, the relative importance of tachinids differs between the two varieties, playing a predominant role as pollinator for the late flowering var. *aestivalis*. The complexity of floral traits and pollinator spectra of *N. ustulata* may indicate a complex, and not yet fully understood, food-deceptive mechanism.

Morphological, Visual, and Chemical Differences Between *N. ustulata* Varieties

Morphological Cues

Flower morphology can play a key role in pollination by promoting reproductive isolation, particularly in highly specialized pollination systems (Schiestl and Schlüter, 2009). For example, in the sexually deceptive orchid genus *Chiloglottis* R. Br., differences in floral morphology have been shown to be enough to achieve pollinator specificity in species relying on the same chemical attractant (de Jager and Peakall, 2016). Variations in floral size can also affect morphological fit and, therefore, pollen/pollinia transfer by their respective pollinators (Anderson and Johnson, 2008; Solís-Montero and Vallejo-Marín, 2017; de Jager and Peakall, 2019). Even relatively subtle differences in flower structures can have an impact on floral isolation such as the position of pollinaria in the moth pollinated *Platanthera bifolia* (L.) Rich. and *P. chlorantha* (Custer) Rchb., which are then attached on different parts of the pollinators (Nilsson, 1983; Schiestl and Schlüter, 2009). Although the differences in size among floral structures of *N. ustulata* var. *ustulata* and var. *aestivalis* were usually quite small, they are consistent even at the population level, and suggest that the two varieties might be morphologically adapted to different pollinator spectra, although

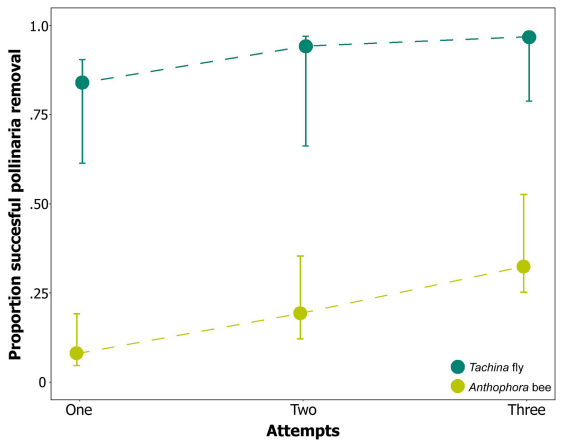

FIGURE 9 | Pollinaria removal success in up to three attempts by the two main pollinator guilds of *Neotinea ustulata*, bees (*Anthophora plumipes*; $n = 12$) and flies (*Tachina fera*; $n = 6$). Back-transformed means and standard errors are shown.

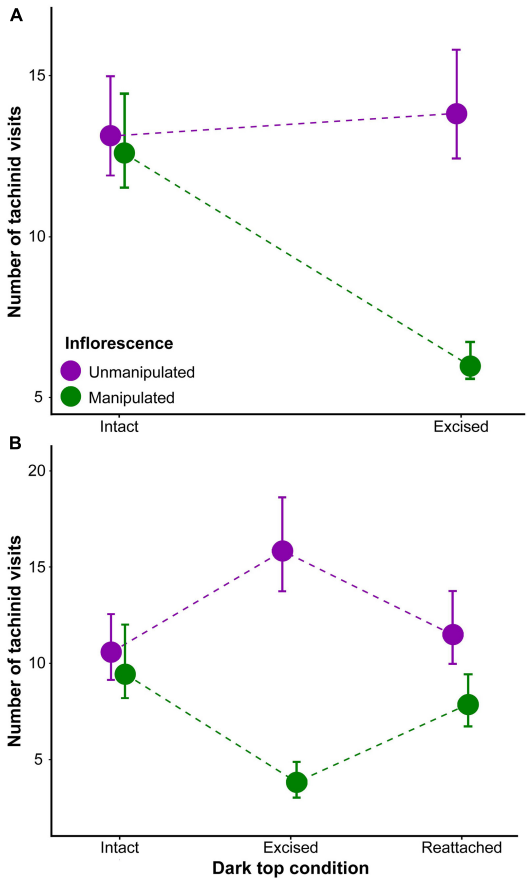

FIGURE 10 | Attractiveness of inflorescence pairs of *Neotinea ustulata* varieties to pollinators: **(A)** *Tachina* aff. *fera* visits to var. *ustulata* inflorescences with and without the dark top excised from one of them. **(B)** *Nowickia ferox* visits to var. *aestivalis* inflorescences with, without and reattached dark top. Note the visits to the inflorescence decrease when dark top is visually absent to pollinators. Back-transformed means and standard errors are shown.

phenotypic plasticity and non-pollinator selection should not be ruled out (Caruso et al., 2019; Sletvold, 2019). For instance, the width of the hood, the length of the lip and the length of the spur could all act as filters for potential pollinators. In fact, a previous study has already shown that the spur length of *N. ustulata* could be under pollinator-mediated selection (Trunschke et al., 2017). The role of flower traits for ensuring mechanical fit has been documented in diverse plant groups (e.g., Steiner and Whitehead, 1990; Muchhala, 2007; Anderson and Johnson, 2008; Pauw et al., 2009), including orchids (e.g., Chapurlat et al., 2015; Gögler et al., 2015; Rakosy et al., 2017; de Jager and Peakall, 2019). The smaller dimensions of *N. ustulata* var. *aestivalis* flowers could thus favor tachinids as pollinators and, conversely, larger flowers with deeper spur could favor a wider range of pollinators and improve pollinaria transfer. Assessment of mechanical fitting and behavioral experiments are necessary to test the role of morphology in *N. ustulata* pollinator filtering.

Visual Cues

Floral color is one of the main traits used by pollinators to identify both rewarding flowering plants and their deceptive mimics (Reverté et al., 2016). While all pollinators are able to respond to flower color signals, their preference for particular color hues and their ability to discriminate between them varies among groups (Reverté et al., 2016). Color reflectance patterns were structured at the population within and between varieties of *N. ustulata*. Although these differences can be related to phenotypic plasticity and non-pollinator-mediated selection (Ellis and Johnson, 2009; Caruso et al., 2019; Sletvold, 2019), pollinator preferences could be also involved. For instance, pollinator preferences have shaped daisy communities, which function as fly pollination specialist (Ellis et al., 2021). Variation in color hue among *N. ustulata* populations can be related to pollinator detectability and environmental conditions such as background context (e.g., coloration of surrounding plants and soil). Independently of the population or variety origin, the stark chromatic contrast between the dark top and the white lips can be discriminated by both bees and flies, and its presence may be result of pollinator selection (see below). This is supported by the occurrence of bright top forms (e.g., light pink or white top) in *N. ustulata* inflorescences, which are not stable and appear from time to time in different populations (Foley, 1990). Color pattern and color contrast have been shown to be important in pollinator attraction (Zhang et al., 2017), and the dark top coloration would play a similar role (see below). Experiments are needed to test the role of color pattern in the pollination success of *N. ustulata* varieties.

Chemical Cues

Both *N. ustulata* varieties produced a rather complex floral scent and the scent bouquets of *N. ustulata* individuals were highly variable. This pattern is not restricted to *N. ustulata* populations from Czech Republic since the floral scent of a var. *ustulata* plant from Sweden was similarly dominated by

α-pinene, β-myrcene, limonene, eucalyptol, and (*E*)-β-ocimene (Kaiser, unpublished data). A study based on a plant from Italy also reported a complex bouquet including eucalyptol and D-limonene (i.e., D'auria et al., 2021); although we are cautious with the identity of some compounds also reported by the authors as they seem to be environmental contaminants. Differences between *N. ustulata* varieties were only detected when comparing compounds with low- and semi-volatility; however, larger scale sampling would be required to confirm that there are not differences between varieties when comparing highly volatile compounds. Differences in hydrocarbon compounds, which were predominant among compounds with lower volatility, could induce differential responses of pollinators once they approach or land on the flowers. Indeed, the function of these compounds, especially alkenes, has been intensively investigated in sexually deceptive orchids (Ayasse et al., 2011; Bohman et al., 2016). In fact, it is extremely unusual for food-deceptive species to produce such a high proportion of alkenes (Schiestl and Cozzolino, 2008), and in particular the production of (*Z*)-11-tricosene, which is extremely rare even among sexually deceptive plants but has been recorded in some tachinids (Martel et al., unpublished data). Therefore, alkenes and other low-volatile compounds could play a major role at short-range attraction in *N. ustulata* by eliciting certain behaviors in tachinid pollinators, while highly volatile compounds and visual cues remain probably the most important stimuli for long-range attraction. Further physiological and behavioral analyses are needed to elucidate the differential importance of *N. ustulata* floral scent in pollination.

Reproductive Success in *N. ustulata* Varieties

As typical for deceptive orchids, fruit set was rather low in most populations of *N. ustulata*. Reproductive success of *N. ustulata* was found to be higher than previously reported (i.e., Neiland, 2001; Harder and Johnson, 2008), but matches with those of some food-deceptive orchids (Gill, 1989; Fritz and Nilsson, 1994; Scopece et al., 2009, 2010; Trunschke et al., 2017). Contrary to previous reports (i.e., Tali et al., 2004; Haraštová-Sobotková et al., 2005), we did find differences in the characters associated with reproductive success between varieties. Furthermore, *N. ustulata* var. *aestivalis* was found to be slightly more successful than var. *ustulata*. Nevertheless, independently of the variety, the bottom and middle section of the inflorescence developed more fruits than the top section. This is consistent with gradual acropetalous opening of *N. ustulata* inflorescences and with tachinid behavior, as they always visit the freshest flowers on the top, but as flies habituate and more buds open, later opening flowers get less visits. This pattern is similar to those recorded in food-deceptive plants pollinated by bumblebees, which contrary to tachinids usually pursue the inflorescence from the bottom toward the top (Nilsson, 1980, 1983, 1984; Fritz, 1990; Jersáková and Kindlmann, 1998) and in sexually deceptive plants pollinated by bees and wasps, which learn to avoid deceitful plants (Peakall, 1990; Peakall and Handel, 1993; Ayasse et al., 2000). Thus, reproductive success pattern of *N. ustulata* indicates that pollinators are initially attracted to visit the plants, but then learn to avoid them.

Pollinator Spectra in *N. ustulata* Varieties

Diverse insect taxa visited flowers of both *N. ustulata* varieties, but only a few acted as true pollinators and the two varieties differ in their main pollinators. Both *Anthophora* and *Tachina* are able to remove pollinaria of *N. ustulata* var. *ustulata* with their proboscis, but *Tachina* flies were more efficient in doing so. Furthermore, while the tachinids were unable to detach the pollinaria from their proboscis, we frequently observed *Anthophora* bees grooming them off. Our observations of *N. ustulata* var. *ustulata* are in line with previous records in which bees and flies have been recorded (Paulus, 2005; Claessens and Kleynen, 2016). In contrast, our observations do not support the assumption that *N. ustulata* var. *aestivalis* is adapted to beetle pollination (see Danesch and Danesch, 1962; Mrkvicka, 1991; van der Cingel, 1995), though beetles can also act as occasional pollinators. While we observed a broad range of beetles, bees and even butterflies visiting the flowers of var. *aestivalis*, only *Nowickia* and *Tachina* were was consistently observed removing pollinaria and occasionally *Bombus* spp. Thus, *N. ustulata* var. *aestivalis* may have become more specialized for fly pollination than var. *ustulata* and this adaptation could be triggered by differences in availability of insects through space and time of flowering, and the higher efficiency of tachinids in transferring pollinia compared to bees. Despite the difference in pollinator spectra, pollen transfer efficiency was similar between both varieties, which would also be linked to their similar degrees of specialization or adaptation to fly pollinators. Our tachinid collection from Austrian and Czech populations suggests that the two *N. ustulata* varieties have one species of *Nowickia* and two of *Tachina* as main pollinators (i.e., *N. ustulata* var. *ustulata* is pollinated by *T. fera* and *T. magnicornis*, and var. *aestivalis* by *T. magnicornis* and *N. ferox*); however, differences in tachinid attraction can also be due to differences in species availability throughout the year and not specific adaption to one of them. *Tachina fera* and *T. magnicornis* are morphologically very similar, can co-occur and can be easily misidentified without a close inspection by a trained person. Although previous reports indicated that *T. magnicornis* flies were visiting *N. ustulata* var. *ustulata* flowers (e.g., Vöth, 1984; Paulus, 2005), some of those flies could have actually been *T. fera* (see **Supplementary Table 6**), which is more abundant and common than *T. magnicornis* (Paulus, pers. obs.). Fly pollination is rather rare among European orchids (Jersáková et al., 2016) and, to the best of our knowledge, no tachinid species has been recorded as the main pollinator of any European orchid. Tachinids have been previously reported in only a couple of specialized pollination systems such as those of *Schizochilus* (van der Niet et al., 2010) and *Telipogon* (Martel et al., 2016). Independently of the identity of their pollinators, both varieties have a similar male success and pollen transfer efficiency, but different female success. Hence, based on pollen transfer efficiency, pollinators of both varieties seem to be very efficient in delivering pollinia (i.e., a ratio of pollinia deposited to pollinia removed above 0.50). The pollinator efficiency is linked to the pollinator constancy as well as pollinator identity (Peter and Johnson, 2009). We therefore suggest that the reported efficiency may be related to tachinids, which seem to be more constant and are more efficient in removing pollinaria.

Role of the Dark Top in *N. ustulata*

We have reliably demonstrated that the dark top is an attractant signal of *N. ustulata* as inflorescences bearing the dark top received significantly more visits of tachinids than inflorescences without it. This increasing attractiveness can be result of a combined effect of color, shape and size of the dark top (i.e., visual stimuli). These findings appear to support the theory put forward almost 90 years ago by Godfery (1933), which considered that the dark top may be an adaptation to attract fly pollinators. The dark top of *N. ustulata* is quite unique among European orchids and although other species can also present a dark top (e.g., *Orchis purpurea* Huds.), they seem to have a far weaker contrast against the lips. Dark spots have also arisen in diverse plant groups such as Apiaceae, Asteraceae, Geraniaceae, Iridaceae, Liliaceae, Papaveraceae and Ranunculaceae (Dafni et al., 1990; Westmoreland and Muntan, 1996; Johnson and Midgley, 1997; van Kleunen et al., 2007; Goulson et al., 2009; Ellis and Johnson, 2010). It was suggested that dark, contrasting spots in flowers and inflorescences might even act as an insect mimic as demonstrated in some beetle- and fly-pollinated plants (e.g., Johnson and Midgley, 1997; van Kleunen et al., 2007; Goulson et al., 2009; Ellis and Johnson, 2010); however, at this point, it is unknown how tachinids are perceiving the contrasting dark top of *N. ustulata*. Although dark spots in flowers promotes pollinator attraction in some plants (e.g., Johnson and Midgley, 1997; van Kleunen et al., 2007; Goulson et al., 2009; Ellis and Johnson, 2010), it does not necessarily involve enhancement of fruit set (e.g., Westmoreland and Muntan, 1996; Johnson and Midgley, 1997; Ellis and Johnson, 2010). However, as dark spots promote visits of the main pollinators, their presence is likely to impact the fruit set of *N. ustulata*. *In situ* manipulation experiments would be needed to know how tachinids are perceiving the contrasting dark and its impact on pollination success.

Pollination Strategy of *N. ustulata*

Neotinea ustulata varieties are visited by generalist insects that seek for nectar and pollen (e.g., bees and flies), and the lip is bright white as in other food-deceptive plants (Johnson and Schiestl, 2016). However, other floral traits such as a strong and complex scent including alkenes, the evolutionary innovation of a dark top on the inflorescence and the predominance of tachinid male visitors (Martel et al., unpublished data) suggest that *N. ustulata* may have evolved a complex food-deceptive pollination system. Based on previous studies (i.e., Vogel, 1972; Bino et al., 1982; Valterová et al., 2007; Scopece et al., 2009), it seems that some orchid species potentially classified as food-deceptive have also evolved signals not usually associated with this strategy (e.g., some kind of chemical mimicry of their pollinators) and their pollinators are gender biased (i.e., attract predominantly female or male pollinators). This may also occur in *N. ustulata* as most abundant alkenes have been also detected in *Tachina* flies (Martel et al., unpublished data). Most floral visitors of *N. ustulata* are only seeking food, but tachinids would in addition perceive alkenes as a signal of other yet unknown resources. For instance, alkenes are key in the chemical mimicry and pollinator attraction of the sexually deceptive *Telipogon peruvianus* T.Hashim. to its tachinid specialist pollinator (Martel et al., 2019). In other complex pollination systems, the fly-pollinated *Gorteria diffusa* Thunb. (Asteraceae) attracts flies seeking food, but male flies are also sexually attracted and try to copulate with the black petal ornamentation (Ellis and Johnson, 2010); similarly, some populations of the beetle-pollinated *Luisia teres* Gaudich attracts nectar-feeding females and male beetles, whereas others sexually attract males (Sugiura et al., 2021). Hence, the combination of traits present in *N. ustulata* indicates that this orchid would exploit not only food-based signals but also sexual ones (e.g., gender-specific pheromones). To address this issue further studies should identify physiologically active scent compounds and carry out behavioral bioassays.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CM, DR, MA, HP, and JJ conceived the study. CM, DR, SD, SJ, MA, HP, and JJ designed the study. CM, DR, HP, LN, HM, and JJ collected the data. CM, DR, SD, SJ, and JJ analyzed the data. CM, DR, and JJ drafted the first version of the manuscript. CM wrote the final version. All authors contributed to manuscript revision and read and approved the submitted version.

FUNDING

This work was partially funded by DAAD and MŠMT to MA and JJ (DAAD-16-13/2016-2017 and 7AMB16DE003, respectively).

ACKNOWLEDGMENTS

We thank Jaromír Vaňhara, Peter Sehnal, and Hans-Peter Tschorsnig for tachinid determination; Ivana Jongepierová, Karel Fajmon, Vlastimil Vlačiha, Dano Dítě for locations of *N. ustulata* populations. We also thank Casper van der Kooi and Ewan Bodenham for the invitation and support to publish our study in this special issue. Roxana Rojas kindly prepared the map shown in Figure 2. The reviewers make very helpful comments that improved an earlier version of the manuscript. DAAD and MŠMT supported this study by a shared Grant to JJ (7AMB16DE003) and MA (DAAD-16-13/2016-2017). CM thanks the Max Planck Partner Group for providing financial supports at PUCP.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.659176/full#supplementary-material

REFERENCES

Adams, R. P. (2017). *Identification of Essential Oil Components by Gas Chromatograph/Mass Spectrometry*, 5th Edn. Carol Stream, IL: Allured Publishing Corporation.

An, L., Neimann, A., Eberling, E., Algora, H., Brings, S., and Lunau, K. (2018). The yellow specialist: dronefly Eristalis tenax prefers different yellow colours for landing and proboscis extension. *J. Exp. Biol.* 221:jeb184788. doi: 10.1242/jeb.184788

Anderson, B., and Johnson, S. D. (2008). The geographical mosaic of coevolution in a plant-pollinator mutualism. *Evolution* 62, 220–225. doi: 10.1111/j.1558-5646.2007.00275.x

Ayasse, M., Schiestl, F. P., Paulus, H. F., Löfstedt, C., Hansson, B. S., Ibarra, F., et al. (2000). Evolution of reproductive strategies in the sexually deceptive orchid Ophrys sphegodes: how does flower-specific variation of odour signals influence reproductive success? *Evolution* 54, 1995–2006. doi: 10.1111/j.0014-3820.2000.tb01243.x

Ayasse, M., Stokl, J., and Francke, W. (2011). Chemical ecology and pollinator-driven speciation in sexually deceptive orchids. *Phytochemistry* 72, 1667–1677. doi: 10.1016/j.phytochem.2011.03.023

Bates, D., Maechler, M., Bolker, B., Walker, S., Bojesen Christensen, R. H., Singmann, H., et al. (2019). *lme4: linear mixed-effects models using 'eigen' and s4. R package version 1.1-21*. Available Online at: https://cran.r-project.org/package=lme4/.

Baumann, H., Blatt, H., Kretzschmar, H., and Eccarius, W. (2005). "Die Orchideentaxa," in *Die Orchideen Deutschlands*, eds H. Blatt, W. Eccarius, and H. Kretzschmar (Uhlstädt-Kirchhasel: Verlag Arbeitskreise Heimische Orchideen), 223–685.

Bino, R. J., Dafni, A., and Meeuse, A. D. J. (1982). The pollination ecology of Orchis galilaea (Bornm. et Schulze) Schltr. (Orchidaceae). *New Phytol.* 90, 315–319. doi: 10.1111/j.1469-8137.1982.tb03263.x

Bohman, B., Flematti, G. R., Barrow, R. A., Pichersky, E., and Peakall, R. (2016). Pollination by sexual deception - it takes chemistry to work. *Curr. Opin. Plant Biol.* 32, 37–46. doi: 10.1016/j.pbi.2016.06.004

Borsos, O. (1962). Geobotanische monographie der orchideen der pannonischen und karpatischen Flora VI. *Sec. Biol.* 5, 27–61.

Carlson, D. A., Mayer, M. S., Silhacek, D. L., James, L. D., Beroza, M., and Bierl, B. A. (1971). Sex attractant pheromone of the house fly: isolation, identification and synthesis. *Science* 174, 76–77. doi: 10.1126/science.174.4004.76

Caruso, C. M., Eisen, K. E., Martin, R. A., and Sletvold, N. (2019). A meta-analysis of the agents of selection on floral traits. *Evolution* 73, 4–14. doi: 10.1111/evo.13639

Chapurlat, E., Ågren, J., and Sletvold, N. (2015). Spatial variation in pollinator-mediated selection on phenology, floral display and spur length in the orchid Gymnadenia conopsea. *New Phytol.* 208, 1264–1275. doi: 10.1111/nph.13555

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543. doi: 10.1007/BF00199331

Chittka, L., and Kevan, P. G. (2005). "Flower colour as advertisement," in *Practical Pollination Biology*, eds A. Dafni, P. G. Kevan, and B. C. Husband (Cambridge, ON: Enviroquest), 157–196.

Claessens, J., and Kleynen, J. (2011). *The Flower of the European Orchid. Form and Function*. Geulle: Jean Claessens and Jacques Kleynen Publishers.

Claessens, J., and Kleynen, J. (2016). *Orchidées d'Europe - Fleur et Pollinisation*. Mèze: Biotope éditions.

Dafni, A., Bernhardt, P., Schmida, A., Ivri, Y., Greenbaum, S., O'Toole, C., et al. (1990). Red bowl-shaped flowers: convergence for beetle pollination in the Mediterranean region. *Israel J. Bot.* 39, 81–92. doi: 10.1080/0021213X.1990.10677134

Danesch, E., and Danesch, O. (1962). *Orchideen Europas, Mitteleuropa*. Bern: Hallwag Verlag.

D'auria, M., Lorenz, R., Mecca, M., Racioppi, R., Romano, V. A., and Viggiani, L. (2021). The scent of Neotinea orchids from basilicata (southern Italy). *Nat. Prod. Res.* 2021, 1–3. doi: 10.1080/14786419.2020.1871343

de Jager, M. L., and Peakall, R. (2016). Does morphology matter? An explicit assessment of floral morphology in sexual deception. *Funct. Ecol.* 30, 537–546. doi: 10.1111/1365-2435.12517

de Jager, M. L., and Peakall, R. (2019). Experimental examination of pollinator-mediated selection in a sexually deceptive orchid. *Ann. Bot.* 123, 347–354. doi: 10.1093/aob/mcy083

Delforge, P. (2006). *Orchids of Europe, North Africa and the Middle East*. London: A and C Black Publishers Ltd.

Ellis, A. G., Anderson, B., and Kemp, J. E. (2021). Geographic mosaics of fly pollinators with divergent color preferences drive landscape-scale structuring of flower color in daisy communities. *Front. Plant Sci.* 12:617761. doi: 10.3389/fpls.2021.617761

Ellis, A. G., and Johnson, S. D. (2009). The evolution of floral variation without pollinator shifts in Gorteria diffusa (Asteraceae). *Am. J. Bot.* 96, 793–801. doi: 10.3732/ajb.0800222

Ellis, A. G., and Johnson, S. D. (2010). Floral mimicry enhances pollen export: the evolution of pollination by sexual deceit outside of the Orchidaceae. *Am. Nat.* 176, E143–E151. doi: 10.1086/656487

Fay, M. F. (2018). Orchid conservation: how can we meet the challenges in the twenty-first century? *Bot. Stud.* 9:16. doi: 10.1186/s40529-018-0232-z

Foley, M. J. Y. (1990). The current distribution and abundance of Orchis ustulata L. in southern England. *Watsonia* 18, 37–48.

Fritz, A., and Nilsson, L. A. (1994). How pollinator-mediated mating varies with population size in plants. *Oecologia* 100, 451–462. doi: 10.1093/beheco/arh147

Fritz, A. L. (1990). Deceit pollination of Neotinea spitzelii (Orchidaceae) on the island of Gotland in the Baltic: a suboptimal system. *Nord. J. Bot.* 9, 577–587. doi: 10.1111/j.1756-1051.1990.tb00548.x

Galizia, C. G., Kunze, J., Gumbert, A., Borg-Karlson, A.-K., Sachse, S., Markl, C., et al. (2004). Relationship of visual and olfactory signal parameters in a food-deceptive flower mimicry system. *Behav. Ecol.* 16, 159–168.

Gill, D. E. (1989). "Fruiting failure, pollinator inefficiency and speciation in orchids," in *Speciation and its Consequences*, eds D. Otte, and J. A. Endler (Sunderland, MA: Sinauer), 458–481.

Godfery, M. J. (1933). *Monograph and Iconograph of Native British Orchidaceae*. Cambridge: Cambridge University Press.

Gögler, J., Stökl, J., Cortis, P., Beyrle, H., Rosaria, M., Lumaga, B., et al. (2015). Increased divergence in floral morphology strongly reduces gene flow in sympatric sexually deceptive orchids with the same pollinator. *Evol. Ecol.* 29, 703–717. doi: 10.1007/s10682-015-9779-2

Goulson, D., McGuire, K., Munro, E. E., Adamson, S., Colliar, L., Park, K. J., et al. (2009). Functional significance of the dark central floret of Daucus carota (Apiaceae) L.; is it an insect mimic? *Plant Species Biol.* 24, 77–82. doi: 10.1111/j.1442-1984.2009.00240.x

Haraštová-Sobotková, M., Jersáková, J., Kindlmann, P., and Čurn, L. (2005). Morphometric and genetic divergence among populations of Neotinea ustulata (Orchidaceae) with different flowering phenologies. *Folia Geobot.* 40, 385–405. doi: 10.1007/BF02804287

Harder, L. D., and Johnson, S. D. (2008). Function and evolution of aggregated pollen in angiosperms. *Int. J. Plant Sci.* 169, 59–78. doi: 10.1086/523364

Hedlund, K., Bartelt, R. J., Dicke, M., and Vet, L. E. M. (1996). Aggregation pheromones of Drosophila immigrans, D. phalerata, and D. suboscura. *J. Chem. Ecol.* 22, 1835–1844. doi: 10.1007/BF02028507

Jacquemyn, H., Brys, R., Hermy, M., and Willems, J. O. (2005). Does nectar reward affect rarity and extinction probabilities of orchid species? An assessment using historical records from Belgium and the Netherlands. *Biol. Conserv.* 121, 226–257. doi: 10.1016/j.biocon.2004.05.002

Jensen, J. M., and Pedersen, H. A. (1999). Ny lokalitet for Bakke-Gögehurt (Orchis ustulata) - med noter om artens fänologiske og morfologiske variation. *Flora og fauna* 105, 29–36.

Jersáková, J., Johnson, S. D., and Kindlmann, P. (2006). Mechanisms and evolution of deceptive pollination in orchids. *Biol. Rev.* 81, 219–235. doi: 10.1017/S1464793105006986

Jersáková, J., Jürgens, A., Smilauer, P., and Johnson, S. D. (2012). The evolution of floral mimicry: identifying traits that visually attract pollinators. *Funct. Ecol.* 26, 1381–1389. doi: 10.1111/j.1365-2435.2012.02059.x

Jersáková, J., and Kindlmann, P. (1998). Patterns of pollinator-generated fruit set in Orchis morio (Orchidaceae). *Folia Geobot.* 33, 377–390. doi: 10.1007/BF02803641

Jersáková, J., Spaethe, J., Streinzer, M., Neumayer, J., Paulus, H., Dötterl, S., et al. (2016). Does Traunsteinera globosa (the globe orchid) dupe its pollinators

through generalized food deception or mimicry? *Bot. J. Linn. Soc.* 180, 269–294. doi: 10.1111/boj.12364

Johnson, S. D., and Midgley, J. J. (1997). Fly pollination of Gorteria diffusa (Asteraceae), and a possible mimetic function for dark spots on the capitulum. *Am. J. Bot.* 84, 429–436. doi: 10.2307/2446018

Johnson, S. D., and Schiestl, F. P. (2016). *Floral mimicry*. Oxford: Oxford University Press.

Kassambara, A., and Mundt, F. (2020). *factoextra: extract and visualize the results of multivariate data analyses. R package version 1.0.7*. Available Online at: https://cran.r-project.org/package=factoextra/.

Kümpel, H., and Mrkvicka, A. C. (1990). Untersuchungen zur Abtrennung der Neotinea ustulata L. subsp. aestivalis (Kümpel) Kümpel and Mrkvicka. *Mitt. Bl. Arbeitskr. Heim. Orch. Baden-Württ.* 22, 306–324.

Lenth, R., Singmann, H., Love, J., Buerkner, P., and Herve, M. (2020). *emmeans: estimated marginal means, aka least-squares means. R package version 1.4.8*. Available Online at: http://cran.r-project.org/package=emmeans/

Maia, R., White, T., Gruson, H., Endler, J., Eliason, C., and Bitton, P.-P. (2021). *pavo: perceptual analysis, visualization and organization of spectral colour data. R package version 2.4.0*. Available Online at: https://cran.r-project.org/package=pavo/.

Martel, C., Cairampoma, L., Stauffer, F. W., and Ayasse, M. (2016). Telipogon peruvianus (Orchidaceae) flowers elicit pre-mating behaviour in Eudejeania (Tachinidae) males for pollination. *PLoS One* 11:e0165896. doi: 10.1371/journal.pone.0165896

Martel, C., Francke, W., and Ayasse, M. (2019). The chemical and visual bases of the pollination of the Neotropical sexually deceptive orchid Telipogon peruvianus. *New Phytol.* 223, 1989–2001. doi: 10.1111/nph.15902

Mrkvicka, A. C. (1991). Bestäuber, Chromosomenzahl und weitere Beobachtungen zu Neotinea ustulata L. subsp. aestivalis (Kümpel) Kümpel and Mrkvicka. *Mitt. Bl. Arbeitskr. Heim. Orch. Baden-Württ.* 23, 331–338.

Muchhala, N. (2007). Adaptive trade-off in floral morphology mediates specialization for flowers pollinated by bats and hummingbirds. *Am. Nat.* 169, 494–504.

Neiland, R. M. (2001). "Neotinea: ecology," in *Genera Orchidacearum 2. Orchidoideae*, Part 1, eds A. M. Pridgeon, P. J. Cribb, M. W. Chase, and F. N. Rasmussen (Oxford, UK: Oxford University Press), 320–323.

Nilsson, L. A. (1980). The pollination ecology of Dactylorhiza sambucina (Orchidaceae). *Bot. Not.* 133, 367–385.

Nilsson, L. A. (1983). Processes of isolation and introgressive interplay between Platanthera bifolia (L.) Rich. and Platanthera chlorantha (Custer) Reichb. (Orchidaceae). *Bot. J. Linn. Soc.* 87, 325–350. doi: 10.1111/j.1095-8339.1983.tb00997.x

Nilsson, L. A. (1984). Anthecology of O. morio (Orchidaceae) and its outpost in the North. *Nova Acta Regiae Soc. Sci. Upsal.* 3, 167–180.

Nunes, C. E. P., Amorim, F. W., Mayer, J. L. S., and Sazima, M. (2016). Pollination ecology of two species of Elleanthus (Orchidaceae): novel mechanisms and underlying adaptations to hummingbird pollination. *Plant Biol.* 18, 15–25. doi: 10.1111/plb.12312

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2019). *vegan: community ecology package. R package version 2.5-6.* Available Online at: https://cran.r-project.org/package=vegan/.

Paulus, H. F. (2005). "Zur Bestäubungsbiologie der Orchideen," in *Die Orchideen Deutschlands*, eds H. Blatt, W. Eccarius, and H. Kretzschmar (Uhlstädt-Kirchhasel: Verlag Arbeitskreise Heimische Orchideen), 98–140.

Pauw, A., Stofberg, J., and Waterman, R. J. (2009). Flies and flowers in Darwin's race. *Evolution* 63, 268–279. doi: 10.1111/j.1558-5646.2008.00547.x

Peakall, R. (1990). Responses of male Zaspilothynnus trilobatus Turner wasps to females and the sexually deceptive orchid it pollinates. *Funct. Ecol.* 4, 159–167. doi: 10.2307/2389335

Peakall, R., and Handel, S. N. (1993). Pollinators discriminate among floral heights of a sexually deceptive orchid: implications for selection. *Evolution* 47, 1681–1687. doi: 10.1111/j.1558-5646.1993.tb01260.x

Peter, C. I., and Johnson, S. D. (2009). Reproductive biology of Acrolophia cochlearis (Orchidaceae): estimating rates of cross-pollination in epidendroid orchids. *Ann. Bot.* 104, 573–581. doi: 10.1093/aob/mcn218

R Core Team. (2017). *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Rakosy, D., Cuervo, M., Paulus, H. F., and Ayasse, M. (2017). Looks matter: changes in flower form affect pollination effectiveness in a sexually deceptive orchid. *J. Evol. Biol.* 30, 1978–1993. doi: 10.1111/jeb.13153

Renner, S. S. (2005). "Rewardless flowers in the angiosperms and the role of insect cognition in their evolution," in *Plant-Pollinator Interactions: from Specialization to Generalization*, eds N. M. Waser, and J. Olerton (Chicago, IL: University of Chicago Press), 123–144.

Renoult, J. P., Valido, A., Jordano, P., and Schaefer, H. M. (2014). Adaptation of flower and fruit colours to multiple, distinct mutualists. *New Phytol.* 201, 678–686. doi: 10.1111/nph.12539

Reverté, S., Retana, J., Gómez, J. M., and Bosch, J. (2016). Pollinators show flower colour preferences but flowers with similar colours do not attract similar pollinators. *Ann. Bot.* 118, 249–257. doi: 10.1093/aob/mcw103

Ripley, B., Venables, B., Bates, D. M., Hornik, K., Gebhardt, A., and Firth, D. (2020). *MASS: support functions and datasets for Venables and Ripley's MASS. R package version 7.3-51.6*. Available Online at: http://cran.r-project.org/package=MASS/

Rogoff, W. M., Gretz, G. H., Jacobson, M., and Beroza, M. (1973). Confirmation of (Z)-9-tricosene as a sex pheromone of the housefly. *Ann. Entomol. Soc. Am.* 66, 739–741. doi: 10.1093/aesa/66.4.739

Schiestl, F. P., and Cozzolino, S. (2008). Evolution of sexual mimicry in the orchid subtribe Orchidinae: the role of preadaptations in the attraction of male bees as pollinators. *BMC Evol. Biol.* 8:27. doi: 10.1186/1471-2148-8-27

Schiestl, F. P., and Schlüter, P. M. (2009). Floral isolation, specialized pollination, and pollinator behavior in orchids. *Annu. Rev. Entomol.* 54, 425–446. doi: 10.1146/annurev.ento.54.110807.090603

Scopece, G., Cozzolino, S., Johnson, S. D., and Schiestl, F. P. (2010). Pollination efficiency and the evolution of specialized deceptive pollination systems. *Am. Nat.* 175, 98–105. doi: 10.1086/648555

Scopece, G., Juillet, N., Müller, A., Schiestl, F. P., and Cozzolino, S. (2009). Pollinator attraction in Anacamptis papilionacea (Orchidaceae): a food or a sex promise? *Plant Species Biol.* 24, 109–114. doi: 10.1111/j.1442-1984.2009.00244.x

Shrestha, M., Dyer, A. G., Dorin, A., Ren, Z. X., and Burd, M. (2020). Rewardlessness in orchids: how frequent and how rewardless? *Plant Biol.* 22, 555–561. doi: 10.1111/plb.13113

Signorell, A. (2020). *DescTools: tools for descriptive statistics. R package version 0.99.36*. Available Online at: http://cran.r-project.org/package= DescTools/

Simón-Porcar, V. I., Silva, J. L., Meeus, S., Higgins, J. D., and Vallejo-Marín, M. (2017). Recent autopolyploidization in a naturalized population of *Mimulus guttatus* (Phrymaceae). *Bot. J. Linn. Soc.* 185, 189–207. doi: 10.1093/botlinnean/box052

Sletvold, N. (2019). The context dependence of pollinator-mediated selection in natural populations. *Int. J. Plant Sci.* 180, 934–943. doi: 10.1086/705584

Solís-Montero, L., and Vallejo-Marín, M. (2017). Does the morphological fit between flowers and pollinators affect pollen deposition? An experimental test in a buzz-pollinated species with anther dimorphism. *Ecol. Evol.* 7, 2706–2715. doi: 10.1002/ece3.2897

Stace, C. A. (2010). *New Flora of the British Isles*, 3rd Edn. Cambridge: Cambridge University Press.

Steiner, K. E., and Whitehead, V. B. (1990). Pollinator adaptation to oil-secreting flowers-Rediviva and Diascia. *Evolution* 44, 1701–1707. doi: 10.2307/2409348

Streinzer, M., Ellis, T., Paulus, H. F., and Spaethe, J. (2010). Visual discrimination between two sexually deceptive Ophrys species by a bee pollinator. *Arthropod-Plant Int.* 4, 141–148. doi: 10.1007/s11829-010-9093-4

Sugiura, N., Matsumura, S., and Yokota, M. (2021). Beetle pollination of Luisia teres (Orchidaceae) and implications of a geographic divergence in the pollination system. *Plant Species Biol.* 36, 52–59. doi: 10.1111/1442-1984.12294

Tali, K. (2004). *Species structure of Neotinea ustulata. Doctoral thesis*. Estonia: Institute of Botany and Ecology, University of Tartu.

Tali, K., Fay, M. F., and Bateman, R. M. (2006). Little genetic differentiation across Europe between early-flowering and late-flowering populations of the rapidly declining orchid Neotinea ustulata. *Biol. J. Linn. Soc.* 87, 13–25. doi: 10.1111/j.1095-8312.2006.00550.x

Tali, K., Foley, M. J. Y., and Kull, T. (2004). Orchis ustulata L. *J. Ecol.* 92, 174–184. doi: 10.1111/j.1365-2745.2004.00858.x

Trunschke, J., Sletvold, N., and Ågren, J. (2017). Interaction intensity and pollinator-mediated selection. *New Phytol.* 214, 1381–1389. doi: 10.1111/nph.14479

Valenta, K., Nevo, O., Martel, C., and Chapman, C. A. (2017). Plant attractants: integrating insights from pollination and seed dispersal ecology. *Evol. Ecol.* 31, 249–267. doi: 10.1007/s10682-016-9870-3

Valterová, I., Kunze, J., Gumbert, A., Luxová, A., Liblikas, I., Kalinová, B., et al. (2007). Male bumble bee pheromonal components in the scent of deceit pollinated orchids; unrecognized pollinator cues? *Arthropod-Plant Int.* 1, 137–145. doi: 10.1007/s11829-007-9019-y

van der Cingel, N. A. (1995). *An Atlas of Orchid Pollination: European orchids*. Rotterdam: A. A. Balkema.

van der Niet, T., Jürgens, A., and Johnson, S. D. (2010). Pollinators, floral morphology and scent chemistry in the southern African orchid genus Schizochilus. *S. Afr. J. Bot.* 76, 726–738. doi: 10.1016/j.sajb.2010.07.004

van Kleunen, M., Nanni, I., Donaldson, J. S., and Manning, J. C. (2007). The role of beetle marks and flower colour on visitation by monkey beetles (Hopliini) in the greater cape floral region, South Africa. *Ann. Bot.* 100, 1483–1489. doi: 10.1093/aob/mcm256

Vereecken, N. J., and Schiestl, F. P. (2009). On the roles of colour and scent in a specialized floral mimicry system. *Ann. Bot.* 104, 1077–1084. doi: 10.1093/aob/mcp208

Vogel, S. (1972). Pollination von Orchis papilionacea L . in den Schwarmbahnen von Eucera tuberculata F. *Jahresber. Naturw. Ver. Wuppertal* 25, 67–74.

Vorobyev, M., and Osorio, D. (1998). Receptor noise as a determinant of colour thresholds. *Proc. R. Soc. Lond. B Biol. Sci.* 265, 351–358. doi: 10.1098/rspb.1998.0302

Vöth, W. (1984). Echinomyia magnicornis Zett. Bestäuber von Neotinea ustulata L. *Die Orchidee* 35, 189–192.

Vöth, W. (1989). Die Bestäuber von Orchis papilionacea L. (Orchidaceae). *Linzer Biol. Beitr.* 21, 391–404.

Westmoreland, D., and Muntan, C. (1996). The influence of dark central florets on insect attraction and fruit production in Queen Anne's lace (Daucus carota L.). *Am. Midl. Nat.* 135, 122–129. doi: 10.1111/nph.13555

Wickham, H., Chang, W., Henry, L., Pedersen, T. L., Takahashi, K., Wilke, C., et al. (2020). *ggplot2: create elegant data visualisations using the grammar of graphics. R package version 3.3.0*. Available Online at: https://cran.r-project.org/package=ggplot2/.

Willis, K. J. (2017). *State of the World's Plants 2017*. London: Royal Botanic Gardens, Kew.

Wucherpfennig, W. (1992). Spätblühende sippen von Orchis ustulata L. *Ber. Bayer. Bot. Ges.* 63, 33–35.

Zhang, C., Vereecken, N. J., Wang, L., Tian, B., Dafni, A., Yang, Y., et al. (2017). Are nectar guide colour changes a reliable signal to pollinators that enhances reproductive success? *Plant Ecol. Divers.* 10, 89–96. doi: 10.1080/17550874.2017.1350763

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer, GS, declared a past co-authorship with several of the authors, CM, MA, to the handling editor.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 25 June 2021
doi: 10.3389/fevo.2021.684175

Honey Bees Can Taste Amino and Fatty Acids in Pollen, but Not Sterols

Fabian A. Ruedenauer[1†], Niklas W. Biewer[2], Carmen A. Nebauer[3], Maximilian Scheiner[2], Johannes Spaethe[3] and Sara D. Leonhardt[1†]*

[1] Department of Life Science Systems, Research Department Life Science Systems, Technical University of Munich, Freising, Germany, [2] Department of Animal Ecology and Tropical Biology, Biocenter, University of Würzburg, Würzburg, Germany, [3] Department of Behavioral Physiology and Sociobiology, Biocenter, University of Würzburg, Würzburg, Germany

The nutritional composition of food is often complex as resources contain a plethora of different chemical compounds, some of them more, some less meaningful to consumers. Plant pollen, a major food source for bees, is of particular importance as it comprises nearly all macro- and micronutrients required by bees for successful development and reproduction. However, perceiving and evaluating all nutrients may be tedious and impair quick foraging decisions. It is therefore likely that nutrient perception is restricted to specific nutrients or nutrient groups. To better understand the role of taste in pollen quality assessment by bees we investigated nutrient perception in the Western honey bee, *Apis mellifera*. We tested if the bees were able to perceive concentration differences in amino acids, fatty acids, and sterols, three highly important nutrient groups in pollen, via antennal reception. By means of proboscis extension response (PER) experiments with chemotactile stimulation, we could show that honey bees can distinguish between pollen differing in amino and fatty acid concentration, but not in sterol concentration. Bees were also not able to perceive sterols when presented alone. Our finding suggests that assessment of pollen protein and lipid content is prioritized over sterol content.

Keywords: nutrient perception, proboscis extension response, plant-pollinator-interactions, resource use, gustation

OPEN ACCESS

Edited by:
Isabel Marques, University of Lisbon, Portugal

Reviewed by:
Sampat Ghosh, Andong National University, South Korea
Ramesh Sagili, Oregon State University, United States

****Correspondence:***
Sara D. Leonhardt
leonhardt@wzw.tum.de

†ORCID:
Fabian A. Ruedenauer
orcid.org/0000-0002-2509-0554
Sara D. Leonhardt
orcid.org/0000-0002-8154-9569

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *22 March 2021*
Accepted: *31 May 2021*
Published: *25 June 2021*

Citation:
Ruedenauer FA, Biewer NW, Nebauer CA, Scheiner M, Spaethe J and Leonhardt SD (2021) Honey Bees Can Taste Amino and Fatty Acids in Pollen, but Not Sterols. Front. Ecol. Evol. 9:684175. doi: 10.3389/fevo.2021.684175

INTRODUCTION

Like other animals, bees need to consume nutrients to maintain their homeostasis and produce progeny (Filipiak et al., 2017). Some nutrients (i.e., non-essential nutrients) can be synthesized by using components of other nutrients as building material. In contrast, essential nutrients cannot be synthesized and need to be ingested with food. Nutrients required in relatively high amounts are termed macronutrients, i.e., carbohydrates, protein, and fat, while micronutrients are required in relatively small amounts, i.e., trace minerals or vitamins (Simpson and Raubenheimer, 2012). Protein consists of amino acids, which are needed for the synthesis of endogenic proteins (Chapman, 1998) and for larval growth (DeGroot, 1953). They additionally provide energy to flight muscles (Micheu et al., 2000). Fat consists of fatty acids, which mostly provide and store energy, but also show antibiotic properties against several pathogens, like the American foulbrood causing agent *Bacillus larvae* (Feldlaufer et al., 1993), and they may enhance cognitive performance (Arien et al., 2015, 2018). Besides fatty acids, sterols represent particularly important lipids and are essential for many insects (Hobson, 1935; Svoboda et al., 1978), since they can act as messengers in the cellular membrane and as precursors for hormones such as the molting hormone (Svoboda et al., 1978). The performance and well-being of bees and other insects does depend on both the quality

(i.e., chemical composition) and quantity (i.e., overall amount) of consumed nutrients. Several studies have shown that deviations from optimal nutrient ratios (Simpson and Raubenheimer, 2012) or over- and under-consumption of specific nutrients can lead to reduced survival and impair reproductive success in honey bees (Altaye et al., 2010; Arien et al., 2018) and bumble bees (Vaudo et al., 2016a,b; Moerman et al., 2017; Grund-Mueller et al., 2020; Ruedenauer et al., 2020).

Bees are unique in that they can obtain all essential and non-essential micro- and macronutrients from floral resources, i.e., mostly pollen and nectar (Haydak, 1970). Nectar is the main source of carbohydrates (i.e., sugars) and only contains low amounts of other nutrients (Baker, 1977; Nicolson and Thornburg, 2007). Pollen, in contrast, provides all other nutrients (DeGroot, 1953; Keller et al., 2005). However, pollen nutrient content can significantly differ within the same and among different plant species (Roulston and Cane, 2000; Roulston et al., 2000; Hanley et al., 2008; Ruedenauer et al., 2019b). As a consequence of such strong variation, bees need to assess the nutritional content of pollen when foraging to ensure an appropriate nutrient intake for themselves and their offspring and thus proper health and development. Nutritional quality assessment may take place directly through pollen nutrient perception at flowers or indirectly through physiological (e.g., nausea) or larval feedback (Behmer, 2009). Direct assessment requires the sense of taste using external chemoreception through chemotactile nutrient receptors, since nutrients are rarely volatile. In fact, bumble bees (*Bombus terrestris*) are able to perceive free amino and fatty acids (Ruedenauer et al., 2019a, 2020), whose concentrations correlate with their respective macronutrients (Ruedenauer et al., 2019b). Honey bees were found to use chemotactile cues to differentiate between pollen of different plant species, indicating that they may also use chemotactile cues to detect variations in nutrient composition (Ruedenauer et al., 2018). It is, however, still unknown, which pollen components honey bees can perceive and thus may use to assess pollen quality. Interestingly, we found that *B. terrestris* does "ignore" specific nutrients, e.g., amino acids, in pollen, even though they can perceive them when presented in isolation (Ruedenauer et al., 2019a). Instead they focused on (i.e., "prioritized" perception of) fatty acids in pollen (Ruedenauer et al., 2020). This finding indicates that bees restrict perception to specific nutrients when faced with a multitude of different compounds in their food resources.

To elucidate which nutrients can be perceived by the honey bee, *Apis mellifera*, we used a classical behavioral assay that conditions the proboscis extension response [PER, Bitterman et al. (1983), Matsumoto et al. (2012), Scheiner et al. (2013)]. PER conditioning makes use of the innate behavior of bees to extend their proboscis in response to sucrose stimulation (Bitterman et al., 1983), and has also been successfully adapted to test chemotactile stimuli like nutrients (Ruedenauer et al., 2015, 2019a, 2020). Differential conditioning of the PER can be used to test if bees are able to differentiate between different stimuli, e.g., different concentrations of the same nutrients. It therefore enables us to test if the bees are able to perceive concentration differences of a specific nutrient or nutrient group through manipulating their concentration, e.g., in pollen.

In this study we investigated whether honey bees are able to perceive concentration differences in amino acids, fatty acids and sterols (presented in isolation or in pollen) by means of chemotactile PER conditioning. Based on our previous results with bumble bees (Ruedenauer et al., 2020), which show similarities to honey bees in foraging behavior and social organization (Michener, 2000), we hypothesized that honey bees can only perceive concentration differences in pollen fatty acids and the structurally similar sterols, but ignore differences in amino acid concentrations, while they may be able to perceive all compound groups when presented in isolation.

MATERIALS AND METHODS

Bee Colonies

All experiments were performed with foragers of the western honey bee (*Apis mellifera carnica*) between June and August 2019 (pollen experiments) and in October 2020 (filter paper experiments). Honey bees were kept in Dadant bee hives at the Biocenter of the University of Würzburg, Germany. The landscape surrounding the hives comprised hedges, gardens, grassland, and orchards, which enabled the colonies to forage on a variety of different plant species (Kriesell et al., 2017). Therefore, colonies were healthy and of normal size. We tested bees from three different hives. In the late morning of sunny and warm days, we collected five departing foragers at the nest entrance of each colony, resulting in a total of fifteen bees tested per day. Bees of each colony were placed in separate containers. We did not differentiate between nectar and pollen foragers, as our aim was to obtain a general overview on nutrient perception in honey bees, though this might have increased overall variation in responses.

Preparation of Stimuli

We used a bee-collected pollen mix (Naturwaren-Niederrhein GmbH, Goch-Asperden, Germany), which was ground in an electronic coffee grinder (CM 800, Graef, Arnsberg, Germany) to produce a powder which ensured homogenization of pollen from different plant species and thorough mixing with the added substances. The pollen mix contains pollen from about fifteen different genera and sustains healthy colony development in honey bees and bumble bees (Ruedenauer et al., 2016). Pollen stimuli were prepared as described in Ruedenauer et al. (2020). For each stimulus, we added 24 g of ground pollen into a petri dish. We then added ten times the natural concentrations of either eleven different amino acids (10x AA), seven fatty acids (10x FA) or five sterols (10x SP), mixed them well in a coffee grinder and added 13 ml (for AA) or 24 ml (for FA and SP) of de-ionized water (henceforth referred to as water) to create nutritionally enriched pollen pastes of similar consistencies (for details of the used AAs, FAs and SPs, see **Supplementary Tables 1–3**). Amino acids were selected to represent a spectrum of different amino acids typically found in pollen of flowers (Ruedenauer et al., 2019b) and representing both essential and

non-essential ones for bees (according to DeGroot, 1953), as well as different chemical characteristics with regard to functional groups, polarity, and acidity. Moreover, these were the same amino acids as already used in a similar experiment with bumble bees (Ruedenauer et al., 2019a). The fatty acids used also corresponded to the ones used in previous experiments (Ruedenauer et al., 2020) and represent fatty acids typically found in pollen (Ruedenauer et al., 2019b). Unfortunately, many sterols, which are frequently found in pollen (Ruedenauer et al., 2019b) cannot be easily purchased; we therefore selected a spectrum of common pollen sterols that were commercially available. Average literature values of nutrient concentrations in pollen of a variety of different plant species were used as a reference to estimate natural concentrations (Manning, 2006; Weiner et al., 2010; Vanderplanck et al., 2014). We used ten times the average natural concentrations as they are still within the natural variation observed in pollen (Ruedenauer et al., 2019b) and found to be differentiated by *Bombus terrestris* in earlier studies (Ruedenauer et al., 2019a, 2020). To create a pure (non-nutritionally enriched) pollen paste, we only added water to the ground pollen. Pollen pastes were frozen at −20°C and allowed to defrost for half an hour before usage.

We found that honey bees were not able to perceive concentration differences of sterols in pollen (see section "Results"). Such a lack of behavioral perception may due to the bees focusing on other substances (than sterols) in the chemically complex pollen mixture, while they may still perceive sterols when presented in isolation [as shown for amino acids in bumble bees, Ruedenauer et al. (2019a, 2020)]. Alternatively, they may not at all be able to perceive sterols. To differentiate between these two possibilities, we additionally tested if honey bees are able to perceive pure sterols, i.e., isolated from other compounds found in pollen. For this, we dissolved all sterols used in the pollen experiment in 1 ml chloroform in their ten-fold natural concentration (10x SC, see **Supplementary Table 3** for amounts of individuals sterols) to obtain the same concentrations as used in the pollen experiment. To prevent concentration changes due to solvent evaporation, the mixture was always prepared on ice directly before usage.

Experimental Procedure

The experimental procedure was based on Sommerlandt et al. (2014) and Ruedenauer et al. (2015). The bees were chilled on ice for 10 min in order to immobilize them, and were then harnessed in plastic tubes (25 mm × 10 mm). Bees were fixed with a 1 mm crepe tape strip behind the head and a 10 mm strip wrapped around the tube to prevent movement except for antennae and proboscis. After 5 min, the harnessed bees were fed 4 μl of a 30% w/w sucrose solution with a micropipette and kept for 3 h in a climate chamber (25°C, 60% humidity, constant darkness).

The experiments were conducted at constant temperature of 22°C and under daylight conditions complemented by artificial light. All experimenters wore gloves during the experiments.

After the 3 h starvation period, bees were tested for a proper PER by touching their antennae with a tooth pick, soaked in 30% w/w sucrose solution. Bees that responded with a PER (ca., 84% of all bees) were allowed to consume a small drop of sucrose solution. Bees not showing a PER were excluded from the experiment. For conditioning, each bee was placed in a rack and left resting for 15 s. We then presented the nutrient stimulus (i.e., conditioned stimulus, CS: either pollen paste or dissolved sterol) on a copper plate [3 mm × 4 mm, Scheiner et al. (1999) and Ruedenauer et al. (2015)] by moving it toward the bee's left antenna. The bee was allowed to scan the stimulus for 6 s and we recorded if it showed a PER to the CS during the stimulation. Nutrient stimuli were prepared by placing 15 mg of pollen paste on a 5 mm × 5 mm wet piece of filter paper (6.8 mg after being soaked in water) or 5 μl of sterol extract on dry filter paper. Filter papers with pollen paste were always prepared directly before the stimulation (to prevent drying), while filter papers with dissolved sterols were prepared 10 min before the experiment to allow for complete evaporation of the solvent. All plates were cleaned in 99% ethanol (Hartenstein, Würzburg, Germany) after each stimulation. Three seconds after presenting the CS to the left antenna, the right antenna was touched with a wooden tooth pick. The tooth pick was either soaked in 50% sucrose solution (representing the unconditioned stimulus, US) as a reward (in CS+ trials) or blank (in CS− trials). With this approach we could test whether the bees learn to differentiate between the rewarded (CS+) and unrewarded (CS−) stimulus. After stimulation, the bee was allowed to rest for 15 s before being replaced by the next bee. After 8 min the same individual was tested again [intertrial interval (ITI), Bitterman et al. (1983)]. We conducted 20 trials for each bee, ten CS+ and ten CS− presentations in a pseudorandomized order. When bees responded with a PER after stimulation with either CS+ or CS−, it was scored as a positive response to the CS (i.e., scored as 1). When bees did not respond to either stimulus with a PER, but only showed a PER to the US (i.e., sugar water), it was scored as a negative response to the CS (i.e., scored as 0). Bees that did not respond to the US were scored with NA. If they did not respond for more than four times in a CS+ trial, they were excluded from the experiment. For bees scoring NA only up to a maximum of four times and then continued to show a PER upon CS, the NA responses were switched to no responses (0) at the end.

When pollen paste was used as stimulus, we always tested the pollen paste enriched with nutrients (10x AA, 10x FA, or 10x SP) against pure pollen paste. Sterols dissolved in chloroform were tested against chloroform only. To control for the effect of stimulus type used as either CS+ or CS−, the same stimuli were always tested with reversed meanings, i.e., each stimulus was once tested as CS+ and once as CS− for two different sets of bees.

Statistical Analyses

To assess learning performance, we tested for differences in the positive PER responses to the stimulus between CS+ and CS−. We used a binomial generalized additive mixed model (GAMM) to test for differences in responses between the two conditioned stimuli CS+ and CS− in relation to "stimulus type" (i.e., pure, 10x AA, 10x FA, 10x SP, or 10x SC). We used "trial" as smoother and "bee colony" and "bee individual" as random factors in the GAMM to take into account colony-specific variation and data dependency as each bee individual contributed with 20 data points (i.e., trials). Additionally, this approach also allowed us to analyze differences between stimulus types while

taking into account variation induced by reversed meanings of the CS. Specifically, if bees showed differences in learning patterns depending on the type of stimulus presented as rewarded (CS+) and unrewarded (CS−), this would result in a significant interaction between CS and stimulus type. If the interaction was not significant, we merged the two datasets for the two reversed meanings. We did not find any significant interactions between conditioned stimulus and "stimulus type" (**Supplementary Table 4**). Therefore, data of both experimental series were merged for all datasets, following the standard procedure for PER conditioning experiments (Laloi et al., 1999; Sommerlandt et al., 2014).

If bees were able to differentiate between the different stimuli and thus different nutrient concentrations presented in the pollen paste (all nutrients) or chloroform (sterols), we considered them able to perceive/taste the tested nutrients. We additionally assessed differences in the number of trials required by bees to significantly differentiate between the two stimuli (CS+ and CS−) using generalized linear mixed effect models (GLMM, with individual bee as random factor) for each trial. All statistical analyses were performed using R v4.0.3 (R Core Team, 2020).

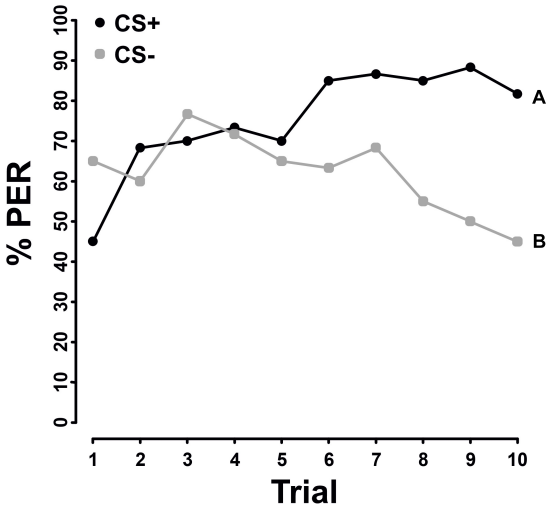

FIGURE 1 | Percentage of proboscis extension responses (% PER) shown by *Apis mellifera* individuals ($N = 60$) in differential chemotactile conditioning to pollen enriched with 10x the natural amino acid concentration (10x AA) against pure pollen over 10 trials. CS+ (black) represents the rewarded conditioned stimulus, CS– (gray) the unrewarded conditioned stimulus. Both, 10x AA and pure pollen were used as CS+ and CS–. As there was no significant difference in learning performance between 10x AA and pure pollen used as CS+ or CS– (**Supplementary Table 4**), data from both groups were combined. Different letters next to each line indicate a significant difference between the two stimuli ($P < 0.05$).

RESULTS

Honey bees were able to learn the difference between pure pollen and 10x AA pollen ($F = 4.398$; $P = 0.036$, **Figure 1**), as well as pure pollen and 10x FA pollen ($F = 21.072$, $P < 0.001$, **Figure 2**). However, PER responses differed significantly between the AA and FA experiment. When presented pure pollen and pollen enriched with FA, the bees significantly differentiated between CS+ and CS− already from the fourth trial onward (GLMM: trial 1–3: ns, trial 4: $\chi^2 = 122.5$, $P < 0.001$, **Figure 2**). However, when the bees were presented with pure pollen and pollen enriched with AA, discrimination was only significant from the sixth trial onward (GLMM: trial 1–5: ns, trial 6: $\chi^2 = 129.58$, $P = 0.006$, **Figure 1**).

In contrast to FA and AA, the bees were not able to discriminate between pollen differing in sterol concentrations, irrespective of whether the sterol stimulus was presented in pollen ($F = 1.940$, $P = 0.164$, **Figure 3**) or chloroform ($F = 2.179$, $P = 0.140$, **Figure 4**).

DISCUSSION

The ability to perceive concentration differences of nutrients in pollen is a prerequisite for assessing the nutritional quality of different pollen sources. Our results suggest that honey bees can perceive and thus taste both amino (AAs) and fatty acids (FAs) but not sterols in pollen when using their antennae. The lack of perception of pollen sterols does not seem to be a consequence of selective perception of specific nutrients (e.g., AAs or FAs) in pollen, as indicated by the experiments with pure sterols. It rather hints at a general inability of *A. mellifera* to perceive this nutrient group via their antennae. Interestingly, these findings contradict our hypothesis that honey bees restrict nutrient perception in pollen to lipids, as has been shown for the bumble bee, *Bombus terrestris* (Ruedenauer et al., 2020). While bumble bees can perceive AAs when presented in isolation (Ruedenauer et al., 2019a), they appear to "ignore" them and only respond to variation in FA content when part of a complex chemical mixture as represented by pollen (Ruedenauer et al., 2020).

The observed difference in perception of pollen nutrients between the two bee species may be related to species-specific differences in nutrient intake regulation. While bumble bees focus on the protein to lipid ratio (P:L-ratio) and specifically regulate fat intake (Vaudo et al., 2016a,b; Ruedenauer et al., 2020), honey bees seem to focus on the protein to carbohydrate ratio (P:C-ratio) and mainly regulate protein intake (Altaye et al., 2010; Pirk et al., 2010; Stabler et al., 2015), possibly in addition to the P:L ratio (Vaudo et al., 2020). The content of free AA in pollen correlates with its protein content (Ruedenauer et al., 2019b). Through assessing pollen AA and FA content, honey bees may consequently be able to regulate both protein and fat intake as well as their ratio.

Interestingly, the honey bees studied seemed to learn differences in pollen FA concentrations faster and more thoroughly than differences in pollen AA concentrations (see **Figures 1** and **2**). This finding suggests that it is easier for them to learn FA concentration differences than AA concentration differences, which might be related to the different effects that these two nutrient groups have on bee performance and thus fitness (Lepage and Boch, 1968; Vaudo et al., 2016b, 2020; Ruedenauer et al., 2020). For example, fat is detrimental to bees at much lower levels of overconsumption than protein (Canavoso et al., 2001; Harrison et al., 2012). It may therefore

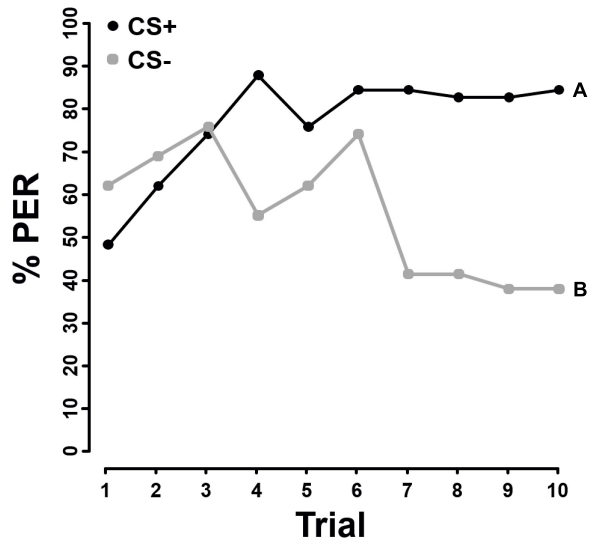

FIGURE 2 | Percentage of proboscis extension responses (% PER) shown by *Apis mellifera* individuals ($N = 58$) in differential chemotactile conditioning to pollen enriched with 10x the natural fatty acid concentration (10x FA) against pure pollen ($N = 58$) over 10 trials. CS+ (black) represents the rewarded conditioned stimulus, CS– (gray) the unrewarded conditioned stimulus. Both, 10x FA and pure pollen were used as CS+ and CS–. As there was no significant difference in learning performance between 10x FA and pure pollen used as CS+ or CS– (**Supplementary Table 4**), data from both groups were combined. Different letters next to each line indicate a significant difference between the two stimuli ($P < 0.05$).

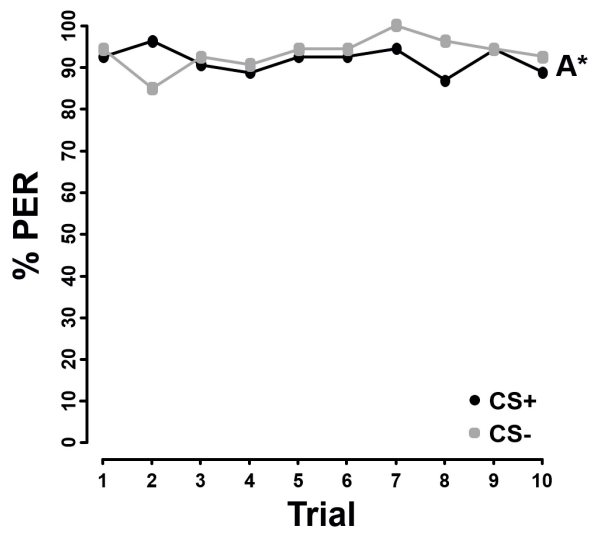

FIGURE 3 | Percentage of proboscis extension responses (% PER) shown by *Apis mellifera* individuals ($N = 53$) in differential chemotactile conditioning to pollen enriched with 10x the natural sterol concentration (10x SP) against pure pollen ($N = 53$) over 10 trials. CS+ (black) represents the rewarded conditioned stimulus, CS– (gray) the unrewarded conditioned stimulus. Both, 10x SP and pure pollen were used as CS+ and CS–. As there was no significant difference in learning performance between 10x SP and pure pollen used as CS+ or CS– (**Supplementary Table 4**), data from both groups were combined. Letters with an asterisk next to the line indicate no significant difference between the two stimuli ($P > 0.05$).

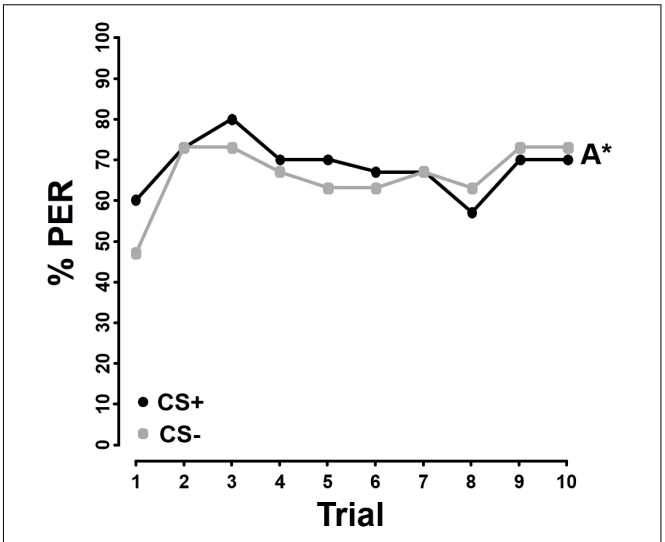

FIGURE 4 | Percentage of proboscis extension responses (% PER) shown by *Apis mellifera* individuals ($N = 30$) in differential chemotactile conditioning to sterols added to pollen in 10x their natural concentration and dissolved in chloroform (10x SC) against pure chloroform over 10 trials. CS+ (black) represents the rewarded conditioned stimulus, CS– (gray) the unrewarded conditioned stimulus. Both, 10x SC and chloroform were used as CS+ and CS–. As there was no significant difference in learning performance between 10x SC and pure chloroform used as CS+ or CS– (**Supplementary Table 4**), data from both groups were combined. Letters with an asterisk next to each line indicate no significant difference between the two stimuli ($P > 0.05$).

be adaptive for bees to be particularly sensitive to fat and strictly avoid its overconsumption. Such a link between nutrient perception and impact on animal fitness has recently also been suggested for *Bombus terrestris* (Ruedenauer et al., 2020).

Interestingly, and in contrast to our hypothesis, honey bees were not able to differentiate between different sterol concentrations or even to perceive pure sterols at all when using their antennae, suggesting that honey bees cannot receive sterols via their antennae. Given the importance of this nutrient group for bees in particular and insects in general (Hobson, 1935; Svoboda et al., 1978), this finding may at first seem surprising. However, sterol concentrations may simply be high enough in pollen of all or at least most plant species to fulfill the demand of honey bees, and/or variation in their concentrations as naturally found in pollen only barely impacts the bees' performance and reproduction. In fact, data from pollen analyzed so far (Vanderplanck et al., 2011; Somme et al., 2015; Roger et al., 2017; Ruedenauer et al., 2019b) shows that sterol contents in pollen vary less among different plant species than protein or fat contents. An alternative explanation for the lack of sterol perception in foragers might be that honey bee nurses alter the sterol composition of food when processing it inside the colony prior to provisioning their larvae. Unfortunately, precise information on the amounts and proportions of sterols required by bees and potential tolerances toward deviations from these are still unknown for the sterols used in our study (Herbert et al., 1980; Chakrabarti et al., 2019).

We can, of course, not exclude that honey bees can perceive other sterols not included in our mixture, such as, e.g., 24-methylene cholesterol, which seems to be highly important for honey bees (Vanderplanck et al., 2014; Chakrabarti et al., 2019). We can also not rule out that bees may perceive sterols by means other than their antennae, i.e., by tarsae or proboscis, or post-ingestively. The observed lack of learning may also be due to other reasons, such as aversiveness to the high concentrations of sterols used. In *B. terrestris*, however, internal, post-ingestive perception appears to complement external, pre-ingestive perception (Ruedenauer et al., 2020), indicating that antennal perception may represent a reliable proxy for overall perception abilities. In fact, post-ingestive perception is mostly used to determine the body's current nutritional needs (Simpson and Raubenheimer, 1996). Pre-ingestive perception, in contrast, may therefore be especially important for polylectic bees, like honey bees and bumble bees, which need to obtain information on individual food/pollen sources collected before mixing and processing it for larval provisioning.

In conclusion, our study reveals bee species-specific differences in pre-ingestive antennal nutrient perception, which may be linked to species-specific differences in nutritional requirements, nutrient regulation and thus in the repertoire of chemical receptors or in the neuronal processing of chemical information. In fact, recent work found species-specific receptor gene expression for different bumble bee species (Sun et al., 2020), indicating that even closely related species that share many life-history traits may differ in their perceptive strategies, likely as a consequence of species-specific nutritional requirements.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://osf.io/w2xbv/?view_only=3713e9ca1fa44448889a33e240f43629 DOI: 10.17605/OSF.IO/W2XBV.

AUTHOR CONTRIBUTIONS

SL, JS, and FR conceived the experimental concepts. NB, CN, and MS performed the experiments. FR, NB, and MS analyzed the data. FR, SL, JS, and MS drafted the manuscript. All authors discussed the results, commented on the draft, and agreed to the final version.

FUNDING

Funding was provided by the Deutsche Forschungsgemeinschaft (DFG project: LE 2750/5-1/2 to SL and SP1380/1-1 and 1-2 to JS).

ACKNOWLEDGMENTS

We are very grateful for the constructive comments provided by the reviewers.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.684175/full#supplementary-material

Supplementary Table 1 | Mean concentrations as naturally found in pollen and stimulus concentrations of amino acids (AA).

Supplementary Table 2 | Mean concentrations as naturally found in pollen and stimulus concentrations of fatty acids (FA).

Supplementary Table 3 | Mean concentrations as naturally found in pollen and stimulus concentrations of sterols (S).

Supplementary Table 4 | Statistical results of the generalized additive mixed effect models (GAMMs) testing for differences in learning performance between different stimuli.

REFERENCES

Altaye, S. Z., Pirk, C. W. W., Crewe, R. M., and Nicolson, S. W. (2010). Convergence of carbohydrate-biased intake targets in caged worker honeybees fed different protein sources. *J. Exp. Biol.* 213, 3311–3318. doi: 10.1242/jeb.046953

Arien, Y., Dag, A., and Shafir, S. (2018). Omega-6:3 ratio more than absolute lipid level in diet affects associative learning in honey bees. *Front. Psychol.* 9:1001. doi: 10.3389/fpsyg.2018.01001

Arien, Y., Dag, A., Zarchin, S., Masci, T., and Shafir, S. (2015). Omega-3 deficiency impairs honey bee learning. *Proc. Natl. Acad. Sci. U.S.A.* 112, 15761–15766. doi: 10.1073/pnas.1517375112

Baker, H. G. (1977). Non-sugar chemical constituents of nectar. *Apidologie* 8, 349–356. doi: 10.1051/apido:19770405

Behmer, S. T. (2009). Insect herbivore nutrient regulation. *Annu. Rev. Entomol.* 54, 165–187. doi: 10.1146/annurev.ento.54.110807.090537

Bitterman, M. E., Menzel, R., Fietz, A., and Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol.* 97, 107–119. doi: 10.1037/0735-7036.97.2.107

Canavoso, L. E., Jouni, Z. E., Karnas, K. J., Pennington, J. E., and Wells, M. A. (2001). Fat metabolism in insects. *Annu. Rev. Nutr.* 21, 23–46.

Chakrabarti, P., Lucas, H. M., and Sagili, R. R. (2019). Evaluating effects of a critical micronutrient (24-Methylenecholesterol) on honey bee physiology. *Ann. Entomol. Soc. Am.* 113, 176–182. doi: 10.1093/aesa/saz067

Chapman, R. F. (1998). *The Insects: Structure and Function*. Cambridge: Cambridge university press.

DeGroot, A. P. (1953). Protein and amino acid requirements of the honey bee (*Apis mellifica* L.). *Physiol. Comp. Oecol.* 3, 197–285.

Feldlaufer, M. F., Knox, D., Lusby, W., and Shimanuki, H. (1993). Antimicrobial activity of fatty acids against *Bacillus larvae*, the causative agent of American foulbrood disease. *Apidologie* 24, 95–99. doi: 10.1051/apido:19930202

Filipiak, M., Kuszewska, K., Asselman, M., Denisow, B., Stawiarz, E., Woyciechowski, M., et al. (2017). Ecological stoichiometry of the honeybee: Pollen diversity and adequate species composition are needed to mitigate limitations imposed on the growth and development of bees by pollen quality. *PLoS One* 12:e0183236. doi: 10.1371/journal.pone.0183236

Grund-Mueller, N., Ruedenauer, F. A., Spaethe, J., and Leonhardt, S. D. (2020). Adding amino acids to a sucrose diet is not sufficient to support longevity of adult bumble bees. *Insects* 11:247. doi: 10.3390/insects11040247

Hanley, M. E., Franco, M., Pichon, S., Darvill, B., and Goulson, D. (2008). Breeding system, pollinator choice and variation in pollen quality in British herbaceous plants. *Funct. Ecol.* 22, 592–598. doi: 10.1111/j.1365-2435.2008.01415.x

Harrison, J. F., Woods, H. A., and Roberts, S. P. (2012). *Ecological and Environmental Physiology of Insects*. Oxford: Oxford University Press.

Haydak, M. H. (1970). Honey bee nutrition. *Annu. Rev. Entomol.* 15, 143–156.

Herbert, E. W., Svoboda, J. A., Thompson, M. J., and Shimanuki, H. (1980). Sterol utilization in honey bees fed a synthetic diet: effects on brood rearing. *J. Insect Physiol.* 26, 287–289. doi: 10.1016/0022-1910(80)90135-3

Hobson, R. P. (1935). On a fat-soluble growth factor required by blow-fly larvae. II. Identity of the growth factor with cholesterol. *Biochem J.* 29, 2023–2026. doi: 10.1042/bj0292023

Keller, I., Fluri, P., and Imdorf, A. (2005). Pollen nutrition and colony development in honey bees: part 1. *Bee World* 86, 3–10. doi: 10.1080/0005772x.2005.11099641

Kriesell, L., Hilpert, A., and Leonhardt, S. D. (2017). Different but the same: bumblebee species collect pollen of different plant sources but similar amino acid profiles. *Apidologie* 48, 102–116. doi: 10.1007/s13592-016-0454-6

Laloi, D., Sandoz, J. C., Picard-Nizou, A. L., and Pham-Delegue, M. H. (1999). Olfactory conditioning of the proboscis extension reflex in the bumble bee Bombus terrestris. *Ann. De La Soc. Entomol. France* 35, 154–158.

Lepage, M., and Boch, R. (1968). Pollen lipids attractive to honeybees. *Lipids* 3, 530–534. doi: 10.1007/bf02530897

Manning, R. (2006). *Fatty Acid Composition of Pollen and The Effect of Two Dominant Fatty Acids (Linoleic and Oleic) in Pollen and Flour Diets on Longevity and Nutritional Composition of Honey Bees (Apis mellifera)*. Ph.D. thesis. Perth, WA: Murdoch University.

Matsumoto, Y., Menzel, R., Sandoz, J. C., and Giurfa, M. (2012). Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: a step toward standardized procedures. *J. Neurosci. Methods* 211, 159–167. doi: 10.1016/j.jneumeth.2012.08.018

Michener, C. D. (2000). *The Bees of the World*. Baltimore, MD: JHU Press.

Micheu, S., Crailsheim, K., and Leonhard, B. (2000). Importance of proline and other amino acids during honeybee flight. *Amino Acids* 18, 157–175. doi: 10.1007/s007260050014

Moerman, R., Vanderplanck, M., Fournier, D., Jacquemart, A.-L., and Michez, D. (2017). Pollen nutrients better explain bumblebee colony development than pollen diversity. *Insect Conserv. Divers.* 10, 171–179. doi: 10.1111/icad.12213

Nicolson, S. W., and Thornburg, R. W. (2007). "Nectar chemistry," in *Nectaries and Nectar*, eds S. W. Nicolson, M. Nepi, and E. Pacini (Berlin: Springer), 215–264. doi: 10.1007/978-1-4020-5937-7_5

Pirk, C. W. W., Boodhoo, C., Human, H., and Nicolson, S. (2010). The importance of protein type and protein to carbohydrate ratio for survival and ovarian activation of caged honeybees (*Apis mellifera* scutellata). *Apidologie* 41, 62–72. doi: 10.1051/apido/2009055

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Roger, N., Michez, D., Wattiez, R., Sheridan, C., and Vanderplanck, M. (2017). Diet effects on bumblebee health. *J. Insect Physiol.* 96, 128–133. doi: 10.1016/j.jinsphys.2016.11.002

Roulston, T. A. H., Cane, J. H., and Buchmann, S. L. (2000). What governs protein content of pollen: pollinator preferences, pollen-pistil interactions, or phylogeny? *Ecol. Monogr.* 70, 617–643. doi: 10.1890/0012-9615(2000)070[0617:wgpcop]2.0.co;2

Roulston, T. H., and Cane, J. H. (2000). Pollen nutritional content and digestibility for animals. *Plant Syst. Evol.* 222, 187–209. doi: 10.1007/978-3-7091-6306-1_10

Ruedenauer, F. A., Leonhardt, S. D., Lunau, K., and Spaethe, J. (2019a). Bumblebees are able to perceive amino acids via chemotactile antennal stimulation. *J. Comp. Physiol. A* 205, 321–331. doi: 10.1007/s00359-019-01321-9

Ruedenauer, F. A., Raubenheimer, D., Kessner-Beierlein, D., Grund-Mueller, N., Noack, L., Spaethe, J., et al. (2020). Best be(e) on low fat: linking nutrient perception, regulation and fitness. *Ecol. Lett.* 23, 545–554. doi: 10.1111/ele.13454

Ruedenauer, F. A., Spaethe, J., and Leonhardt, S. D. (2015). How to know which food is good for you: bumblebees use taste to discriminate between different concentrations of food differing in nutrient content. *J. Exp. Biol.* 218, 2233–2240. doi: 10.1242/jeb.118554

Ruedenauer, F. A., Spaethe, J., and Leonhardt, S. D. (2016). Hungry for quality—individual bumblebees forage flexibly to collect high-quality pollen. *Behav. Ecol. Sociobiol.* 70, 1209–1217. doi: 10.1007/s00265-016-2129-8

Ruedenauer, F. A., Spaethe, J., Van Der Kooi, C. J., and Leonhardt, S. D. (2019b). Pollinator or pedigree: which factors determine the evolution of pollen nutrients? *Oecologia* 191, 349–358. doi: 10.1007/s00442-019-04494-x

Ruedenauer, F. A., Wöhrle, C., Spaethe, J., and Leonhardt, S. D. (2018). Do honeybees (*Apis mellifera*) differentiate between different pollen types? *PLoS One* 13:e0205821. doi: 10.1371/journal.pone.0205821

Scheiner, R., Abramson, C. I., Brodschneider, R., Crailsheim, K., Farina, W. M., Fuchs, S., et al. (2013). Standard methods for behavioural studies of *Apis mellifera*. *J. Apicult. Res.* 52, 1–58. doi: 10.16984/saufenbilder.300635

Scheiner, R., Erber, J., and Page, R. E. (1999). Tactile learning and the individual evaluation of the reward in honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 185, 1–10. doi: 10.1007/s003590050360

Simpson, S. J., and Raubenheimer, D. (1996). Feeding behaviour, sensory physiology and nutrient feedback: a unifying model. *Entomol. Exp. Appl.* 80, 55–64. doi: 10.1007/978-94-009-1720-0_13

Simpson, S. J., and Raubenheimer, D. (2012). *The Nature of Nutrition: A Unifying Framework from Animal Adaptation to Human Obesity*. Princeton: Princeton University Press.

Somme, L., Vanderplanck, M., Michez, D., Lombaerde, I., Moerman, R., Wathelet, B., et al. (2015). Pollen and nectar quality drive the major and minor floral choices of bumble bees. *Apidologie* 46, 92–106. doi: 10.1007/s13592-014-0307-0

Sommerlandt, F. M. J., Rossler, W., and Spaethe, J. (2014). Elemental and non-elemental olfactory learning using PER conditioning in the bumblebee, *Bombus terrestris*. *Apidologie* 45, 106–115. doi: 10.1007/s13592-013-0227-4

Stabler, D., Paoli, P. P., Nicolson, S. W., and Wright, G. A. (2015). Nutrient balancing of the adult worker bumblebee (*Bombus terrestris*) depends on the dietary source of essential amino acids. *J. Exp. Biol.* 218, 793–802. doi: 10.1242/jeb.114249

Sun, C., Huang, J., Wang, Y., Zhao, X., Su, L., Thomas, G. W. C., et al. (2020). Genus-wide characterization of bumblebee genomes provides insights into their evolution and variation in ecological and behavioral traits. *Mol. Biol. Evol.* 38, 486–501. doi: 10.1093/molbev/msaa240

Svoboda, J. A., Thompson, M. J., Robbins, W. E., and Kaplanis, J. N. (1978). Insect steroid metabolism. *Lipids* 13, 742–753. doi: 10.1007/bf02533755

Vanderplanck, M., Michez, D., Vancraenenbroeck, S., and Lognay, G. (2011). Micro-quantitative method for analysis of sterol levels in honeybees and their pollen loads. *Anal. Lett.* 44, 1807–1820. doi: 10.1080/00032719.2010.526271

Vanderplanck, M., Moerman, R., Rasmont, P., Lognay, G., Wathelet, B., Wattiez, R., et al. (2014). How does pollen chemistry impact development and feeding behaviour of polylectic bees? *PLos One* 9:e86209. doi: 10.1371/journal.pone.0086209

Vaudo, A. D., Patch, H. M., Mortensen, D. A., Tooker, J. F., and Grozinger, C. M. (2016a). Macronutrient ratios in pollen shape bumble bee (Bombus impatiens) foraging strategies and floral preferences. *Proc. Natl. Acad. Sci. U.S.A.* 113, E4035–E4042.

Vaudo, A. D., Stabler, D., Patch, H. M., Tooker, J. F., Grozinger, C. M., and Wright, G. A. (2016b). Bumble bees regulate their intake of the essential protein and lipid pollen macronutrients. *J. Exp. Biol.* 219, 3962–3970.

Vaudo, A. D., Tooker, J. F., Patch, H. M., Biddinger, D. J., Coccia, M., Crone, M. K., et al. (2020). Pollen protein: lipid macronutrient ratios may guide broad patterns of bee species floral preferences. *Insects* 11:132. doi: 10.3390/insects11020132

Weiner, C. N., Hilpert, A., Werner, M., Linsenmair, K. E., and Blüthgen, N. (2010). Pollen amino acids and flower specialisation in solitary bees. *Apidologie* 41, 476–487. doi: 10.1051/apido/2009083

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 28 June 2021
doi: 10.3389/fevo.2021.676520

Geographical Variation in Flower Color in the Grassland Daisy *Gerbera aurantiaca*: Testing for Associations With Pollinators and Abiotic Factors

Isabel M. Johnson[1], Trevor J. Edwards[2] and Steven D. Johnson[1]*

[1] *Centre for Functional Biodiversity, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa,*
[2] *Department of Agriculture and Water Resources, Tullamarine, VIC, Australia*

Geographical variation in flower color of a plant species may reflect the outcome of selection by pollinators or may reflect abiotic factors such as soil chemistry or neutral processes such as genetic drift. Here we document striking geographical structure in the color of capitula of the endemic South African grassland daisy *Gerbera aurantiaca* and ask which of these competing explanations best explains this pattern. The color of capitula ranges from predominantly red in the southwest to yellow in the center, with some northern populations showing within-population polymorphism. Hopliine scarab beetles were the most abundant flower visitors in all populations, apart from a yellow-flowered one where honeybees were frequent. In a mixed color population, yellow, orange and red morphs were equally attractive to hopliine beetles and did not differ significantly in terms of fruit set. Beetles were attracted to both red and yellow pan traps, but preferred the latter even at sites dominated by the red morph. We found no strong associations between morph color and abiotic factors, including soil chemistry. Plants in a common garden retained the capitulum color of the source population, even when grown from seed, suggesting that flower color variation is not a result of phenotypic plasticity. These results show that flower color in *G. aurantiaca* is geographically structured, but the ultimate evolutionary basis of this color variation remains elusive.

Keywords: common garden, pollinator color preference, abiotic factors, beetle pollination, flower color polymorphism, honeybees

OPEN ACCESS

Edited by:
Casper J. Van Der Kooi,
University of Groningen, Netherlands

Reviewed by:
Kelsey Jaye Ruth Paularena Byers,
John Innes Centre, United Kingdom
Montserrat Arista,
Seville University, Spain
Yuval Sapir,
Tel Aviv University, Israel

****Correspondence:***
Isabel M. Johnson
isabelmarionjohnson@gmail.com

Specialty section:
This article was submitted to Coevolution, a section of the journal Frontiers in Ecology and Evolution

Received: *05 March 2021*
Accepted: *14 May 2021*
Published: *28 June 2021*

Citation:
Johnson IM, Edwards TJ and Johnson SD (2021) Geographical Variation in Flower Color in the Grassland Daisy Gerbera aurantiaca: Testing for Associations With Pollinators and Abiotic Factors. Front. Ecol. Evol. 9:676520. doi: 10.3389/fevo.2021.676520

INTRODUCTION

Color is one of the most important cues used by pollinators to locate, recognize and discriminate between flowers (Menzel and Shmida, 1993; Schiestl and Johnson, 2013). There is good evidence for differences among pollinator functional groups in their perceptions of colors, their degree of innate attraction to certain colors and their abilities to learn to associate colors with rewards (Chittka and Menzel, 1992; Chittka and Raine, 2006). As a result, deployment of color signals is considered a key part of plant advertising strategies that influence pollination outcomes (Schiestl and Johnson, 2013; Reverté et al., 2016). This is reflected in the broad associations between various animal groups and the colors of flowers they visit (Fenster et al., 2004; Renoult et al., 2014). Some phylogenetic

studies have shown that macro-evolutionary transitions in flower color are statistically associated with pollinator shifts (Tripp and Manos, 2008) while others have found no relationship between pollination system and flower color (Smith et al., 2008). The role of selection in generating macro-evolutionary patterns in flower color has been highlighted by experimental studies using hybrid arrays with introgressed flower colors (Bradshaw and Schemske, 2003). These experiments, in which the effects of color and morphological signals on pollinator attraction can be uncoupled, have shown that pollinators frequently discriminate among plants according to flower colors (Bradshaw and Schemske, 2003; Hoballah et al., 2007). Similarly, experiments using arrays of model flowers that vary only in color have shown strong discrimination according to color by flower-visiting animals (Campbell et al., 2010).

Geographical variation in the availability of pollinators with different color preferences would be expected to lead to intraspecific divergence in flower color among plant populations (Stebbins, 1970). For example, the floral color polymorphism in *Drosera cistiflora* may be accounted for by the spatiotemporal variation in pollinator assemblages which display different color preferences (Johnson et al., 2020). This micro-evolutionary process could account for macro-evolutionary pattern if populations that differ in flower color were to diverge to the point of speciation (Johnson, 2006).

As an alternative to the hypothesis that changes in pollinator composition account for geographical variation in flower color, recent studies have identified geographical variation in color preferences of the same pollinator as a basis of selection (Newman et al., 2012; Whitehead et al., 2018). This can occur when color preferences of a pollinator vary as a result of conditioning due to associations between colors and rewards in local plant communities (Campbell et al., 2010; Whitehead et al., 2018). A much less likely possibility, given the conserved visual systems of insects, is that differences in color preferences among populations of a single insect species could be hard-wired (Raine and Chittka, 2007).

The relative importance of biotic and abiotic drivers of flower color polymorphisms within and between populations are not well understood (Strauss and Whittall, 2006; Dalrymple et al., 2020; Sapir et al., 2021) and there is considerable debate about the extent to which both within and between population flower color polymorphisms represent the outcome of pollinator-mediated selection, as opposed to other mechanisms (Hannan, 1981; Strauss and Whittall, 2006; Rauscher, 2008; Paine et al., 2019; Dafni et al., 2020). Flower color transitions have been attributed to pollinators (Fenster et al., 2004), herbivory (Carlson and Holsinger, 2013), rainfall and sunlight (Schemske and Bierzychudek, 2007; Arista et al., 2013; Vaidya et al., 2018) or neutral processes (Edh et al., 2007; Wang et al., 2016). Plant pigments responsible for flower color, in particular anthocyanins, have a number of functions other than pollinator attraction (Bohm and Stuessy, 2001), and may be associated with abiotic factors such as soil chemistry (Mogford, 1974; Schemske and Bierzychudek, 2007; Koski and Ashman, 2016) drought and heat stress (Strauss and Whittall, 2006; Arista et al., 2013), as well as interactions such as herbivory, seed parasitism and fitness (Coberly and Rauscher, 2008; Carlson and Holsinger, 2013). Flower color may also evolve in response to pollen and nectar robbers that visit flowers and deplete their resources without effecting pollination and fertilization. Red color, for example, may reduce visits by pollen-depleting honeybees (Lunau et al., 2011; Santamaría and Rodríguez-Gironés, 2015). Since plant populations occur in intricate communities and habitats with complex interactions, traits may be the result of several interacting selective factors (Strauss and Whittall, 2006; Carlson and Holsinger, 2013). While flower color polymorphism between populations has been relatively well-researched, within-population variation, which may be either continuous or discrete, has been less well studied (Sapir et al., 2021).

Here we investigated striking patterns of variation in capitulum color among and within populations of the daisy *Gerbera aurantiaca* and examined biotic and abiotic factors that could be correlated with this variation. Capitula of *G. aurantiaca* are typically bright red with a dark center, but several recently discovered populations are either entirely yellow-flowered (Johnson et al., 2005) or color polymorphic (ranging from yellow through orange to red). The combination of a red display and black center is typical of a guild of plants attractive to glaphyrid scarab beetles in the Mediterranean region (Dafni et al., 1990; Streinzer et al., 2019) and is also found in some members of various guilds of South African plants which are pollinated solely or primarily by hopliine scarab beetles (Bernhardt, 2000; Goldblatt and Manning, 2011; Johnson et al., 2020). The primary pollinators in red-flowered population of *G. aurantiaca* are hopliine scarab beetles, but honeybees are frequent visitors in yellow-flowered populations (Johnson et al., 2004; IM Johnson unpublished data). Hopliine scarab beetles use visual cues and are readily attracted to artificial flowers (Picker and Midgley, 1996; Johnson and Midgley, 2001; Van Kleunen et al., 2007), making them excellent experimental subjects for studies of pollinator-mediated selection on flower color.

Here we hypothesized that flower color polymorphism in *G. aurantiaca* is maintained by natural selection from pollinator preference or from differences in soil or climatic characteristics. We asked the following questions: (1) Does capitulum color variation in *G. aurantiaca* show a geographic distribution pattern? (2) Is morph color associated with abiotic factors? (3) What are the color preferences of the main pollinators? and (4) Do pollinators display a preference for color morphs in polymorphic populations of *G. aurantiaca*?

MATERIALS AND METHODS

Study Species and Sites

Gerbera aurantiaca Sch.Bip. (Asteraceae: Mutisieae) is a long-lived perennial herb endemic to the mistbelt grasslands of KwaZulu-Natal and Mpumalanga in eastern South Africa (Johnson et al., 2005). The species is known from approximately 25 scattered, isolated populations ranging in size from a few to hundreds of clones, and is listed as endangered due to habitat transformation resulting from commercial forestry, agriculture and urban development (Raimondo et al., 2009). The populations

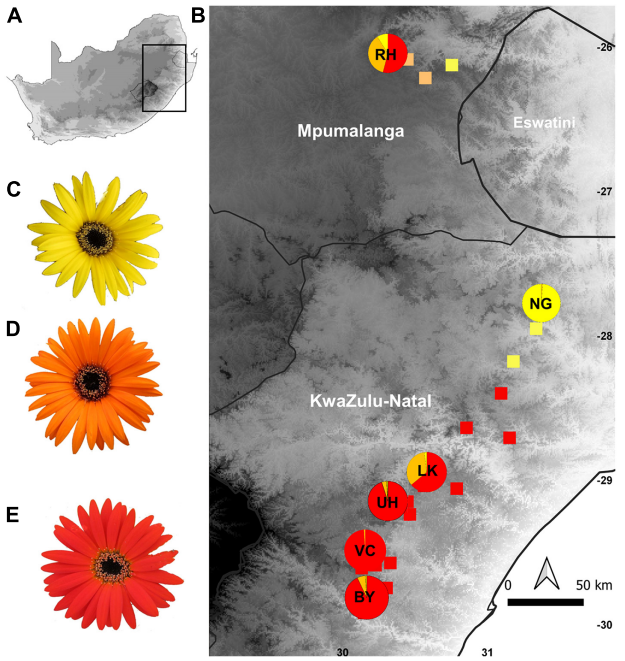

FIGURE 1 | Map showing the geographic distribution of capitulum color morphs in populations of *Gerbera aurantiaca* along its latitudinal range. **(A)** Map of South Africa; **(B)** color forms of *G. aurantiaca* in eastern South Africa; **(C)** *G. aurantiaca* yellow capitulum; **(D)** *G. aurantiaca* orange capitulum; **(E)** *G. aurantiaca* red capitulum. BY, VC, UH, LK, NG, and RH are the six study populations selected and the pie charts indicate the capitulum color proportions of these populations.

occur in grasslands scattered along a roughly north-south axis between –26° and –30° S latitude along the eastern region of SA at elevations of between 900 and 1,700 m (**Figure 1**). *G. aurantiaca* populations are found on or near rocky outcrops in organic–rich topsoil overlying well-drained, acidic, nutrient-deficient sub-soils associated with a high degree of leaching typical of the high rainfall and cool temperatures of mistbelt grasslands (Fey, 2010).

Reproduction of *G. aurantiaca* is both clonal, by means of underground stems, and sexual with flowering taking place in austral spring (September to November). The radiate capitula contain about 200 florets, with approximately 100 female ray florets in three outer whorls, and with the central disc florets hermaphrodite but functionally male. Capitula are not nyctinastic and remain open throughout flowering. The species is largely self-incompatible and highly dependent on pollinators for fruit set (Johnson et al., 2004). Populations are typically predominantly red-flowered, but both completely yellow-flowered and color polymorphic populations with yellow, orange and red-flowered inflorescences also occur (Johnson et al., 2005). The chemical basis of capitulum coloration of *G. aurantiaca* has not been analyzed as far as we know but the flower color of *G. jamesonii* a sister species, and the related commercially important ornamental *Gerbera* hybrids, is derived from carotenoids which cause yellow and orange coloration, and the flavonoids, pelargonidin and cyanidin, which are responsible for the red coloration (Valadon and Mummery, 1967; Tyrach and Horn, 1997). A feature of *G. aurantiaca* capitula is the dark center during the initial female stages of flowering when the disc florets are covered by the overarching dark purple pappus hairs and dark anther caps. Pollen is the primary floral reward as the florets produce little or no nectar and have no discernible scent.

The hairy monkey beetle *Eriesthis vulpina* Burmeister (Coleoptera: Scarabaeidae: Hopliini), appears to be the most important pollinator of *G. aurantiaca* (Johnson et al., 2004). Its use of color as a cue for locating flowers was evidenced by experiments in which large numbers of these beetles were captured in red plastic dishes placed in populations (Johnson et al., 2004). *Eriesthis* beetles almost completely ignored blue plastic dishes placed alongside red dishes in these populations (c. 100 beetles attracted to red dishes vs. one beetle attracted to blue dishes; SD Johnson, unpublished data), suggesting that they strongly prefer red over blue colors. The honeybee, *Apis mellifera scutellata* Lepeletier (Hymenoptera: Apidae) is also a common visitor in the yellow-flowered population.

Geographical Distribution of Color Morphs

We recorded the proportions of capitulum color morphs (as apparent in human vision) along transects across each of 23 populations ranging from southern KwaZulu-Natal to Mpumalanga (**Figure 1** and **Supplementary Table 1**). At least one hundred clones were counted in larger populations and all clones were counted in the smaller populations. To minimize subjective bias in color allocation, one person (IMJ) carried out all counts. Ray floret samples were matched to a flower color chart (RHS, 2007) and broadly assigned to orange, red or yellow. Geographic position (south latitude and east longitude, WGS 84) and elevation (m.a.s.l) were measured using a handheld Garmin Etrex GPS. A generalized linear model (GLM) with a binomial error distribution, logit link function, and correction for overdispersion was applied to model the proportion of clones with red capitula in each population against latitude, longitude and elevation and we used likelihood ratios to test significance of fixed effects. All GLMs in this study were implemented in SPSS ver. 27 (IBM corp.).

Spectral Reflectance and Floral Morphological Trait Measurements

We selected six populations representative of the color variation across the distribution range (**Figure 1**) to investigate pollinator color preferences; of these three (BY, VC and UH) were predominantly red-flowered, one (NG) almost entirely yellow-flowered and two (RH and LK) polymorphic with a mixture of red, orange and yellow-flowered plants (**Table 1**). Here we measured spectral reflectance (300–700 nm) of the ray florets using an Ocean Optics S2,000 spectrometer (Ocean Optics Inc., Dunedin, Fla.), Ocean Optics DT-mini deuterium tungsten halogen light source and fibre optic reflection probe (QR-400-7-UVVIS; 400 lm) held at 45° to the object surface in a probe holder (RPH-1). An Ocean Optics WS-1 diffuse reflectance standard was used to calibrate the spectrometer (Johnson and Andersson, 2002). Spectral reflectance readings were averaged from the mid-adaxial surface of three ray florets

TABLE 1 | Site name, abbreviation (abbr.), geographical co-ordinates (dd), area (ha), approximate number of clones and percentage of clones with red orange and yellow capitula at the six *Gerbera aurantiaca* study sites.

Site name	Abbr	Lat S; Long E	Area (ha)	No. of clones (approx.)	Capitulum color (%)		
					Red	Orange	Yellow
Byrne	BY	–29.81; 30.19	6.5	250	93	6	1
Victoria Club	VC	–29.57; 30.33	2	100	99	1	0
Umvoti Heights	UH	–29.18; 30.38	1.6	150	95	4	1
Lookout	LK	–29.04; 30.58	6.2	250	64	34	2
Ngome	NG	–27.84; 31.35	100	>1,000	0	1	99
Rooihoogte	RH	–26.05; 30.37	4	250	54	37	9

from at least 20 different plants from each population. All spectral reflectance curves are available from the Floral Reflectance Database (Arnold et al., 2010). Since we were interested in color as a floral signal and insect flower visitors most readily perceive rapid changes in spectral reflectance (Chittka and Menzel, 1992; Dyer et al., 2012) we used inflection or marker points which identify the wavelengths where change in spectral reflectance is maximal. We used the online *Spectral MP* (Dorin et al., 2020) to class capitulum color as yellow (inflection points from 520 to 530 nm), orange (inflection points from 530 to 600 nm) or red (inflection points from 600 to 625 nm). We recorded the positions of the different color morphs in the polymorphic RH population with a handheld Garmin Etrex GPS to investigate spatial structuring.

To test whether other floral traits that might influence pollinator attraction were associated with ray floret color we measured the capitulum diameter and ray floret length and ray floret inflection points for inflorescences from 50 clones collected randomly in the polymorphic RH population and tested for trait correlations using Pearson tests. We used finite mixture analysis allowing for unequal variance and with BIC criteria implemented in the r package mclust 5.4.6 (Scrucca et al., 2016) to assess whether the distribution of inflection points in populations were best explained by a single Gaussian distribution or by two or more Gaussian distributions. Both equal variance and unequal variance models were tested. We also tested for significant deviations from unimodality of inflection points in each population using Hartigan's diptest implemented in the r package diptest (Maechler, 2016).

The Influence of Abiotic Factors

We sourced the bioclimatic variables temperature (MAT: mean annual temperature °C) and precipitation (MAP: mean annual precipitation mm.) from www.worldclim.org/bioclim (Hijmans et al., 2005) for 23 populations across the distribution range of *G. aurantiaca* (**Supplementary Table 1**). A generalized linear model (GLM) with a binomial error distribution, logit link function, and correction for overdispersion was applied to model the proportion of clones with red capitula in each population against temperature and precipitation.

Since flower color in polymorphic species may be associated with differences in edaphic factors (soil characteristics) (Horovitz, 1976; Rajakaruna and Bohm, 1999) we collected soil samples from 14 population locations of *G. aurantiaca*. For each population 15 augered subsamples of the top 15 cm layer taken randomly across the population patch were combined, air dried, stored at room temperature and analyzed for pH (KCL), exchange acidity, total cations, acid saturation, organic carbon (C), calcium (Ca), copper (Cu), potassium (K), magnesium (Mg), manganese (Mn), nitrogen (N_2), phosphorus (P), zinc (Zn), and clay content (**Supplementary Table 2**) by the Soil Fertility and Analytical Services Laboratory, KwaZulu-Natal Dept. of Agriculture (Manson and Roberts, 2000). We used Principal Components Analysis (Braak and Smilauer, 2002) to assess the relationship between the 14 edaphic variables (standardized to avoid using different scales for comparative purposes). Population scores were plotted onto the principal components axes and correlation vectors were used to examine relationships between the population samples and the first two PCA axes of the soil ordination. Linear regression was carried out using the first four regression factor scores and the proportion of red clones in the 14 populations.

Common Garden Experiment

To test whether the capitulum color of *G. aurantiaca* plants from different color morph populations changes when they are grown together under identical climatic and edaphic conditions, we cultivated both ramets and seeds collected from red and yellow flowered populations in a common garden at the KwaZulu-Natal National Botanical Garden in Pietermaritzburg where conditions (soil characteristics, temperature and precipitation) were identical. We did not include orange morphs since we were not aware of their extent in the polymorphic populations at the start of this experiment. Sample sizes were limited due to permit restrictions relating to the threatened status of the species. We collected flowering ramets from each of five widely spaced clones growing in red (BY) and yellow (NG) source populations and transplanted these into the common garden nursery bed. Ramets were used as they provided a baseline spectral reflectance and because they take less time to flower in cultivation than seed raised plants. In addition, seeds were collected from clones at least five meters apart in the red-flowered LM and the yellow-flowered NG populations and marked seedlings from each population were transplanted randomly into the common garden nursery bed. Reflectance spectra of ray florets from each individual were

measured at collection (in the case of ramets) and at intervals once flowering had occurred for both groups over a 5 year period, We compared mean inflection points of the spectral reflectance curves (Dorin et al., 2020) of ray florets for both ramets and seed grown plants using a GLM with normal distribution with source population and year as independent variables.

Color Choice Behavior of Insect Visitors

Pan traps have been widely used for passive sampling of flower-visiting insects and may give an indication of their color preferences as well as their abundance (Picker and Midgley, 1996; Leong and Thorp, 1999; Shrestha et al., 2019). Here we tested color discrimination of potential pollinators using colored pan trap arrays at the six study populations. Red and yellow traps were chosen as they represent the extremes of capitulum colors that occur naturally in the range of *G. aurantiaca.* We did not test for orange due the unavailability of traps with suitable reflectance spectra. Spectral reflection curves were used to calculate the inflection points (Dorin et al., 2020) of the traps and compare these to those of *G. aurantiaca* capitula. We used twenty sets of red and yellow plastic traps (11 cm in diameter and 8.5 cm deep) filled with 150 ml of water and placed randomly in pairs 15 cm apart amongst flowering *G. aurantiaca* plants. We carried out these experiments during peak flowering on sunny days between 07:00 and 16:00 h in late October when insects were most active. Captured insects were identified at least to family level and counted. Beetles appeared to be unaffected by being trapped and most were released after recording on account of their role as important pollinators of this threatened study species. Pan trap catches of the dominant insect visitors, *E. vulpina* beetles and honeybees, were analyzed using a GLM with negative binomial distribution and log link with trap color and population site as independent variables. For the analysis of catches, one value at sites where no insects were caught in traps had to be adjusted from a choice for yellow to one for red in order for the model to converge (Zuur et al., 2009).

Since hymenopteran vision is well-researched we used the bee color hexagon (Chittka and Menzel, 1992; Peitsch et al., 1992) to assess how honeybees would perceive the color of capitula of *G. aurantiaca* and the pan traps. The mean reflectance spectrum calculated from three ray florets from each of 20 individuals of each color form and those from the red and yellow pan traps was plotted in the bee color hexagon. Background color was calculated from the spectra of *G. aurantiaca* leaves. Coleopteran vision has been less well studied and due to this we did not assess their perception of capitulum color.

Capitulum Visitation and Fruit Set in the Color Polymorphic Population

To determine if *E. vulpina* hopliine scarab beetles, the dominant insect visitors to *G. aurantiaca*, exhibit color preferences in a polymorphic population, we recorded the frequency of these beetles visiting capitula of different color morphs at the RH population along three 200 m transects during peak flowering in three separate years. As the intensity of flowering varied from year to year, we recorded different total inflorescence numbers per transect (2008 N = 155, 2011 N = 471, 2013 N = 291). The proportion of capitula of different colors (yellow, orange, and red) that were occupied by beetles was compared using a logistic GLM with binomial distribution and logit link function.

We measured fecundity (mean fruit set per capitulum) in relation to morph color in the polymorphic RH population. In 2011 (N = 61: yellow = 19, orange = 28, red = 24), and 2013 (N = 56: yellow = 9, orange = 13, red = 34) capitula were bagged and labeled at the end of the male phase and harvested after 3 weeks. Filled fruit, easily distinguished by their larger size, darker color and firmness were counted for each bagged capitulum. Fruit set per capitulum for each year was analyzed using a GLM with a negative binomial distribution and log link function. Color morph, year and the interaction of color morph and year were fixed factors in this model.

RESULTS

Geographical Distribution of Color Morphs

In human color vision 15 of the 23 *G. aurantiaca* populations measured had predominantly red-flowered clones, 5 were predominantly yellow-flowered and 3 were polymorphic with mixed yellow, orange and red-flowered clones. The red flowered populations occur mainly in the southern part of the distribution range with one polymorphic population (LK), the yellow cluster in the center, and northern populations are mainly color polymorphic with varying proportions of red, orange and yellow capitula (**Figure 1** and **Supplementary Table 1**). The proportion of clones with red capitula in the 23 populations was significantly correlated with latitude (χ^2 = 9.460, df = 22, P = 0.002), and longitude (χ^2 = 6.791, df = 22, P = 0.009) but not with elevation (χ^2 = 1.805, df = 22, P = 0.179).

Spectral Reflectance and Floral Morphological Trait Measurements

Mean inflection points of the reflectance spectra of the six representative populations were between 609 and 615 nm for the red-flowered populations (BY 615 nm, VC 611 nm, UH 609 nm, LK 600 nm) populations, 524 nm for the yellow-flowered NG population and 597 nm for the polymorphic RH population (**Figures 2A–F** and **Supplementary Table 2**). No UV reflectance (300–400 nm) was recorded (**Figure 2**). Although the northern color polymorphic RH and central LK populations showed a wide range of color variation we were able to assign plants to particular color classes as the distribution of inflection points in this population was broad (**Figures 2D,F**), with most inflection points clustered around 620 nm with a smaller peak around 540 nm for RH and 600–620 with a smaller cluster at 530–570 nm for LK. The frequency of inflection points in the NG, BY, VC and UH populations fitted a single Gaussian distribution better than two (ΔBIC range = 2.24–7.80), but the distribution of inflection points fitted two Gaussian distributions much better than one Gaussian distribution in the RH population (ΔBIC = 39.8) and LK population (ΔBIC = 23.5). Fits to three

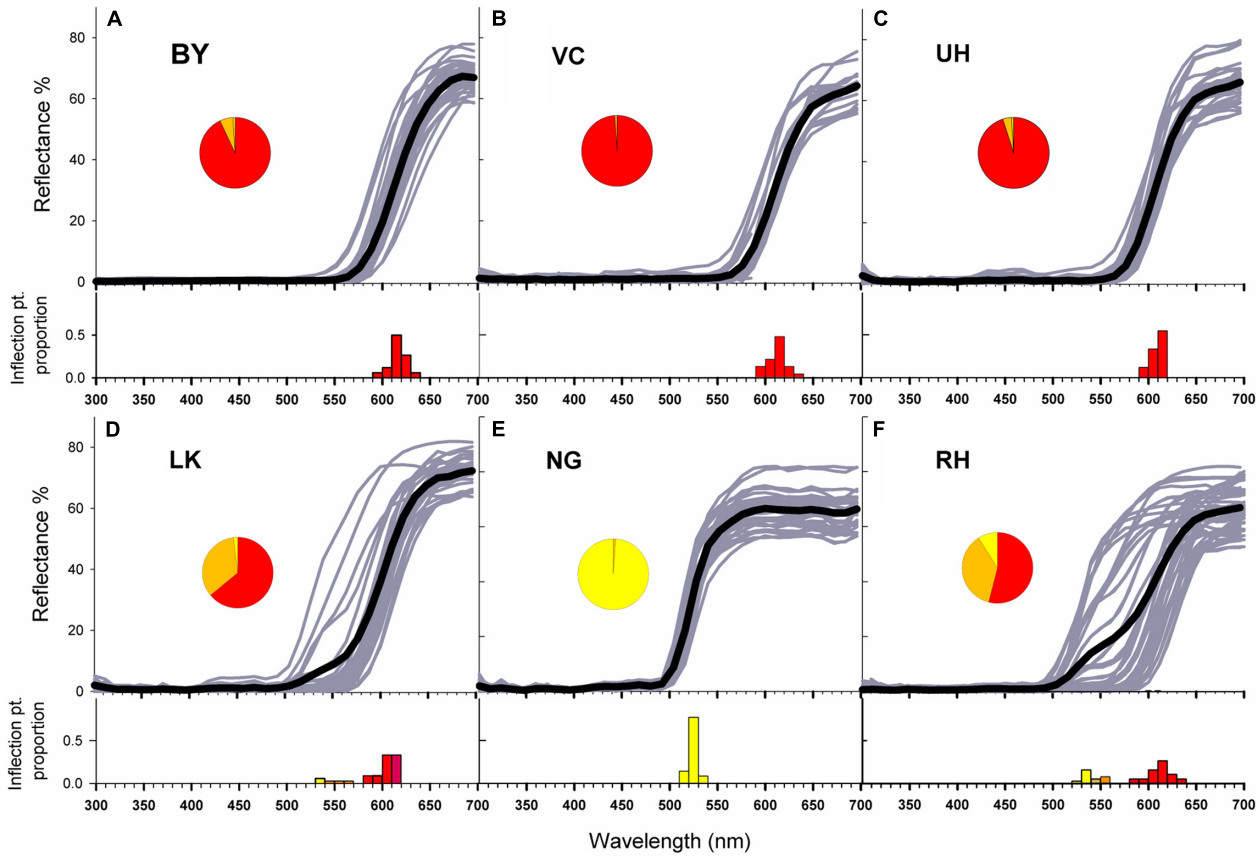

FIGURE 2 | Reflectance spectra between wavelengths of 300 and 700 nm and proportion of inflection points in each 10 nm bin for *Gerbera aurantiaca* ray florets from the six study populations **(A–F)**. Darker lines indicate population means. Pie charts show the proportions of yellow, orange and red capitula in each population.

or more Gaussian distributions were not supported for any population, but in the RH and LK populations, models with fits to three Gaussian distributions fitted better than models with fits to a single distribution (ΔBIC = 16.4–33.6) but were still supported less than were models fitted to two Gaussian distributions (ΔBIC = 6.2–7.1). However, we found no significant deviations from unimodality for the distributions of inflection points in any of the populations ($P > 0.095$). We also found no significant correlations between inflection points and capitulum diameter ($r = -0.15$, $P = 0.289$) or ray length ($r = -0.029$, $P = 0.845$) (**Supplementary Table 3**).

The Influence of Abiotic Factors

Environmental conditions were similar across the distribution range of *G. aurantiaca* with climatic variables for the 23 population localities ranging from 14.0 to 17.6°C for mean annual temperature (MAT), 878-1057 mm for mean annual precipitation (MAP) (**Supplementary Table 1**). The proportion of clones with red capitula in populations was not significantly correlated with temperature ($\chi^2 = 0.662$, df = 22, $P = 0.416$) or precipitation ($\chi^2 = 2.702$, df = 22, $P = 0.1$).

Differences in soil chemistry between sites were small (pH ranged from 3.94 to 4.69, N from 0.12 to 0.6% and organic C content 2.5–6%) (**Supplementary Table 4**). Principal components of the edaphic variables were summarized into the first and second axes of the principal component analysis (PCA), which accounted for 64.9% of the total variation and were mainly explained by pH-acidity gradient (axis 1) and Org C/N gradient (axis 2) (**Supplementary Figure 1** and **Supplementary Table 5**). The PCA biplot (**Figure 3**) shows an apparent pH-acidity gradient, and the two northernmost sites on the top left stand out because of their more weathered soils with higher Mg and lower than average organic carbon content. pH and Organic C can be used as largely independent surrogate variables to represent variation of other associated variables along these two typical soil chemistry gradients. We found no significant relationship between the proportion of red clones in a population and PCA Factor 1 ($r^2 = 0.037$, $P = 0.512$), Factor 2 ($r^2 = 0.24$, $P = 0.596$), Factor 3 ($r^2 = 0.008$, $P = 0.977$) or Factor 4 ($r^2 = 0.05$, $P = 0.441$) using linear regression. We further observed no spatial structure in the polymorphic RH population with different color morphs scattered randomly (**Figure 4A**) and often growing less than one meter from each other (**Figure 4E**).

Common Garden Experiment

Both ramets and seed-raised plants retained the capitulum color of the parent populations when grown in a common garden

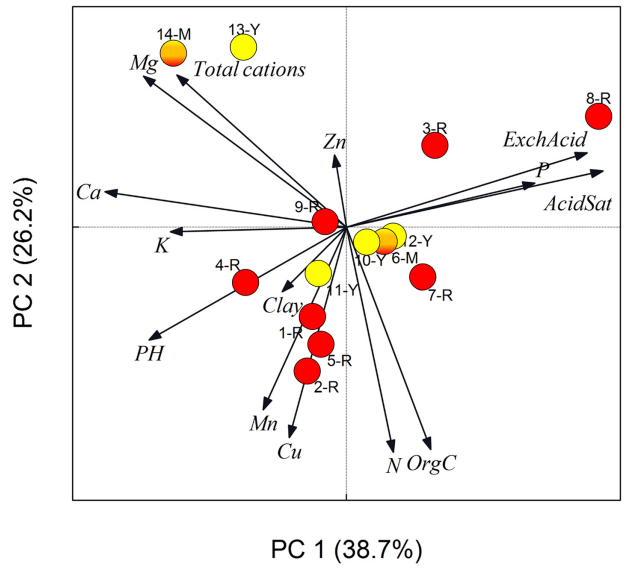

FIGURE 3 | Principal Components Analysis biplot with vectors overlaid showing relationships of *Gerbera aurantiaca* populations and soil chemical variation across 14 sampling sites. Dominant capitulum color (red, mixed and yellow) of the populations are indicated by the circles (population codes and details of chemical analyses are listed in Supporting information **Supplementary Table 4**).

over a 5-year period. Flowering ramets collected in the red-flowered BY (mean inflection point 616.0 ± 1.7 nm, $N = 5$) and yellow-flowered NG (mean inflection point 525.6 ± 1.8, $N = 5$) populations showed no change from their original ray floret morph color ($\chi^2 = 3397.82$, df = 1, $P < 0.0001$) with no significant change in morph color recorded over the 5-year observation period ($\chi^2 = 1.319$, df = 1, $P = 0.517$) and no significant interaction between population and year ($\chi^2 = 1.530$, df = 3, $P = 0.465$) (**Figure 5A**).

Seed-raised plants flowered 4 years after germination and displayed the predominant capitulum color of the source populations: LM (red with mean inflection point 606.80 ± 1.8 nm, $N = 18$) and NG (yellow with mean inflection point 523.4 ± 1.9 nm, $N = 18$). There was a significant difference between the mean inflection points of the two seed populations ($\chi^2 = 2012.457$, df = 1, $P < 0.0001$) but there was no significant effect of time ($\chi^2 = 0.51$, df = 1, $P = 0.821$) or interaction between seed population and year ($\chi^2 = 0.003$, df = 2, $P = 0.957$) (**Figure 5B**).

Color Choice Behavior of Insect Visitors

A total of 682 *E. vulpina* beetles, and 39 honeybees, as well as some insects from other taxonomic groups were caught in the red and yellow pan traps. At all populations where beetles were present (UH, LK, NG, and RH) significantly more individuals of *E. vulpina* were caught in yellow than red traps (**Figure 6A** and **Supplementary Table 6**). The highest number of beetles overall was trapped at the red-flowered UH population, followed by RH (flower color polymorphic), NG (yellow), and LK (predominantly red), while no individuals of *E. vulpina* were trapped at the two southernmost sites, BY and VC (red). We recorded significant effects of population ($\chi^2 = 97.951$, df = 5, $P = 0.001$), and of trap color ($\chi^2 = 14.904$, df = 1, $P = 0.001$) on the number of *E. vulpina* beetles caught, but the interaction between population and trap color was not significant ($\chi^2 = 7.182$, df = 6, $P = 0.207$) suggesting that there were no marked differences in beetle color preference between populations.

Very few honeybees were captured in pan traps, but despite this low statistical power we detected a slight significant effect of trap color ($\chi^2 = 5.172$, df = 1, $P = 0.023$), but not of population ($\chi^2 = 9.464$, df = 5, $P = 0.092$). There was a significant interaction between population and trap color ($\chi^2 = 15.352$, df = 6, $P = 0.009$) with more bees trapped in yellow than red traps at four of the populations (BY, VC, UH, and LK), equal numbers at NG and none trapped at RH although they were frequently observed on other plants at the site (**Figure 6B** and **Supplementary Table 6**).

When plotted in the model of hymenopteran color perception, loci of *G. aurantiaca* ray floret reflectance spectra were positioned primarily in the green and UV green segments of the hexagon with the florets of the red color forms clustered more closely to the origin of the hexagon than the yellow forms, suggesting that the yellow capitula are more visible to bees than the red. The loci of orange forms were intermediate between red and yellow in bee color space (**Figure 6C**). The spectral reflectances of the pan traps (**Supplementary Figure 2**) were similar to those of the red and yellow ray florets in terms of hymenopteran color perception (**Figure 6C**) and inflection points (red trap = 601 nm, floret = 608 nm; yellow trap = 519 nm, floret = 524).

Capitulum Visitation and Fruit Set in the Color Polymorphic Population

Analysis of the incidence of *E. vulpina* beetles present on orange, red and yellow colored capitula (**Figures 4B–D**) at the mixed color site (RH) during transect surveys in three separate years indicated that the beetles did not discriminate among color morphs, with no significant differences found between the proportions of orange, red and yellow capitula that were occupied by beetles ($\chi^2 = 0.410$, df = 2, $P = 0.814$). The overall proportion of capitula occupied by beetles varied significantly among years ($\chi^2 = 24.90$, df = 2, $P < 0.0001$) with a higher incidence of beetles on capitula in 2013 than in 2008 or 2011 (**Figure 7A**). There was no interaction between year and color morph ($\chi^2 = 0.643$, df = 4, $P = 0.958$), implying that responses to colors by beetles did not vary among years.

We did not detect any significant differences in mean fruit set among color morphs in 2011 or 2013 ($\chi^2 = 0.349$, df = 2, $P = 0.840$) or year ($\chi^2 = 292$, df = 1, $P = 0.589$). There was no significant interaction between year and color morph ($\chi^2 = 0.216$, df = 3, $P = 0.898$) (**Figure 7B**).

DISCUSSION

This study reveals a pattern of geographical structure of variation in capitulum color of *G. aurantiaca* along the north-south axis of its range with most southern populations being predominantly

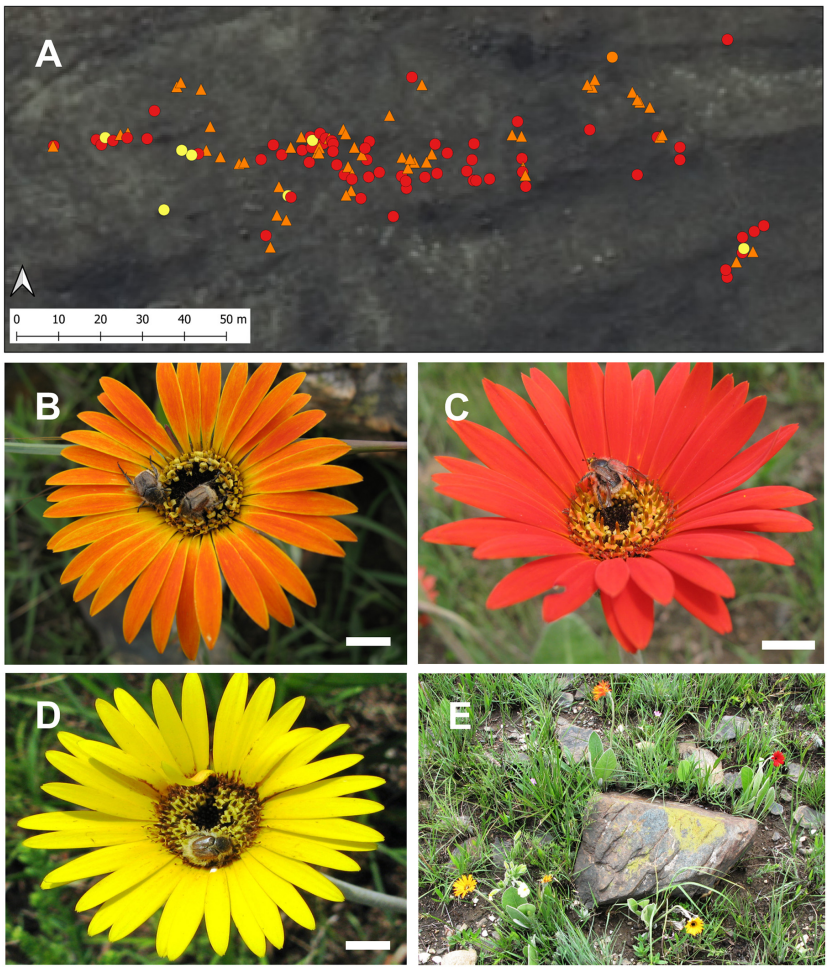

FIGURE 4 | (A) Map showing gps positions of *Gerbera aurantiaca* capitulum color forms in the polymorphic RH population, **(B–D)** *Eriesthis vulpina* beetles visiting orange, red and yellow capitula (Scale bars = 1 cm), and **(E)** different color forms growing in close proximity in the RH population.

red, the central cluster being predominantly yellow, and four populations, mainly in the north displaying a pattern of within population color polymorphism. Color variation in two polymorphic populations was continuous, but with inflection points clustering in two groups, as supported by the finite mixture analysis. We found no clear association between capitulum color and various ecogeographic and edaphic factors although a more detailed analysis of nitrogen in the forms utilized by plants should be carried out in future studies to confirm this. We recorded no spatial segregation of color morphs in the polymorphic RH population with plants of different inflorescence color growing intermixed in close proximity, suggesting that climate and soils do not influence floral color. Floral color was maintained over a 5-year period in a common garden experiment, both in ramet- and seed-grown plants, further indicating that the color polymorphism is not a response to soil or climatic variation (de Villemereuil et al., 2016). While the lack of a clear association with abiotic factors points to a biotic explanation for the evolutionary divergence in flower color among populations, the role of pollinators as drivers of the color change in *G. aurantiaca* is not entirely clear. Pollination studies have shown that a single hopliine scarab beetle species, *E. vulpina*, is responsible for the majority of all floral visits to *G. aurantiaca* (Johnson et al., 2004), unlike the color polymorphic *Drosera cistiflora* in the western Cape region of South Africa, where several different species of hopliine scarab beetles discriminate between different color forms in the same population (Johnson et al., 2020). Our study did not provide clear evidence that *E. vulpina* beetles prefer any particular capitulum color within a population as they were recorded visiting capitula in both the red and yellow–flowered populations, and they did not appear to discriminate between *G. aurantiaca* color morphs at the northern color polymorphic RH site. This was further supported by the lack of significant differences in fecundity of the three color morphs at the RH population, where capitula were almost exclusively visited by *E. vulpina* beetles.

Native honeybees are frequent visitors to the flowers of many plant species of the eastern South African grasslands (Hepburn and Radloff, 1995; Stanley et al., 2020) but there is some debate regarding their ability to easily detect red

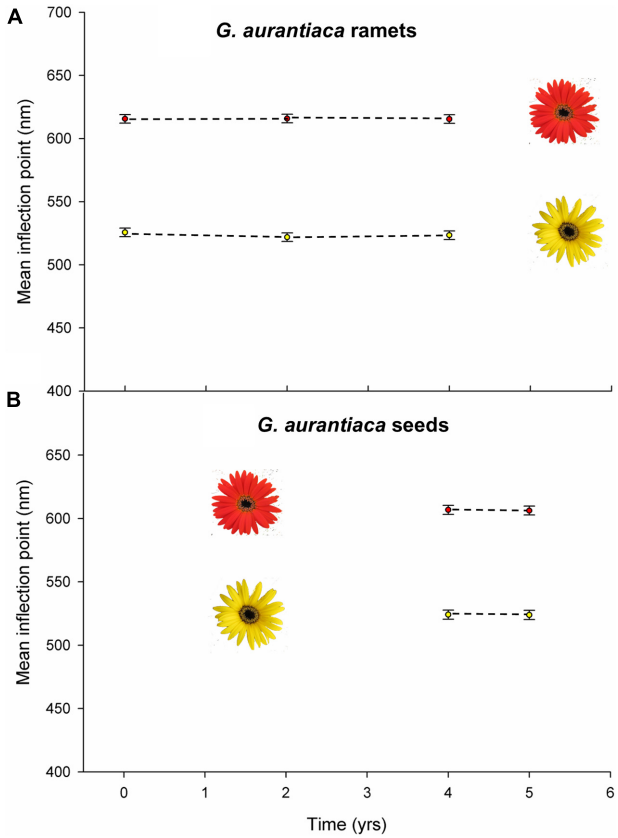

FIGURE 5 | Mean inflection points of the ray florets of *Gerbera aurantiaca* plants cultivated in a common garden of **(A)** ramets from the BY and NG populations at collection and after 2 and 5 years, and **(B)** from seed collected from the LM and NG populations after 4 and 5 years. Vertical bars indicate 95% confidence intervals.

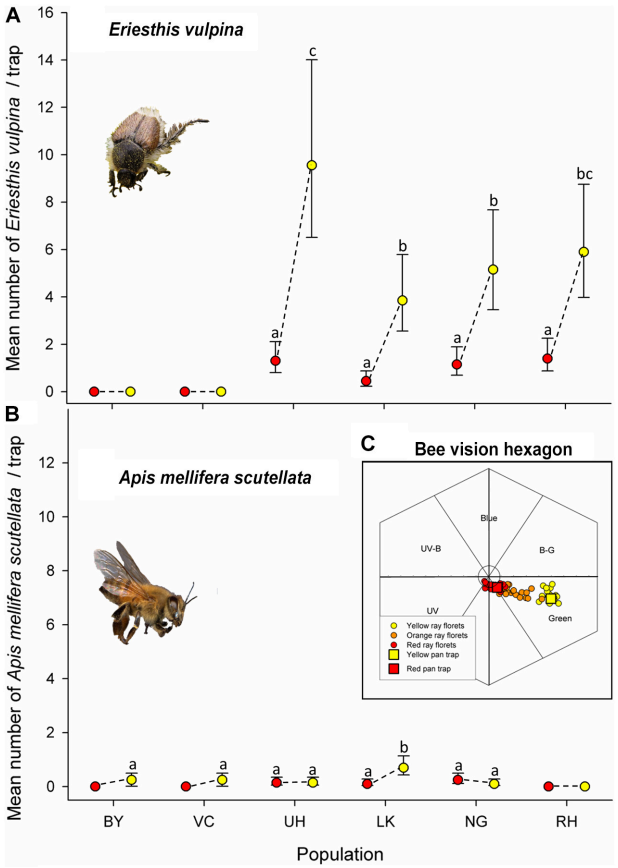

FIGURE 6 | Mean numbers of hopliine scarab beetles and honeybees caught in yellow and red pan traps at the six study sites during an 8-h period. **(A)** *Eriesthis vulpina* (no individuals were trapped at the BY and VC sites) and **(B)** *Apis mellifera scutellata*, and **(C)** ray floret colors of the yellow, orange and red forms of *Gerbera aurantiaca* and red and yellow pan trap spectra as loci in the hymenopteran visual space hexagon. The six segments represent the six categories of bee color perception. Distance of the loci from the origin gives an indication of the ability of bees to perceive the color and distance between loci the ability to discriminate between the colors. Mean Euclidean distances from origin ± SD: yellow 0.52 ± 0.048 N = 16, 0.27 ± 0.11 N = 10 for orange and 0.107 ± 0.036 N = 20 for red. Vertical bars indicate 95% confidence intervals. Means sharing letters are not significantly different.

flowers (Chittka and Waser, 1997; Lunau et al., 2011). Although honeybees were observed visiting other flower species in the color polymorphic RH population and were frequently seen on yellow capitula in the monomorphic NG population, none were recorded on red *G. aurantiaca* capitula during the transect surveys. The relative importance of monkey beetles and honeybees as pollinators of yellow populations of *G. aurantiaca* is difficult to determine without further research into pollen deposition efficiency (King et al., 2013).

While *E. vulpina* preferred yellow over red in the color choice experiments using pan traps at all populations where it was present (including the predominantly red-flowered), approximately 25% of the total catch was from red traps. Based on this and the frequent presence of *E. vulpina* on red capitula it appears that red-colored flowers are easily detected by this beetle. Although beetles preferred yellow over red plastic bowls, this may reflect the particular spectral properties of the plastic bowls in this experiment. Attraction of hopliine scarab beetles in the Western Cape to red bowl-shaped flowers has been recorded in previous studies (Johnson and Midgley, 2001; Johnson et al., 2004; Van Kleunen et al., 2007), but these studies indicate that trap color choices do not necessarily reflect the flower colors favored by these beetles (Picker and Midgley, 1996; Mayer et al., 2006; Van Kleunen et al., 2007). We hesitate to conclude that beetles generally prefer yellow over red flower color as such a preference was not evident in our survey of beetles on capitula in polymorphic populations. This survey suggests that in natural polymorphic populations, red, orange and yellow capitula have equal probabilities of being visited by *E. vulpina*. This could also indicate that additional cues other than color, such as suitability of larger capitula for socializing, may be used by these beetles for recognition and choice of floral host plants (Dafni, 1997). We found no association between capitulum and ray floret size and flower color inflection points in the RH polymorphic population suggesting that capitulum color is not linked to morphological

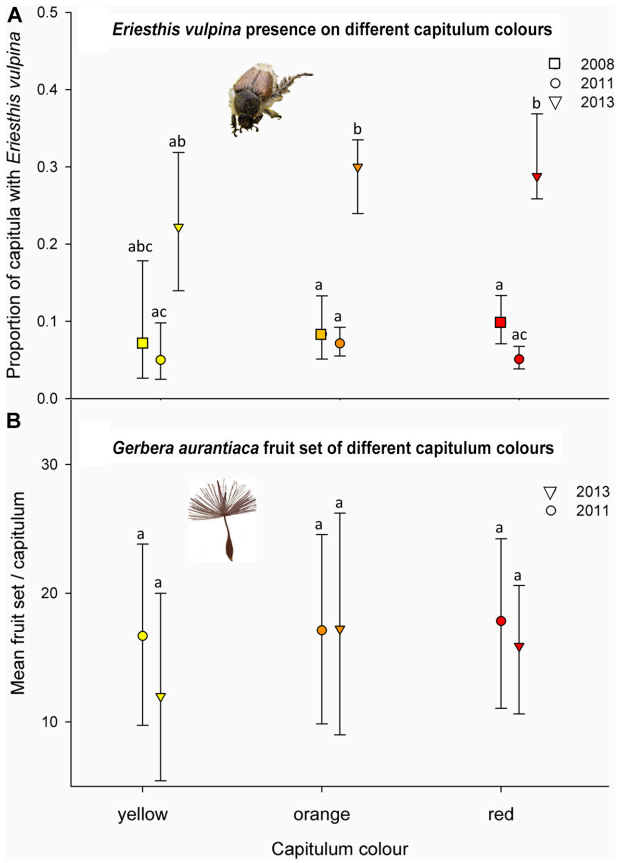

FIGURE 7 | (A) *In-situ* hopliine scarab beetle (*Eriesthis vulpina*) capitulum color preference in the color polymorphic RH population: Proportion of yellow, orange and red *Gerbera aurantiaca* capitula with *E. vulpina* individuals present during 2008, 2011, and 2013 flowering seasons. **(B)** Mean fruit set per capitulum of the yellow, orange and red color forms at the RH population in 2011 and 2013. Vertical bars indicate 95% confidence intervals. Means sharing letters are not significantly different.

traits and thus unlikely to evolve through pleiotropic effects of morphological evolution. We did not use a vision model to determine how *E. vulpina* beetles may perceive the ray floret and pan trap colors as the spectral sensitivity of photoreceptors of this species is not known. Coleopteran vision has been less well studied than hymenopteran vision but recent studies suggest that their visual capability can extend into longer wavelengths than that of Hymenoptera (Martínez-Harms et al., 2012; Lebhardt and Desplan, 2017; Streinzer et al., 2019).

We caught relatively fewer honeybees than hopliine scarab beetles in the pan traps, but even our small sample suggested that bees showed a marked preference for yellow over red at most sites. When plotted in the bee color hexagon, the loci for red ray floret limbs of *G. aurantiaca* capitula and for the red pan traps used here are close to those for the vegetative background, and are therefore likely to be indistinct to honeybees, while the loci for yellow ray florets and for the yellow pan traps place further away from that of the background providing a heightened contrast and are therefore more visibly distinct to hymenopterans. This may explain why bees seldom visit the red color forms of *G. aurantiaca* but were frequently observed visiting capitula in the yellow NG population, although, curiously, not in the polymorphic RH population. The yellow-flowered population of *G. aurantiaca* may thus have a bimodal pollination system involving both beetles and honeybees.

Red flowers that are not bird pollinated are uncommon in these mistbelt grasslands and as far as we are aware there are no insect pollinators that specialize on visiting red flowers in the region other than the butterfly *Aeropetes tulbaghia* (Johnson et al., 2009) which is a nectar feeder and unlikely to visit *G. aurantiaca*. The hopliine scarab beetles which pollinate the *G. aurantiaca* flowers appear to be attracted to all color forms equally and in red-flowered populations where they are absent fruit set is extremely low (Johnson et al., 2004) making the dominance of red capitula in these southernmost populations enigmatic. One possible scenario is that the red form of *G. aurantiaca* was historically associated with a pollinator which selected for the red color, but is no longer present (Cooley et al., 2008; Hopkins and Rausher, 2012) and that the long-lived clonal growth form of *G. aurantiaca* would allow the red color form to persist long after the selective preference of the now absent specialist pollinator had disappeared. A second possibility, yet to be tested, is that pollen robbers such as honeybees have a net negative effect on plant fitness because of their efficient collection and transfer of pollen to their corbiculae without depositing it on appropriate stigmas and therefore causing pollen limitation (Hargreaves et al., 2010). It has been proposed that red flower color could be a strategy to avoid pollen robbing by bees (Lunau et al., 2011; Santamaría and Rodríguez-Gironés, 2015). In this case, the red color of some morphs may represent a compromise between attraction and defense, in that red capitula are clearly attractive to beetle pollinators while at the same time are relatively inconspicuous to pollen-robbing insects such as honeybees. While this strategy appears effective in the case of *G. aurantiaca* since honeybees were seldom observed visiting red flowers, the high fruit set in the yellow NG population where honeybees were frequent recorded suggests that they may contribute to fruit set. Experimental observations of the relative success of single visits on fruit set by honeybees and comparisons of the efficiency of pollen transfer by beetles vs. honeybees at this site may help to clarify this.

A further possibility to consider is that the color polymorphic populations in the northern part of the range may be the result of red and yellow forms meeting and interbreeding. Preliminary crosses indicate that the red and yellow color forms of *G. aurantiaca* breed true, with inter-red and inter-yellow crosses producing entirely red and yellow F1 offspring, respectively. Crosses between red and yellow forms (both yellow pollen onto red mothers and red pollen onto yellow mothers) produce almost entirely orange and yellow F1 offspring (IM Johnson, unpublished data). However, we are not aware of red-flowered populations in the northern part of the distribution range of *G aurantiaca*, which casts doubt on the idea that within-population color polymorphism reflects hybridization.

Since the capitulum color variation across the distribution range of *G. aurantiaca* described here does not appear be influenced by pollinator color preference or climatic and

soil characteristics it provides an ideal system for testing whether there is a pattern of neutral evolution that reflects simple isolation by distance (Wright, 1943; Schemske and Bierzychudek, 2001, 2007). We are currently analyzing the population genetic structure across the geographic and color variation range using molecular markers to gain a better understanding of the genetic relationships among populations and color morphs.

In conclusion, we found that the geographic pattern of capitulum color distribution in *G. aurantiaca* populations does not appear to be associated with abiotic factors or pollinator color preferences. The idea that the divergence between red- and yellow-flowered population reflects a shift between beetle and bee populations is appealing, but we did not obtain clear evidence that hopliine scarab beetles favor red over orange or yellow inflorescences in the color polymorphic population. Further work needs to be conducted to explore the effects of capitulum color on interactions between *G. aurantiaca* and various antagonists, such as pollen thieves, herbivores and seed predators, as well as detailed genetic studies of within and between population structure in order to better understand the mechanisms driving color variation in this species.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

This manuscript is based on a Ph.D. thesis by IMJ who collected the data and wrote up the results under the supervision of SDJ and TJE. All authors contributed to the article and approved the submitted version and participated in the design of the study.

FUNDING

This study was funded by the Botanical Society of South Africa and the National Research Foundation of South Africa (Grant 46372 to SDJ).

ACKNOWLEDGMENTS

We thank Ezemvelo KZN Wildlife and Mpumalanga Parks and Tourism for research permits (EKZNW 2554/2003; 3021/2005, 394/2010), Mondi (Ltd.) and SAPPI Forestry Companies, and private landowners for permission to work on their properties and John and Sandi Burrows for alerting us to the polymorphic population in Mpumalanga. The KwaZulu-Natal National Botanical Garden (South African National Biodiversity Institute) provided nursery facilities for the common garden experiment and Craig Morris (ARC) gave assistance with statistical analysis of soil variables. Three reviewers and the editor provided extremely insightful comments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.676520/full#supplementary-material

Supplementary Figure 1 | Scree plot showing eigenvalues of principal components for edaphic variables of 14 *G. aurantiaca* population.

Supplementary Figure 2 | Reflectance spectra of red and yellow pan traps.

Supplementary Table 1 | Location, geographical and climatic conditions of *G. aurantiaca* populations across their distributional range.

Supplementary Table 2 | Mean inflection points of *Gerbera aurantiaca* ray florets from the six study populations.

Supplementary Table 3 | Floral morphological traits measurements (inflection points, capitulum diameter and ray floret length) from the color polymorphic RH population of *Gerbera aurantiaca*.

Supplementary Table 4 | Soil chemical properties for 14 sites across the geographical range of *Gerbera aurantiaca.*

Supplementary Table 5a | Total variance of explained soil variable components.

Supplementary Table 5b | Component matrix for *G. aurantiaca* soil variables.

Supplementary Table 5c | Regression factor scores for soils PCA of 14 *G. aurantiaca* populations.

Supplementary Table 6 | Pan trap catches: numbers of *Eriesthis vulpina* and *Apis mellifera* individuals caught in red and yellow pan traps.

REFERENCES

Arista, M., Talavera, M., Berjano, R., and Ortiz, P. L. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *J. Ecol.* 101, 1613–1622. doi: 10.1111/1365-2745.12151

Arnold, S., Faruq, S., Savolainen, V., MacOwen, P., and Chittka, L. (2010). FReD: The floral reflectance database — A web portal for analyses of flower colour. *PLoS One* 5:e14287. doi: 10.1371/journal.pone.0014287

Bernhardt, P. (2000). Convergent evolution and adaptive radiation of beetle-pollinated angiosperms. *Plant Syst. Evol.* 222, 293–320. doi: 10.1007/978-3-7091-6306-1_16

Bohm, B. A., and Stuessy, T. F. (2001). *Flavonoids of the Sunflower Family (Asteraceae)*. New York: Springer.

Braak, C. J. F. T., and Smilauer, P. (2002). *CANOCO: Reference Manual and CanoDraw for Windows User's Guide: Software for Canonical Community Ordination (version 4.5)*.

Bradshaw, H. D., and Schemske, D. W. (2003). Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* 426, 176–178. doi: 10.1038/nature02106

Campbell, D. R., Bischoff, M., Lord, J. M., and Robertson, A. W. (2010). Flower color influences insect visitation in alpine New Zealand. *Ecology* 91, 2638–2649. doi: 10.1890/09-0941.1

Carlson, J. E., and Holsinger, K. E. (2013). Direct and indirect selection on floral pigmentation by pollinators and seed predators in a color polymorphic South African shrub. *Oecologia* 171, 905–919. doi: 10.1007/s00442-012-2453-2

Chittka, L., and Menzel, R. (1992). The evolutionary adaptation of flower colours and insect pollinators colour vision. *J. Comp. Physiol. A* 171, 171–181.

Chittka, L., and Raine, N. E. (2006). Recognition of flowers by pollinators. *Curr. Opin. Plant Biol.* 9, 428–435. doi: 10.1016/j.pbi.2006.05.002

Chittka, L., and Waser, N. M. (1997). Why red flowers are not invisible to bees. *Israel J. Plant Sci.* 45, 169–183. doi: 10.1080/07929978.1997.10676682

Coberly, C., and Rauscher, M. D. (2008). Pleiotropic effects of an allele producing white flowers in Ipomea purpurea. *Evolution* 62, 1076–1085. doi: 10.1111/j.1558-5646.2008.00355.x

Cooley, A. M., Carvallo, G., and Willis, J. H. (2008). Is floral diversification associated with pollinator divergence? Flower shape, flower colour and pollinator preference in Chilean Mimulus. *Ann. Bot.* 101, 641–650. doi: 10.1093/aob/mcn014

Dafni, A. (1997). The response of Amphicoma spp. (Coleoptera; Glaphyridae) beetles to red models differing in area, shape and symmetry. *Israel J. Plant Sci.* 45, 247–254. doi: 10.1080/07929978.1997.10676688

Dafni, A., Bernhardt, P., Schmida, A., Ivri, Y., Greenbaum, S., O'Toole, C., et al. (1990). Red bowl-shaped flowers: convergence for beetle pollination in the Mediterranean region. *Israel J. Bot.* 39, 81–92.

Dafni, A., Tzohari, H., Ben-Shlomo, R., Vereecken, N. J., and Ne'eman, G. (2020). Flower colour polymorphism, pollination modes, breeding system and gene flow in Anemone coronaria. *Plants* 9, 1–18. doi: 10.1093/oxfordjournals.aob.a084448

Dalrymple, R., Kemp, D., Flores-Moreno, H., Laffan, S., White, T., Hemmings, F., et al. (2020). Macroecological patterns in flower colour are shaped by both biotic and abiotic factors. *New Phytol.* 228, 1972–1985. doi: 10.1111/nph.16737

de Villemereuil, P., Gaggiotti, O., Mouterde, M., and Till-Bottraud, I. (2016). Common garden experiments in the genomic era: new perspectives and opportunities. *Heredity* 116, 249–254. doi: 10.1038/hdy.2015.93

Dorin, A., Shrestha, M., Herrmann, M., Burd, M., and Dyer, S. G. (2020). Automated calculation of spectral-reflectance marker-points to enable analysis of plant colour-signalling to pollinators. *MethodsX* 7:100827. doi: 10.1016/j.mex.2020.100827

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G. P., Simonov, V., and Wong, B. B. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. B* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Edh, K., Widén, B., and Ceplitis, A. (2007). Nuclear and chloroplast microsatellites reveal extreme population differentiation and limited gene flow in the Aegean endemic Brassica cretica (Brassicaceae). *Mol. Ecol.* 16, 4972–4983. doi: 10.1111/j.1365-294x.2007.03585.x

Fenster, C. B., Scott Armbruster, W., Wilson, P., Dudash, M. R., and Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Ann. Rev. Ecol. Evol. Syst.* 35, 375–403.

Fey, M. (2010). *Soils of South Africa. Their distribution, properties, classification, genesis, use and environmental significance*. Cape Town: Cambridge University Press.

Goldblatt, P., and Manning, J. C. (2011). Hopliine beetles (Scarabaeidae: Rutelinae: Hopliini), specialized pollinators of the southern African flora. *Curtis's Bot. Magazine* 28, 238–259. doi: 10.1111/j.1467-8748.2011.01752.x

Hannan, G. L. (1981). Flower color polymorphism and pollination biology of Platystemon californicus Benth. (Papaveraceae). *Am. J. Bot.* 68, 233–243. doi: 10.1002/j.1537-2197.1981.tb12383.x

Hargreaves, A. L., Harder, L. D., and Johnson, S. D. (2010). Native pollen thieves reduce the reproductive success of a hermaphroditic plant, Aloe maculata. *Ecology* 91, 1693–1703. doi: 10.1890/09-0792.1

Hepburn, H. R., and Radloff, S. E. (1995). First approximation to a phenology of the honeybees (Apis mellifera) and flora of Africa. *Oecologia* 101, 265–273. doi: 10.1007/bf00328811

Hijmans, R. J., Cameron, E. E., Parra, J. L., Jones, P. G., and Jarvis, A. (2005). Very high resolution interpolated climate surfaces for global land areAS. *Int. J. Climatol.* 25, 1965–1978. doi: 10.1002/joc.1276

Hoballah, M. E., Gubitz, T., Stuurman, J., Broger, L., Barone, M., Mandel, T., et al. (2007). Single gene–mediated shift in pollinator attraction in Petunia. *Plant Cell* 19, 779–790. doi: 10.1105/tpc.106.048694

Hopkins, R., and Rausher, M. D. (2012). Pollinator-mediated selection on flower color allele drives reinforcement. *Science* 335, 1090–1092. doi: 10.1126/science.1215198

Horovitz, A. (1976). Edaphic factors and flower colour distribution in the Anemoneae (Ranunculaceae). *Plant Syst. Evol.* 126, 239–242. doi: 10.1007/bf00983363

Johnson, I. M., Edwards, T. J., and Condy, G. (2005). Gerbera aurantiaca. *Flowering Plants of Africa* 59, 134–139.

Johnson, S. D. (2006). "Pollinator-driven speciation in plants," in *Ecology and evolution of flowers*, ed. L. D. B. Harder (Oxford: Oxford University Press), 295–306.

Johnson, S. D., and Andersson, S. (2002). A simple field method for manipulating ultraviolet reflectance of flowers. *Can. J. Bot.* 80, 1325–1328. doi: 10.1139/b02-116

Johnson, S. D., Collin, C. L., Wissman, H. J., Halvarsson, E., and Agren, J. (2004). Factors contributing to variation in seed production among remnant populations of the endangered daisy Gerbera aurantiaca. *Biotropica* 36, 148–155. doi: 10.1646/q1569

Johnson, S. D., Harris, L. F., and Procheş, Ş. (2009). Pollination and breeding systems of selected wildflowers in a southern African grassland community. *South Afr. J. Bot.* 75, 630–645. doi: 10.1016/j.sajb.2009.07.011

Johnson, S. D., and Midgley, J. J. (2001). Pollination by monkey beetles (Scarabaeidae: Hopliini): do colour and dark centres of flowers influence alighting behaviour? *Environ. Entomol.* 30, 861–866. doi: 10.1603/0046-225x-30.5.861

Johnson, S. D., von Witt, C. G., and Anderson, B. (2020). Floral color variation in Drosera cistiflora is associated with switches in beetle pollinator assemblages. *Front. Plant Sci.* 11:606259. doi: 10.3389/fpls.2020.606259

King, C., Ballantyne, G., and Willmer, P. (2013). Why flower visitation is a poor proxy for pollination: measuring single-visit pollen deposition, with implications for pollination networks and conservation. *Methods Ecol. Evol.* 4, 811–818. doi: 10.1111/2041-210x.12074

Koski, M. H., and Ashman, T.-L. (2016). Macroevolutionary patterns of ultraviolet floral pigmentation explained by geography and associated bioclimatic factors. *New Phytol.* 211, 708–718. doi: 10.1111/nph.13921

Lebhardt, F., and Desplan, C. (2017). Retinal perception and ecological significance of color vision in insects. *Curr. Opin. Insect Sci.* 2017:24.

Leong, J. M., and Thorp, R. W. (1999). Colour-coded sampling: the pan trap colour preferences of oligolectic and nonoligolectic bees associated with a vernal pool plant. *Ecol. Entomol.* 24, 329–335. doi: 10.1046/j.1365-2311.1999.00196.x

Lunau, K., Papiorek, S., Eltz, T., and Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *J. Exper. Biol.* 214, 1607–1612. doi: 10.1242/jeb.052688

Maechler, M. (2016). *diptest: Hartigan's Dip Test Statistic for Unimodality. R package version 0.75-7*.

Manson, A., and Roberts, V. (2000). *Analytical methods used by the soil fertility and analytical services section*. KwaZulu-Natal: Dept Agriculture & Rural Development.

Martínez-Harms, J., Vorobyev, M., Schorn, J., Shmida, A., Keasar, T., Homberg, U., et al. (2012). Evidence of red sensitive photoreceptors in *Pygopleurus israelitus* (Glaphyridae: Coleoptera) and its implications for beetle pollination in the southeast Mediterranean. *J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol.* 198, 451–463. doi: 10.1007/s00359-012-0722-5

Mayer, C., Soka, G., and Picker, M. (2006). The importance of monkey beetle (Scarabaeidae: Hopliini) pollination for Aizoaceae and Asteraceae in grazed and ungrazed areas at Paulshoek, Succulent Karoo, South Africa. *J. Insect Conserv.* 10, 323–333. doi: 10.1007/s10841-006-9006-0

Menzel, R., and Shmida, A. (1993). The ecology of flower colours and the natural colour vision of insect pollinators: The Israeli flora as a study case. *Biol. Rev.* 68, 81–120. doi: 10.1111/j.1469-185x.1993.tb00732.x

Mogford, D. J. (1974). Flower colour polymorphism in Cirsium palustre. 2. *Pollination. Heredity* 33, 257–263. doi: 10.1038/hdy.1974.91

Newman, E., Anderson, B., and Johnson, S. D. (2012). Flower colour adaptation in a mimetic orchid. *Proc. R. Soc. Lond. B* 279, 2309–2313. doi: 10.1098/rspb.2011.2375

Paine, K. C., White, T. E., and Whitney, K. D. (2019). Intraspecific floral color variation as perceived by pollinators and non-pollinators: evidence for pollinator-imposed constraints? *Evol. Ecol.* 33, 461–479. doi: 10.1007/s10682-019-09991-2

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* 170, 23–40. doi: 10.1007/bf00190398

Picker, M., and Midgley, J. J. (1996). Pollination by monkey beetles (Coleoptera: Scarabaeidae: Hopliini): flower and colour preferences. *Afr. Entomol.* 4, 7–14.

Raimondo, D., Von Staden, L., Foden, W., Victor, J. E., Helme, N. A., Turner, R. C., et al. (2009). *Red list of South African plants 2009*. Pretoria: South African National Biodiversity Institute.

Raine, N. E., and Chittka, L. (2007). The adaptive significance of sensory bias in a foraging context: floral colour preferences in the bumblebee Bombus terrestris. *PLoS One* 2:e556. doi: 10.1371/journal.pone.0000556

Rajakaruna, N., and Bohm, B. (1999). The edaphic factor and patterns of variation in Lasthenia californica (Asteraceae). *Am. J. Bot.* 86, 1576–1596. doi: 10.2307/2656795

Rauscher, B. J. (2008). Evolutionary transitions in flower color. *Int. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Renoult, J. P., Blüthgen, N., Binkenstein, J. N. C., Weiner, M., Werner, M., and Schaefer, H. M. (2014). The relative importance of color signaling for plant generalization in pollination networks. *Oikos* 124, 347–354. doi: 10.1111/oik.01361

Reverté, S., Retana, J., Gómez, J. M., and Bosch, J. (2016). Pollinators show flower colour preferences but flowers with similar colours do not attract similar pollinators. *Ann. Bot.* 118, 249–257. doi: 10.1093/aob/mcw103

RHS. (2007). *RHS Colour Chart*. London: RHS.

Santamaría, L., and Rodríguez-Gironés, M. A. (2015). Are flowers red in teeth and claw? Exploitation barriers and the antagonist nature of mutualisms. *Evol. Ecol.* 29, 311–322. doi: 10.1007/s10682-015-9757-8

Sapir, Y., Gallagher, M., and Senden, E. (2021). What maintains flower colour variation within populations? *Trends Ecol. Evol.* 36, 507–519. doi: 10.1016/j.tree.2021.01.011

Schemske, D. W., and Bierzychudek, P. (2001). Perspective: Evolution of flower color in the desert annual Linanthus parryae: Wright revisited. *Evolution* 55, 1269–1281. doi: 10.1554/0014-3820(2001)055[1269:peofci]2.0.co;2

Schemske, D. W., and Bierzychudek, P. (2007). Spatial differentiation for flower colour in the desert annual Linanthus parryae: was wright right? *Evolution* 61, 2528–2543. doi: 10.1111/j.1558-5646.2007.00219.x

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Scrucca, L., Fop, M., Murphy, T. B., and Raftery, A. E. (2016). mclust 5: Clustering, classification and density estimation using gaussian finite mixture models. *R J.* 8, 289–317. doi: 10.32614/rj-2016-021

Shrestha, M., Garcia, J. E., Chua, J. H. J., Howard, S. H., Tscheulin, T., Dorin, A., et al. (2019). Fluorescent pan traps affect the capture rate of insect orders in different ways. *Insects* 10:40. doi: 10.3390/insects10020040

Smith, D. S., Ané, C., and Baum, D. A. (2008). The role of pollinator shifts in the floral diversification of Iochroma (Solanaceae). *Evolution* 62, 793–806. doi: 10.1111/j.1558-5646.2008.00327.x

Stanley, D. A., Msweli, S. M., and Johnson, S. D. (2020). Native honeybees as flower visitors and pollinators in wild plant communities in a biodiversity hotspot. *Ecosphere* 11, 1–12.

Stebbins, G. L. (1970). Adaptive radiation of reproductive characteristics in angiosperms, I: Pollination mechanisms. *Ann. Rev. Ecol. Syst.* 1, 307–326. doi: 10.1146/annurev.es.01.110170.001515

Strauss, S. Y., and Whittall, J. B. (2006). "Non-pollinator agents of selection on floral traits," in *Ecology and Evolution of Flowers*, ed. L. D. B. Harder (New York, NY: Oxford University Press).

Streinzer, M., Roth, N., Paulus, H. F., and Spaethe, J. (2019). Color preference and spatial distribution of glaphyrid beetles suggest a key role in the maintenance of the color polymorphism in the peacock anemone (Anemone pavonina, Ranunculaceae) in Northern Greece. *J. Compar. Physiol.* 205, 735–743. doi: 10.1007/s00359-019-01360-2

Tripp, E. A., and Manos, P. S. (2008). Is floral specialization an evolutionary dead-end? Pollination system transitions in Ruellia (Acanthaceae). *Evolution* 62, 1712–1737. doi: 10.1111/j.1558-5646.2008.00398.x

Tyrach, A., and Horn, W. (1997). Inheritance of flower colour and flavonoid pigments in Gerbera. *Plant Breed.* 116, 377–381. doi: 10.1111/j.1439-0523.1997.tb01015.x

Vaidya, P., McDurmon, A., Mattoon, E., Keefe, M., Carley, L., Lee, C., et al. (2018). Ecological causes and consequences of flower color polymorphism in a self-pollinating plant (Boechera stricta). *New Phytol.* 218, 380–392. doi: 10.1111/nph.14998

Valadon, L., and Mummery, R. (1967). Carotenoids of certain Compositae flowers. *Phytochemistry* 6, 983–988. doi: 10.1016/s0031-9422(00)86050-1

Van Kleunen, M., Nanni, I., Donaldson, J. S., and Manning, J. C. (2007). The role of beetle marks and flower colour on visitation by monkey beetles (Hopliini) in the greater Cape floral region, South Africa. *Ann. Bot.* 100, 1483–1489. doi: 10.1093/aob/mcm256

Wang, H., Talavera, M., Min, Y., Flaven, E., and Imbert, E. (2016). Neutral processes contribute to patterns of spatial variation for flower colour in the Mediterranean Iris lutescens (Iridaceae). *Ann. Bot.* 117:mcw036.

Whitehead, M. R., Gaskett, A. C., and Johnson, S. D. (2018). Floral community predicts pollinators' color preference: implications for Batesian floral mimicry. *Behav. Ecol.* 30, 213–222. doi: 10.1093/beheco/ary138

Wright, S. (1943). Isolation by distance. *Genetics* 28, 114–138.

Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A., and Smith, G. M. (2009). *Mixed effects models and extensions in ecology with R*. New York: Springer.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 13 July 2021
doi: 10.3389/fevo.2021.680463

Variation in *Manduca sexta* Pollination-Related Floral Traits and Reproduction in a Wild Tobacco Plant

Julia Bing[1†], Xiang Li[2†], Alexander Haverkamp[3], Ian T. Baldwin[1], Bill S. Hansson[2], Markus Knaden[2] and Felipe Yon[4]*

[1] Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Jena, Germany, [2] Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, Jena, Germany, [3] Laboratory of Entomology, Department of Plant Sciences, Wageningen University, Wageningen, Netherlands, [4] Grupo Entomologia, Instituto de Medicina Tropical, Universidad Peruana Cayetano Heredia, Lima, Peru

OPEN ACCESS

Edited by:
Casper J. Van Der Kooi, University of Groningen, Netherlands

Reviewed by:
Anna Lisa Stöckl, Julius Maximilian University of Würzburg, Germany
Katarzyna Roguz, University of Warsaw, Poland

***Correspondence:**
Felipe Yon
felipe.yon.t@upch.pe

†Present address:
Julia Bing, Department for Systematic Botany, Institute for Ecology and Evolution, Friedrich Schiller University, Jena, Germany
Xiang Li, PartiQla GmbH, Jena, Germany

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *14 March 2021*
Accepted: *10 June 2021*
Published: *13 July 2021*

Citation:
Bing J, Li X, Haverkamp A, Baldwin IT, Hansson BS, Knaden M and Yon F (2021) Variation in Manduca sexta Pollination-Related Floral Traits and Reproduction in a Wild Tobacco Plant. Front. Ecol. Evol. 9:680463. doi: 10.3389/fevo.2021.680463

Most flowering plants depend on animal pollination for successful sexual reproduction. Floral signals such as color, shape, and odor are crucial in establishing this (often mutualistic) interaction. Plant and pollinator phenotypes can vary temporally but also spatially, thus creating mosaic-like patterns of local adaptations. Here, we investigated natural variation in floral morphology, flower volatile emission, and phenology in four accessions of a self-compatible wild tobacco, *Nicotiana attenuata*, to assess how these traits match the sensory perception of a known pollinator, the hawkmoth *Manduca sexta*. These accessions differ in floral traits and also in their habitat altitudes. Based on habitat temperatures, the accession occurring at the highest altitude (California) is less likely to be visited by *M. sexta*, while the others (Arizona, Utah 1, and Utah 2) are known to receive *M. sexta* pollinations. The accessions varied significantly in flower morphologies, volatile emissions, flower opening, and phenology, traits likely important for *M. sexta* perception and floral handling. In wind tunnel assays, we assessed the seed set of emasculated flowers after *M. sexta* visitation and of natural selfed and hand-pollinated selfed flowers. After moth visitations, plants of two accessions (Arizona and Utah 2) produced more capsules than the other two, consistent with predictions that accessions co-occurring with *M. sexta* would benefit more from the pollination services of this moth. We quantified flower and capsule production in four accessions in a glasshouse assay without pollinators to assess the potential for self-pollination. The two Utah accessions set significantly more seeds after pollen supplementation compared with those of autonomous selfing flowers, suggesting a greater opportunistic benefit from efficient pollinators than the other two. Moreover, emasculated flowers of the accession with the most exposed stigma (Utah 2) produced the greatest seed set after *M. sexta* visitation. This study reveals intraspecific variation in pollination syndromes that illuminate the potential of a plant species to adapt to local pollinator communities, changing environments, and altered pollination networks.

Keywords: ***Manduca sexta*, *Nicotiana attenuata*, floral trait, pollination, plant reproduction, local adaptation**

INTRODUCTION

Flowers exploit the sensory bias of insect pollinators to improve their chance of receiving outcrossed (Balamurali et al., 2015) or at least geitonogamous pollinations (Vaughton and Ramsey, 2010; Sukumaran et al., 2020), the opportunities for which differ considerably depending on plant densities, interpopulation distances, and phenologies (Carvalheiro et al., 2014; Kantsa et al., 2018). Attracting (or luring) pollinators is achieved *via* different types of rewards or attractants offered by the plant. In some cases, these signals can vary among plants from different habitats and populations in response to the specific needs of pollinators that can maximize the reproductive output of the plant (Gómez et al., 2009a).

Most species are assemblages of different genotypic and phenotypic populations within an ecologically complex landscape. The geographic mosaic theory of coevolution incorporates this fact and postulates that populations are under different evolutionary selective pressures over large geographic distances due to spatial variation (Thomson, 2005). In a pollinator community context that would mean that due to spatial variations over the habitats of its range, a plant species can be either adapted or maladapted to certain pollinator(s). This suggests that when a plant species with a specialized pollination syndrome occurs outside the range of the optimal pollinator, many of the adaptations might become maladaptations as they could hinder the attraction of other potential pollinators. In contrast, if a plant has a generalist pollinator community, certain floral traits can be considered as local adaptations over its range to maximize the service of the most optimal local pollinators (Gómez et al., 2009a).

Scent is an important factor for speciation or pollinator fidelity to a certain plant species (Balamurali et al., 2015; Kantsa et al., 2018; Souto-Vilarós et al., 2018) in relation to the pollinator community. The loss of a specific major scent compound can be interpreted as a release from a main pollinator, particularly if the scent at high concentrations acts also as a repellent to herbivores (Baldwin et al., 1997; Kessler et al., 2019). Furthermore, pollinators can also act as herbivores for the same plant species. For example, besides searching for nectar, female moths are also in the search of oviposition sites. Both nectaring and oviposition behavior have been shown to be linked in the hawkmoth *Manduca sexta*, bearing the risk for the plant to be consumed by the caterpillars of its pollinator (Kessler et al., 2012; Smith et al., 2017).

Other studies addressing outcrossing versus selfing point out that self-compatible populations at edges of a native range will have a larger proportion of selfing compared with outcrossed. Plants growing at the edges of the native range may have fewer pollinators available that can perceive and interact successfully with the flowers, this being even more so for specialist plant–pollinator systems. Thus, in order to colonize new habitats and avoid Allee effects (Morgan et al., 2011), plants in edge populations would have to rely more on selfing rather than outcrossing. Over time, different floral traits can be (de)selected as those are no longer exploiting a specialized perception of a pollinator to increase the reproductive output of the plants. The reduction of investment on floral advertisement can lead to a shorter flower presentation time and faster self-pollination.

Additionally, there are physical–mechanical–morphological floral traits that can facilitate the visualization of certain traits such as the timing of flower opening and flower movement (Aizen, 2003; van Doorn and Van Meeteren, 2003; Hodges et al., 2004; Yon et al., 2016). Flower opening conditions the perception of a corolla display by a pollinator, as the pigments located in the front and back of the petals can be different, as well as allowing the visualization of anthers, pollen, and pistil. As studies have shown on *Aquilegia* and artificial flowers (Hodges et al., 2004; Sprayberry and Suver, 2011), the orientation of flowers can render the visual organs of many pollinators useless. In these cases, the obverse of the corolla and its features are not exposed to the pollinator, due to particular flying and foraging approaches to the flowers (Hodges et al., 2004; Ushimaru and Hyodo, 2005; Haverkamp et al., 2019).

Nicotiana attenuata is a solanaceous plant species mainly occurring in the Southwest United States, where it inhabits a broad range of elevations at least between 800 and 2,300 meters above sea level (masl) (Haverkamp et al., 2018). This represents a temperature variation between locations at the warmest month July of 7.5°C at high elevation to 19.9°C at lower elevation during the night (WorldClim database). *N. attenuata* is assumed to have a hawkmoth pollination syndrome, with white long tubular flowers, night opening, night upright orientation, and strong night scent that exploit the sensory bias of the hawkmoth *M. sexta*. Previous studies examining the interaction between *M. sexta* and *N. attenuata* have shown the importance of one particular floral volatile compound, benzyl acetone (BA; Kessler et al., 2015; Haverkamp et al., 2016b). The presence of this compound increases the foraging success of the moth, as well as the reproductive output of the plant (Haverkamp et al., 2016b).

At the same time, the larvae of this hawkmoth is a voracious herbivore on *N. attenuata*, and its attraction as pollinator therefore brings a risk of damage (Kessler et al., 2010, 2015; Reisenman et al., 2010; Kessler, 2012). This might select against exploiting *M. sexta* sensory bias and pollination service in some natural populations under less beneficial growth conditions (Gómez et al., 2009b). Besides *M. sexta*, also other hawkmoths such as *Hyles lineata* or *Manduca quinquemaculata*, but also day-active hummingbirds (e.g., *Archilochus alexandri*) and bee species (e.g., *Apis mellifera*, *Lasioglossum* spp.) have been observed to visit flowers of *N. attenuata*, which feed on nectar and pollen, respectively. In field experiments, emasculated flowers open during the day produced capsules, thus showing the capability of day-active pollinators to provide the plant with pollen (Kessler et al., 2015), especially when plants are attacked by *M. sexta* caterpillars (Kessler et al., 2010). *N. attenuata* plants that are under attack of *M. sexta* caterpillars produce flowers with reduced BA emissions that open in the morning and are preferred by day-active hummingbirds (Kessler et al., 2010). Thus, plants reduce herbivore pressure by switching from *M. sexta* pollination, which involves the risk of caterpillar-feeding damage, to hummingbird pollination.

We recently showed that *N. attenuata* originating from different populations are differentially attractive to the pollinator

M. sexta (Haverkamp et al., 2018). In this follow-up study, we investigate how this relates to the reproductive success of four *N. attenuata* accessions. Moreover, we propose that some accessions are more specialized on *M. sexta* pollination than others, following the idea of local specialization.

MATERIALS AND METHODS

Seed Germination and Plant Cultivation

The *N. attenuata* Torr. (Solanaceae) seeds for all experiments were sterilized and germinated on Petri dishes with Gamborg's B5 media as described in Krügel et al. (2002). The seeds were maintained under 16 h:8 h light:dark conditions in a growth chamber with temperature set to 28°C in light and 26°C in dark (Percival, Perry, IA, United States) for 10 days. Afterwards, seedlings were transferred to TEKU pots (TEKU JP 3050 104 pots, Poppelmann, Lohne, Germany) with Klasmann plug soil (Klasmann-Deilmann, Saterland, Germany) in a glasshouse [16 h:8 h light:dark, humidity: 50–60%, temperature: 23–25°C (light), and 19–21°C (dark)]. After 10 days, plants were transferred from TEKUs to 1 L pots for glasshouse and wind tunnel experiments at the Max Planck Institute for Chemical Ecology (MPI CE), Jena, Germany.

In this study, we focused on genetically fixed differences among different accessions rather than phenotypic plasticity. Therefore, plants were inbred for several generations and raised under uniform growth conditions in the glasshouse. Plants originated from four different wild-type *N. attenuata* native populations: "Ut1" from the D.I. Ranch (Santa Clara, UT, United States; inbred for 31 generations), "Az" from Townsend Winona Road (east Flagstaff, AZ, United States; inbred for five generations), "Ca" from Benton Crossing Road (west Benton Hot Springs, CA, United States; inbred for five generations), and "Ut2" from Lytle Ranch Preserve (Lytle Ranch Preserve Rd, UT, United States; inbred for seven generations). The locations from which the seeds of the different accessions originated are shown in **Figure 2**.

Manduca sexta Rearing

Manduca sexta moths used for wind tunnel experiments were obtained from a colony maintained at the MPI CE. Moths were reared as previously described in Koenig et al. (2015). Eggs for the colony were collected from female moths, which were allowed to freely oviposit on *N. attenuata* plants. Caterpillars were fed on artificial diet at 27°C ambient temperature, 70% relative humidity, and 16:8 light:dark regime. As soon as caterpillars reached the wandering stage and stopped feeding, they were transferred into wooden blocks for pupation. Pupae were sexed 1 week before hatching, and male and female pupae were transferred in separate flight cages (15.5 h daylight with 25°C and 70% relative humidity, 7.5 h dim light at 0.5 lx with 20°C and 60% relative humidity). Between both phases (daylight and dim phase), a transition time of 30 min each was used. For experiments, male moths were used 3 days after hatching. Since *M. sexta* females frequently lay eggs while foraging on *N. attenuata* (Kessler et al., 2012), we used male moths to exclude the oviposition behavior from our experiments.

Flower Morphology and Opening

Flower morphology and opening of four *N. attenuata* accessions was investigated by measuring corolla limb diameter and area, pistil and corolla tube length, and flower opening of single freshly open flowers from seven independent plants per accession. The corolla limb diameter was measured between the most outer tips after flowers fully opened, and the area was measured from scanned calibrated images of the surfaces of corollas.

Flowers from seven independent plants per accession were cut open using forceps to measure the pistil and corolla tube length by hand with a ruler. The length of the pistil was measured from the flower base to the stigma. The corolla tube length was measured from the flower base to the tip of the open corolla petals. The length ratio of the corolla tube and the pistil within a flower was calculated by dividing the first over the second, where values above 1 means the pistil is shorter and the stigma is within the corolla tube and values below 1 means that the stigma is protruding from the corolla tube.

Flower opening (flower aperture) was recorded at 1/h acquisition intervals using a time-lapse imaging setup, composed of a webcam (Logitech Europe S.A., Lausanne, Vaud, Switzerland) connected to a laptop to automatically acquire and store the photos. To quantify the opening, the inner distance between opposite lobes was measured in pixels and converted to millimeter using the software IMAGE TOOLS v.3.0 (UTHSCSA, San Antonio, TX, United States). Later, the aperture values were converted to percentage of opening by taking the maximum value in millimeters from the fully open flower as denominator for all other intermediate values.

Pollen Count

For evaluating the number of pollen grains for four different natural accessions, anther heads were collected separately before anthesis (8–10 a.m.) into 2 ml reagent tubes and stored in a desiccator for 24 h until opening. For each accession, all five anthers from nine flowers of five plants were collected. After anthers opened, 250 µl of 2% sodium chloride solution was added to each tube and vortexed for approximately 1 min. The number of pollen grains per anther was counted under a microscope using a Neubauer cell counting chamber with a depth of 0.1 mm (Neubauer improved, Superior Marienfeld, Lauda-Königshofen, Germany). To ensure an equal distribution of pollen within the sample, we shortly vortexed each tube again directly before adding 10 µl to the chamber. For each sample, five large squares (5×1 mm^2) were counted for estimation of the total number of pollen grains per anther following the Neubauer chamber formula:

$$\frac{\textit{Number of pollen}}{\textit{grains per anther}} = \frac{\sum \textit{pollen counted} \times 250\ \mu\text{l}}{5\ \text{mm}^2 \times 0.1\ \text{mm}}$$

Floral Benzyl Acetone Emissions

Benzyl acetone was measured as described in Haverkamp et al. (2018) using polydimethylsiloxane (PDMS) as traps and

GC-MS for quantification. Here, we compare total overnight emissions of four flowers from different plants of the Ut2 accession with data for Ut1, Az, and Ca accessions taken from Haverkamp et al. (2018).

Nectar and Sugar Measurements

The amount of nectar was measured by carefully removing the corolla tube and collecting the nectar with a capillary (Brand, Wertheim, Germany) and measuring the length and dividing by its conversion factor 3.2. We used six plants per accessions and one flower per plant. The composition of sugars in the nectar was measured on a LC-Triple Quadrupole-MS instrument, Bruker EVOQ Elite (Bruker, Billerica, MA, United States), employing an HESI ion source as described in Schäfer et al. (2016). The quantification of glucose, fructose, and sucrose was done relative to the internal standard sorbitol.

Phenology Measurements

To examine the total number of flowers and capsules produced over the lifespan of the plants, we used five plants of each wild-type accession in the glasshouse. Since flowers remain open for approximately 3 days, the number of open flowers was recorded every 3 days in the morning (8–10 a.m.) to avoid multiple counting of the same flower. Additionally, all capsules were counted at each time point. At the end, the number of flowers from all time points was summed to estimate the total number of flowers and capsules as well as the total flower to total capsule ratio produced for each plant. Flower and capsule counts were recorded until plant senescence.

Map of Accessions and *M. sexta* Presence Prediction

The map of accessions was generated using the WorldClim database, referenced location of the accessions, *M. sexta* referential presence data in Utah (Yon et al., 2017b), and environmental temperature required for flying of *M. sexta* (Heinrich, 1971). The digital elevation model was used to generate altitude contour lines at 500 masl intervals using QGIS v.2.18. The predicted distribution map of *M. sexta* was generated using temperature as the proxy parameter for determining the likeliness of presence in southwestern United States using the function Bioclim modeling of the software DIVA-GIS v.7.5.

Wind Tunnel Bioassays

Behavioral assays with *M. sexta* moths were performed in a plexiglass wind tunnel (250 × 90 × 90 cm) at the MPI CE. The laminar flow of charcoal-filtered air was set to 0.37 m/s, which is similar to those conditions that hawkmoths commonly experience while foraging (Riffell et al., 2008). Climate conditions were adjusted to 25°C air temperature and 70% relative humidity. At the latest 1 h before the experiment, plants and moths were transferred to separate chambers with similar climate conditions as the wind tunnel.

To measure pollination rates of flowers of four natural accessions after *M. sexta* visitation, one flower per plant was emasculated before anthesis (daylight morning cycle) to exclude self-pollination (Kessler et al., 2008), and all other flowers were removed. Pollen of fresh flowers from plants not used in the wind tunnel was collected in the corresponding morning. In order to measure the output of pollen delivery, pollen was gently rubbed on the hawkmoth proboscis using a fine brush prior to its release in the wind tunnel (Haverkamp et al., 2016b). For each trial, another plant was placed at one side of the wind tunnel with the flower at a position 25 cm from the front end, 45 cm distant from both side walls, and approximately 70 cm from the ground of the wind tunnel. Moths were kept in individual mesh cages (15 cm × Ø13 cm) until being placed on a platform at the rear end of the wind tunnel opposite of the plant (10 cm from the rear end, 45 cm from both side walls, and 30 cm from the wind tunnel floor). Moths, which did not initiate wing fanning within 5 min, were excluded from the experiment. After the moths were taking off, they were allowed to fly freely for 4 min in the wind tunnel. The foraging behavior was observed *via* a video camera situated behind the flower at the beginning of the wind tunnel (Logitech C615, United States, infrared filter removed). The camera was recording at 30 Hz with a resolution of 800 × 600 pixels.

Flower approach was scored as flower contact of the moth with its proboscis or front legs. Flower handling time was counted from the first contact until the moth had no more contact with the flower for more than 1 s. Only the first flower approach was used for statistical analyses in order to exclude learning effects. Foraging success was evaluated by measuring the amount of residual nectar after an apparent successful moth contact with the flower, where a foraging event was scored as successful when nectar was fully removed as described in Haverkamp et al. (2018, 2019).

Pollination Experiments

All hand pollinations were performed in the evening between 7 and 9 p.m. with freshly open flowers, and the pollen was collected immediately before the start of the experiment from freshly open flowers. Hand pollinations with pollen of the same accession (self) and a pollen mixture of all four accessions (mix) were performed using emasculated flowers on five plants per accession. Therefore, anthers have been removed before anthesis in the morning between 5 and 6 a.m. of the day of flower opening as described in Kessler et al. (2008). For self hand pollination, we collected freshly open anthers of the accessions in separate 0.5 ml PCR reaction tubes. For mix hand pollination, the same number of freshly open anthers from the different accessions was collected with clean forceps into a 0.5-ml PCR reaction tube and mixed by tapping multiple times against the walls. Anther heads were carefully removed from the reaction tube prior to avoid any damage when applying pollen to the stigma. For each hand pollination treatment (self and mix), we used 20 flowers on five plants per accession. The same plants were used for pollen collection, self and mix hand pollination. Only capsules that produced seeds have been used for statistical analysis.

Additionally, we used nonemasculated flowers to evaluate if the seed set of autogamously selfing flowers (autogamous selfing) differs from that of flowers supplemented with pollen from the same flowers (selfing hand). Therefore, we removed

all open flowers in the morning of the experiment to ensure the use of fresh flowers. For both treatments, 10 flowers on five plants per accession were used, whereas only flowers that produced capsules containing seeds have been considered for statistical analysis.

In all hand pollination experiments, pollen was applied to experimental flowers by homogeneously covering the stigma using a wooden tooth pick. Afterwards, the flowers' pedicels were carefully labeled using a colored string with each treatment being assigned to a different color. Capsules of all pollination experiments were collected shortly before opening approximately 14–20 days after pollination and dried in a desiccator for 2 days before being further processed. The number of seeds from capsules obtained from hand and wind tunnel pollinations have been automatically counted using ImageJ2 (Fiji; Schindelin et al., 2012; Rueden et al., 2017).

Statistical Analysis

The data were analyzed using the software R version 4.0.4 (The R Foundation for Statistical Computing, 2021) and, additionally, the following R packages "pairwiseCI" v.0.1-27, "lsmeans" v.2.3, "multcomp" v.1.4-16, and "agricolae" v.1.3-3. Data normality was evaluated with Shapiro tests and additionally visualized with Q-Q plots. When data were normally distributed, we employed an analysis of variance (ANOVA) model; when data were not normally distributed, we employed a generalized linear model, with family adjustments depending on data type (Gaussian for continuous values and quasi-binomial for proportions). Direct pairwise comparisons, when not required one of the previous models, were analyzed with a Holm-corrected Fisher's exact test for normally distributed data and a Wilcoxon test for non-normally distributed data. Additionally, in order to evaluate difference in floral trait variance among accessions, we used the Fligner test for pairwise variance analysis with Holm adjustment.

RESULTS

Flower Morphology and Opening

The corolla limb area was analyzed using the "Momocs" R package and compared between accessions using a generalized linear model followed by *post hoc* Tukey with Holm adjustment for multiple comparison showing the greatest corolla limb area (**Figure 1A** and **Supplementary Table 1**) and corolla tube length with an ANOVA followed by the honestly significant difference (HSD) test (**Figure 1B** and **Supplementary Table 1**) for flowers of the accession Ut2. To evaluate differences in the position of the stigma relative to the corolla tube, the ratio of tube/pistil length was compared between the accessions (GLM followed by pairwise Wilcoxon test), whereas values <1 indicate a stigma located outside of the corolla tube (Ut2) and values >1 a stigma located inside the corolla tube (Ut1, Az, and Ca). The tube/pistil length ratio differed significantly between Ca and Ut2, whereas Ut1 and Az did not differ from any accession (**Figure 1C** and **Supplementary Table 1**). For these three morphological traits, the size of their variance was not significantly different when tested with the Fligner tests (corolla limb area $p = 0.5315$, tube length $p = 0.9891$, tube/pistil length $p = 0.2999$).

Flowers of Ut2 and Ca started opening earlier but slower than Ut1 and Az, which results in a similar flower opening when *M. sexta* activity period starts at 20 h (**Figure 1F**). The progressive opening of the corolla limb (millimeters open expressed as percentage of its full opening) was analyzed with a GLM [accessions $F = 16.736$, $p < 0.0001$; time (as factor) $F = 146.059$, $p < 0.0001$] followed by *post hoc* least square means Tukey for pairwise comparison between accessions (**Supplementary Table 1**). We observed that flowers of Ca and Ut2 started opening significantly earlier than flowers of Az or Ut1, while the latter two were not different between themselves.

Pollen Count

The number of pollen grains per anther (**Figure 1D** and **Supplementary Table 1**) was compared between the accessions. Due to its normal distribution, ANOVA followed by the HSD test was performed, showing that Ut2 has significantly more pollen grains per anther than Ca ($p = 0.0007$) and Ut1 ($p = 0.0445$), but does not differ from Az ($p = 0.4514$). No significant difference was found between the variance size with the Fligner test ($p = 0.752$).

Floral Benzyl Acetone Emissions

Furthermore, analysis of total BA emission in flowers of the four accessions was performed using a GLM followed by a multicomparison HSD test (**Figure 1E**). Flowers of Ut1 emit the greatest amount of BA, being only significantly different from Ca ($p = 0.0221$), which is originating from a population outside of the predicted *M. sexta* range (**Figure 2A**). No significant difference was found between the variance size with the Fligner test ($p = 0.3208$).

Nectar and Sugar Measurements

Nectar volume differed significantly among the four accessions (GLM $p < 0.0001$) and was the highest in Ca, followed by Ut2. The other two accessions presented similar low nectar volumes (**Supplementary Figure 1A**). When analyzing the sugar composition, Az had the highest concentrations of glucose, fructose, and sucrose compared with the other accessions (GLM followed by a multicomparison HSD test, glucose: $p = 0.0044$, fructose: $p = 0.0031$, sucrose: $p = 0.0139$), but was not significantly different for each pairwise comparison (**Supplementary Figures 1B–D**).

Flower and Capsule Count

Five plants per accessions were observed regarding their flower and capsule kinetics. Az plants started flowering around 28 days after potting, which is 2.4 to 4.8 days earlier than the other accessions. Moreover, Az plants attained their flowering peak 7.8 to 9 days earlier than Ca, Ut1, and Ut2. The flower kinetic was analyzed with a GLM with factors accession and a four term polynomial fitting for days after potting (dap) (accession $p = 0.0014$ and dap $p < 0.0001$). Followed by a least square means analysis for pairwise comparison, overall, the flowering kinetic of Az differed significantly from all other accessions (**Figure 3A** and **Supplementary Table 2**).

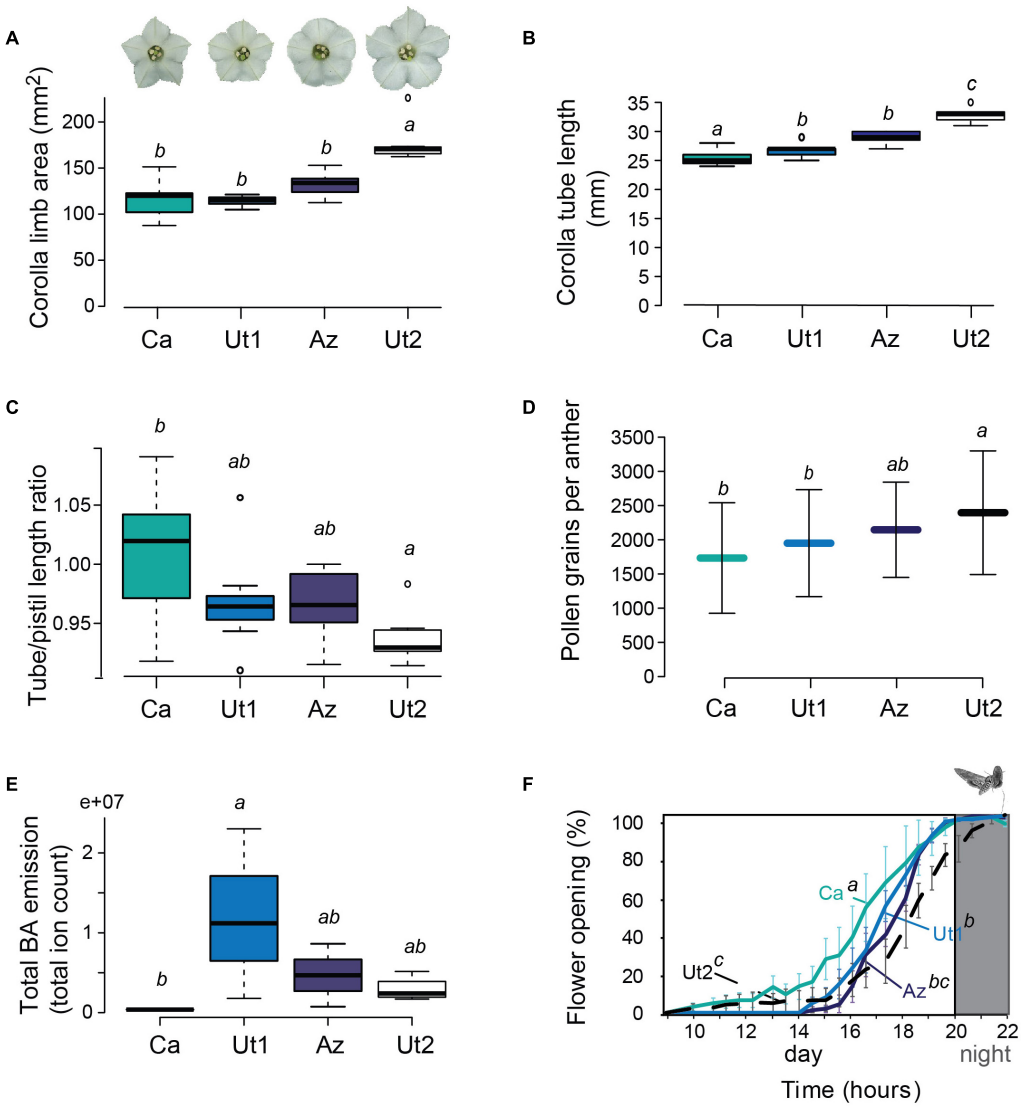

FIGURE 1 | Floral morphology and pollen number of four native *Nicotiana attenuata* accessions. **(A)** Boxplot showing the corolla limb area analyzed using the R package "Momocs" [$N = 7$, generalized linear model followed by *post hoc* Tukey with Holm adjustment, different letters indicate significant differences ($p < 0.001$)]. **(B)** Boxplot of corolla tube length [$N = 7$, ANOVA model followed by HSD test with Holm adjustment, different letters indicate significant differences ($p < 0.005$)]. **(C)** Boxplot showing the corolla tube/pistil length ratio. Values above 1 indicate a stigma which is shorter than the corolla tube, whereas a value below 1 indicates a stigma located outside of the corolla tube [$N = 7$, generalized linear model followed by pairwise Wilcoxon test with Holm adjustment, different letters indicate significant differences ($p < 0.05$)]. **(D)** Number of pollen grains (mean ± SD) per anther [$N = 45$, ANOVA followed by HSD test, different letters indicate significant differences ($p < 0.05$)]. **(E)** Boxplot showing the total BA emission of flowers [$N = 4$, generalized linear model followed by HSD test, different letters indicate significant differences ($p < 0.05$)]. **(F)** Flower opening (mean ± SEM) between 9 and 22 h [$N = 4$, generalized linear model followed by HSD test with Holm adjustment, different letters indicate significant differences ($p < 0.05$)]. The flower opening refers to the flower aperture measured in millimeters and expressed as percentage of opening. The gray bar represents the activity period of *M. sexta* within the time span measured. Boxplots show the median (thick bar), the first and the third quartiles [box and 1.5 times the interquartile range (whiskers)]. Abbreviations for accessions: Az, Arizona; Ca, California; Ut1, Utah 1; Ut2, Utah 2.

Furthermore, the capsule kinetic, with the same statistical approach as the flower kinetic, showed significant differences between Az and all other accessions (**Figure 3B** and **Supplementary Table 2**), with Az starting to set capsules 4.2 to 8.4 days ahead of Ca, Ut1, and Ut2. The number of capsules produced per flower over the lifespan of the plant was compared as capsule-to-flower ratio between accessions (**Figure 3C** and **Supplementary Table 1**). Arizona plants showed a significantly greater capsule-to-flower ratio, being approximately 20% higher than those of the other accessions.

Manduca sexta Flower Handling and Pollination Output

The duration of flower contact was measured for the first flower approached by *M. sexta*. Solely, Ut1 and Ca showed a statistically

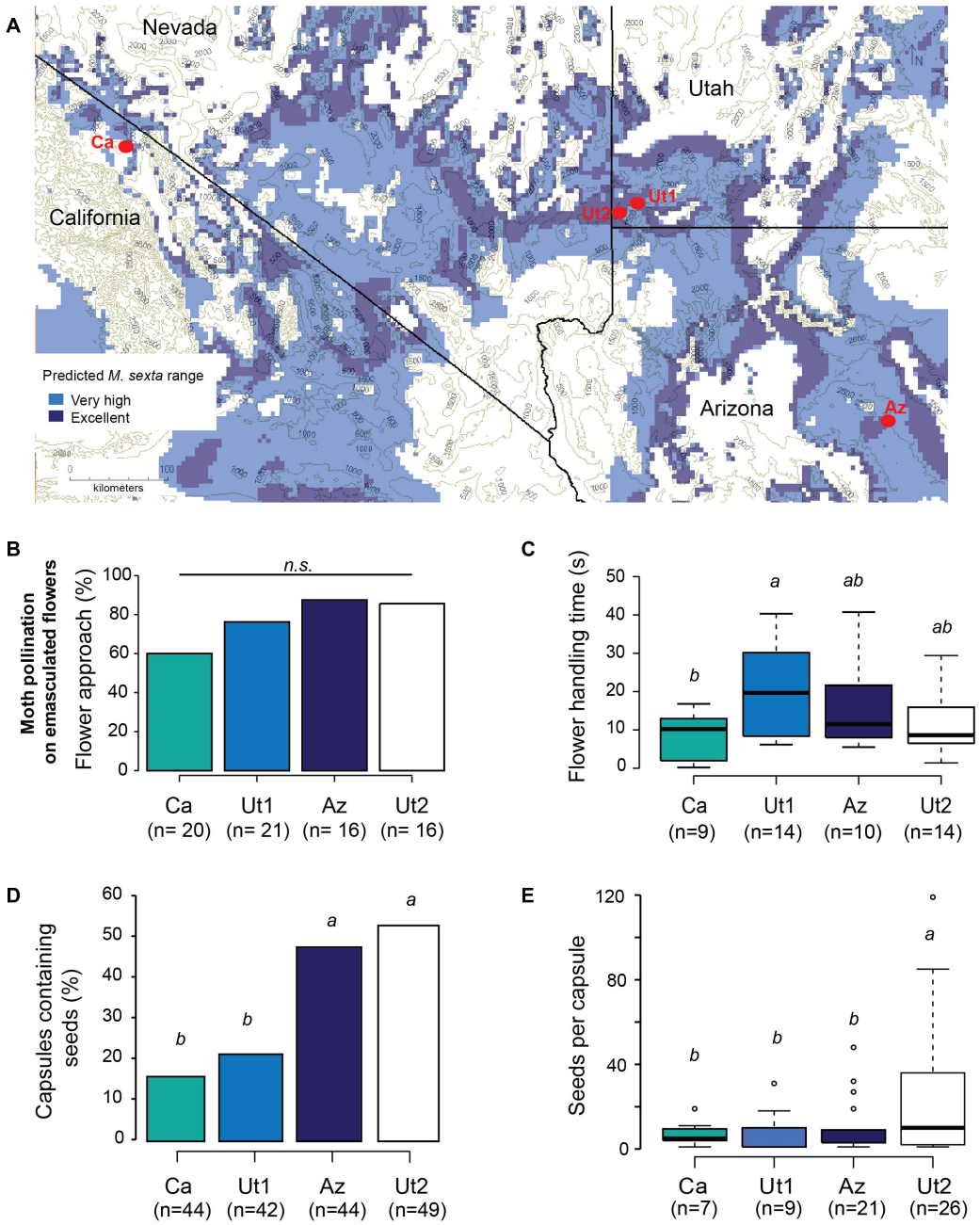

FIGURE 2 | Map of *Manduca sexta* predictions based on temperature range and pollination output of four natural accessions. **(A)** Predicted distribution map of *M. sexta* with temperature as the proxy parameter in the Great Basin Desert, United States. Red circles show the seed collection sites. **(B)** Total percentage of emasculated flowers of *N. attenuata* approached by *M. sexta* moths in a wind tunnel assay [$p = 0.1326$, Holm-corrected Fisher's exact test, data published for Ca, Az, and Ut1 (Haverkamp et al., 2019)]. **(C)** Flower contact time (mean ± SD) for first flower approached [ANOVA followed by Holm-corrected least significant differences, data published for Ca, Az, and Ut1 (Haverkamp et al., 2019)]. **(D)** Total percentage of capsules containing seeds formed after *M. sexta* visitation (ANOVA followed by pairwise HSD test with Holm adjustment). **(E)** Seed output of emasculated flowers resulting from moth pollination (generalized linear model followed by least squares pairwise comparison). Numbers below the *x*-axis represent replicate numbers. Different letters indicate significant differences ($p < 0.05$). n.s., non-significant difference. Whiskers of the boxplots in panels **(C,E)** indicate 1.5 times the interquartile range; boxes depict first and third quartiles.

significant difference in flower contact time, while Az and Ut2 did not differ from any accession (**Figure 2C** and **Supplementary Table 1**). When foraging on flowers of Ca, moths have the lowest foraging success (20%) compared with the other three accessions (Ut1: 66.67%, Az: 56.25%, and Ut2: 50%), although in a pairwise comparison, only Ca and Ut1 were significantly different (**Supplementary Figure 2**). The foraging success of the moth did not differ between Ut1, Ut2, and Az.*Manduca sexta* approached

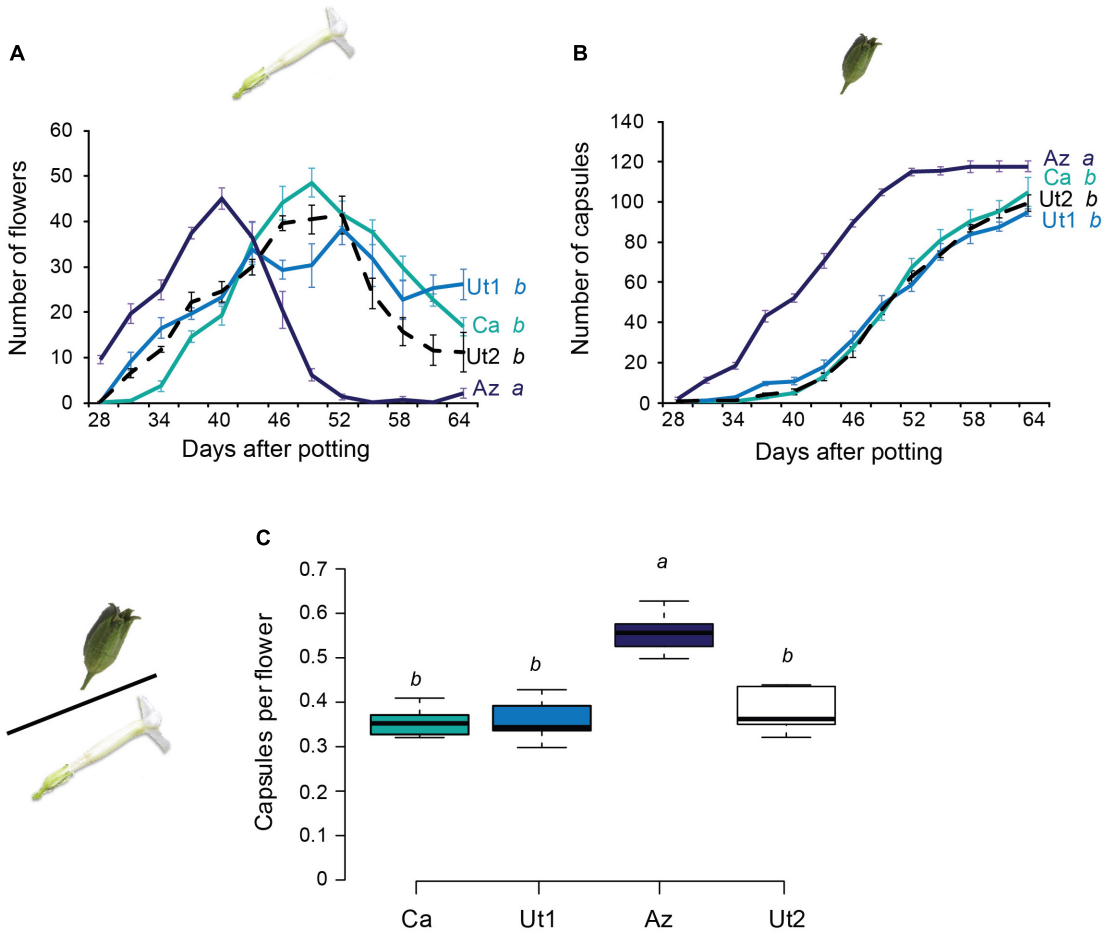

FIGURE 3 | Flower and capsule count over the lifespan of four *N. attenuata* accessions. **(A)** Mean ± SEM number of flowers per plant ($N = 5$, generalized linear model with polynomial fitting with four terms, followed by least square means for pairwise comparison with Tukey adjustment). **(B)** Mean ± SEM accumulative number of capsules produced per plant ($N = 5$, generalized linear model with polynomial fitting with four terms, followed by least square means for pairwise comparison with Tukey adjustment). **(C)** Proportion of capsules formed per total number of flowers per plant [$N = 5$, generalized linear model (quasi-binominal family) followed by a least square mean pairwise comparison]. Error bars of the boxplot in panel **(C)** indicate 1.5 times the interquartile range; boxes depict first and third quartiles. Different letters indicate significant differences ($p < 0.05$).

a similar percentage of emasculated flowers in the wind tunnel ($p = 0.1326$ Holm-corrected Fisher's exact test, **Figure 2B**). We used the capsules formed by the approached flowers for seed counts to estimate the output of *M. sexta* pollination on emasculated flowers. Not all of the capsules contained fully formed seeds and the accessions differed in this percentage of capsules, where Az and Ut2 formed a significantly greater number of capsules containing seeds than Ca and Ut1 (**Figure 2D** and **Supplementary Table 1**; Ca: 15.91%, Ut1: 21.43%, Az: 47.73%, and Ut2: 53.06%).

To analyze the number of seeds per capsule that result from *M. sexta* pollinations, we only used capsules containing fully formed seeds. Capsules of Ut2 contained a greater number of seeds than Ca, Ut1, and Az, even though being only significantly different with Az (**Figure 2E** and **Supplementary Table 1**). The seed output presented a significant difference of the variance size with the Fligner test ($p = 0.0061$). Followed by a pairwise Fligner test comparison (with Holm adjustment) of the size of variances, a difference was found only between the pair Az–Ut2 ($p = 0.022$).

Hand Pollination Experiments

We compared the seed set of emasculated flowers hand pollinated either with self pollen or with mix pollen containing all four accessions (**Figure 4A**). The number of seeds produced per capsule did not differ significantly between both treatments in Ca ($p = 0.096$), Ut1 ($p = 0.680$), and Ut2 ($p = 0.449$). Only Az produced a significantly greater amount of seeds per capsule when pollinated with mix pollen ($p = 0.024$). No significant difference was found for the variance size between accessions either for self pollen or mix pollen with the Fligner tests ($p = 0.6127$ and $p = 0.3775$, respectively).

To assess possible pollen limitation resulting from the lack of autogamous selfing, we used nonemasculated flowers. We

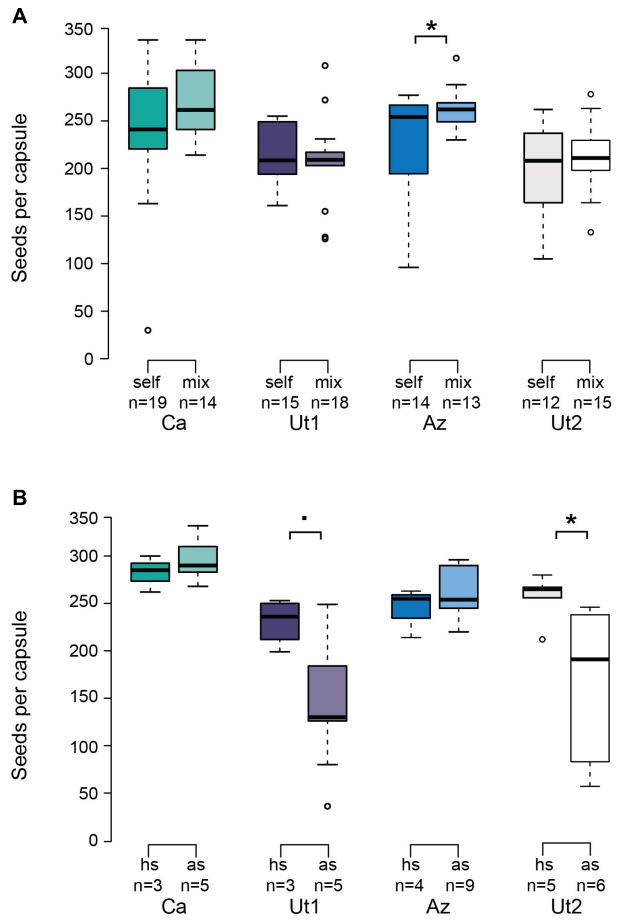

FIGURE 4 | Seed output after natural selfing and hand pollination with self and mixed pollen. **(A)** Seed output of emasculated flowers hand pollinated with self pollen ("self," left box) or a pollen mixture of all four accessions ("mix," right box). **(B)** Seed output of natural selfing flowers (autogamous selfing "as," right bars) in comparison with nonemasculated flowers hand pollinated with additional self pollen (selfing hand "hs," left bars). Whiskers of the boxplots indicate 1.5 times the interquartile range; boxes depict first and third quartiles; centerline indicates median. Statistics for both data sets were performed using a generalized linear model followed by a pairwise Wilcoxon test to compare between both treatments of the same accession. Numbers below the *x*-axis represent the number of replicates. Asterisks indicate statistically significant differences ($p < 0.05$); dot indicates near statistically difference ($p < 0.051$).

compared the seed set between flowers allowed to naturally self (autogamous selfing) and flowers supplemented with pollen of the same plant (selfing hand, **Figure 4B**). In Ut2 ($p = 0.0173$), nonemasculated flowers additionally hand pollinated with self pollen (selfing hand) showed a significantly greater seed set compared with naturally selfing flowers (autogamous selfing), whereas no difference was detected for Ca ($p = 0.5714$) and Az ($p = 0.7857$). In Ut1, we observed the same tendency as in Ut2, even though the difference between both treatments was not significant ($p = 0.0503$). No significant difference was found for the variance size between accessions either with self pollen or mix pollen with the Fligner tests ($p = 0.1699$ and $p = 0.9517$, respectively).

DISCUSSION

Specializing on a certain pollinator guild has been argued to restrict the range distribution of a plant due to limited pollination service outside the habitat of the optimal pollinators. Here, we investigated this hypothesis for *N. attenuata* by measuring flower traits and the ability for self-pollination and outcross pollination in plant populations with full access to one of their major pollinators and in a population at the edge of the habitat of pollinators.

Nicotiana attenuata is a plant species with high phenotypic plasticity of floral traits involved in pollinator attraction (Kessler et al., 2015; Zhou et al., 2017; Guo et al., 2020), and many of its flower characteristics seem to point toward a specialization for hawkmoth pollination. Previous studies have, for example, shown that flowers of *N. attenuata* are synchronized in flower opening, flower movement, and BA emission (Yon et al., 2017a; Haverkamp et al., 2019) with the activity of *M. sexta*. Therefore, the question can be raised if *N. attenuata* accessions varying in the prediction of *M. sexta* occurrence differ in floral traits that are playing a major role in the synchronization with the activity period of the moth and its ability to perceive the flowers.

Abiotic factors such as, for example, soil composition, temperature, water, and light availability can influence plant traits. Here, the accessions were grown under uniform conditions in the greenhouse to focus on genetically fixed differences and to avoid, e.g., herbivory, which has been shown to influence flower phenology (Kessler et al., 2010). Nevertheless, the growing conditions in the greenhouse differ from those in the natural habitat of accessions (e.g., temperature, soil, light). To what extent abiotic factors can alter traits that were measured in our study is not known for *N. attenuata*, and our conclusions were made based on the data collected from plants raised under the same conditions.

The floral volatile compound BA has been shown to be crucial to ensure a successful plant–pollinator interaction between *N. attenuata* and *M. sexta*, i.e., the presence of that compound is not only necessary to enable the moth to remove the nectar from the flowers, but it also increases the female reproduction success of the plant (Kessler et al., 2015; Haverkamp et al., 2016b). Interestingly, BA, which is a species-specific volatile compound of *N. attenuata* (Euler and Baldwin, 1996; Guo et al., 2020), can be perceived by the moth *via* olfactory receptors on its antennae but furthermore through specific chemical receptors on its proboscis (Haverkamp et al., 2016b). Thus, the presence of BA is not only important for long-distance attraction, but also increases flower handling time. Flower handling time directly correlated with the foraging success of the moth and the reproductive output of the plant in experiments were BA emissions had been genetically silenced, but other flower traits were left unaltered (Haverkamp et al., 2016b). Among the four *N. attenuata* accessions, BA emission ranged from high levels in Ut1 to no detectable emission for Ca (**Figure 1E**). Interestingly, Ca originated from a population at above 2,000 masl, where *M. sexta* is not expected to be present as a potential pollinator (**Figure 2A**). The lack of BA in Ca seems to be associated with a lower foraging success (**Supplementary Figure 2**) and is to a certain degree also associated with a

decreased reproductive success of flowers visited by *M. sexta* (**Figures 2D,E**).

During foraging on *N. attenuata* flowers, *M. sexta* moths are hovering in front of the flower and have to navigate their approximately 7.5-cm-long proboscis (Haverkamp et al., 2016a) into the much shorter corolla tube for reaching the nectar at its base (**Figure 1B**). This challenging task might be facilitated by a greater corolla limb area, which offers more surface for the proboscis of the moth to land and, therefore, increases handling effectiveness (Deora et al., 2021). In our study, the flowers of Ut2 have the greatest corolla limb area (**Figure 1A**). Interestingly, Ut2 also had a relatively low flower handling time (**Figure 2C**), while maintaining a relatively high foraging success rate (**Supplementary Figure 2**). This suggests that the moths are able to handle these flowers rather effectively in spite of its low BA emissions (**Figure 1E**). In addition to the corolla limb size, also the corolla tube length affects foraging efficiency in *M. sexta*. Haverkamp et al. (2016a) tested the energy balance of *M. sexta* when foraging on flowers of different *Nicotiana* species varying in corolla tube length. The results of this study revealed a close correlation between the energetic foraging costs and the match between the proboscis length to the flower tube length, with better matching flowers leading to lower energetic costs. Consequently, it can be assumed that *M. sexta* would forage energetically and more efficiently on *N. attenuata* accessions with longer corolla tubes. In our study, flowers of the four accessions varied significantly in corolla tube length (**Figure 1B**) with the shortest flowers being measured for the accession originating from a population outside of the *M. sexta* range prediction (Ca, **Figure 2A**). The longest flowers were measured for Ut2, which does not only originate from a location with excellent prediction of *M. sexta* (**Figure 2A**) but also produced the highest seed set after *M. sexta* visitation in the wind tunnel (**Figure 2E**). In spite of its relatively low BA emissions, the Ut2 accession might therefore be an attractive flower for *M. sexta* due to its morphological properties, which likely facilitate a higher energy gain for the moths during foraging.

After having successfully reached the flower nectar, its quality and quantity are being evaluated by taste receptors on the proboscis and stretch receptors in the gut (Dethier, 1976). The outcome of this assessment will decide whether the moth will learn the particular flower and subsequently visit other flowers of the same type. Hawkmoth pollination has often been linked to high sucrose concentrations in the nectar followed by a preference for fructose but not for glucose (Kelber, 2003). Interestingly, sucrose amounts in the nectar of *N. attenuata* were very low for all accessions in comparison with glucose and fructose (Haverkamp et al., 2018). In spite of these low amounts, we did find a tendency for higher sucrose concentrations in those accessions that occur in the range of *M. sexta* (**Supplementary Figure 1**). Taste neurons on the moth proboscis respond to sucrose concentration as low as $\leq$5 mM, potentially enabling the moths to discriminate between the nectar of the different accessions tested here (Haverkamp et al., in prep.). Baker and Baker (1983) found sucrose contents of about 50% of the total sugars in hawkmoth-pollinated flowers. Nonetheless, later studies do not report such high proportions of sucrose for moth-pollinated flowers (Galetto et al., 1998; Perret et al., 2001). Differences in the perception of the nectar reward could then further impact the way moths learn and remember the flowers of a certain accession (Wright et al., 2009). In contrast to our prediction, the largest volume of nectar was found in the Ca accession (**Supplementary Figure 1**); however, this nectar was also the most diluted (Haverkamp et al., 2018), which might make these flowers less attractive to nectar-robbing carpenter bees, which often damage flowers during foraging (Kessler et al., 2008). Besides sugars, secondary metabolites are often found in floral nectar and have been argued as a mechanism to exclude unwanted flower visitors (van der Kooi et al., 2021). Nicotine is the most important defensive secondary metabolite in *N. attenuata* and is also found in the flower nectar; however, in a previous study, no differences in the nicotine concentration between *N. attenuata* accessions were found (Haverkamp et al., 2018). In addition to this, many more secondary compounds have been detected in the nectar of the Ut1 accession, some of which differentially attracted or repelled hawkmoth and hummingbird pollinators (Kessler and Baldwin, 2007), and it could be speculated that these compounds are also regulated as an adaption to the local pollinator community.

Assuming that *N. attenuata* is specialized on *M. sexta* (Haverkamp et al., 2016b), plant populations at higher elevations, where the nights are colder and the warm periods are shorter, face a dilemma concerning their reproductive strategy since *M. sexta* moths require temperatures not lower than 12.5°C to fly (Heinrich, 1971). Under these circumstances, switching to generalist pollinators available during day time would be a strategy to loosen the dependence on *M. sexta*. Alternative pollinators for *N. attenuata* are day-active bees (e.g., *A. mellifera*, "sweat bees" such as *Lasioglossum* spp.) or hummingbirds. When using transgenic *N. attenuata* lines altered in circadian clock or in BA emission, hummingbirds were identified as pollen vectors (Kessler et al., 2015; Yon et al., 2017b), showing the possibility of *N. attenuata* to switch to these generalist pollinators. Most of these day-active pollinators have been observed to be able to access the flowers as soon as the corolla starts opening. Additionally, *H. lineata*, a generalistic hawkmoth active at dusk, has been shown to pollinate *N. attenuata* flowers, although BA does not seem to be crucial in establishing the interaction with *H. lineata* or hummingbirds (Kessler et al., 2015).

The flowers of the Ca and Ut2 accessions open earlier during the day compared with those of Az and Ut1 (**Figure 1F**), which might allow for an early recruitment of day pollinators. *N. attenuata* flowers already contain nectar before they are fully open and keep producing it throughout the night until 4 a.m. (Kessler, 2012). Although the timing of nectar production seems to be synchronized with *M. sexta* activity period, due to the early nectar production, it is possible that hummingbirds visiting earlier open flowers, such as Ca and Ut2, would be rewarded. Furthermore, Bhattacharya and Baldwin (2012) showed that the stigma is already receptive to pollen before flowers are fully open in the evening. Thus, pollen deposited on the stigma during the day could result in successful fertilization. We observed that hummingbirds and sweat bees can visit flowers shortly after they

start opening; subsequently, it could be assumed that flowers of Ca and Ut2 are accessible already in the morning hours, whereas flowers of Ut1 and Az could be accessed from around 15 h onwards (**Figure 1F**). However, it is unknown if flowers that are less open are pollinated as efficiently as fully open flowers by the different day-active pollinators. At the time when *M. sexta* is active, the flower opening is not different among the accessions (**Figure 1F**); therefore, we would rather expect differences in flower accessibility for day pollinators such as sweat bees and hummingbirds. Whether accessions whose flowers open earlier (Ut2, Ca) really benefit from early visitations during the day remains to be tested.

Plants of the Az accession start earlier to produce flowers and seed capsules and, furthermore, show the highest flower-to-capsule ratio in the absence of pollen vectors (**Figure 3C**). The flowers of Az invest more into seed production in the presence of outcross pollen, resulting in a higher seed set (**Figure 4A**). The same is observed for Ca flowers that show a similar tendency for investing into a higher seed production when outcross pollen is present (**Figure 4A**). This opportunistic behavior might optimize the reproduction cost of the plant by investing more only in the presence of outcross pollen. This could be beneficial, since not every pollinator visit might result in the deposition of outcross pollen but rather in the transfer of pollen from flowers within the same plant (geitonogamy). Especially if outcrossing occurs only occasionally, resources can be saved by investing not all energy in every flower, but rather in those that contribute to a greater genetic diversity of the offspring. Opposite to that, Ut1 and Ut2 seem to always invest in seed production (**Figure 4A**). This strategy would make sense, if an ample abundance of pollinators can be expected that provide the flower with outcross pollen. This might be true for Ut1 and Ut2 since previous studies performing field experiments with *N. attenuata* plants in Utah have identified outcrossing rates of above 30% (Sime and Baldwin, 2003; Kessler et al., 2008, 2012). The pollination service of hummingbirds might be restricted, for example by requirements such as nesting sites, and also most bees forage within a small radius, whereas hawkmoths are assumed to travel over long distances (Kawahara et al., 2013). It could be speculated that bees and hummingbirds might provide mainly pollination service within plants due to visiting multiple flowers on the same plant. Pollinators who visit multiple flowers within one plant might mainly contribute to geitonogamy instead of transferring pollen between conspecific plants (Vaughton and Ramsey, 2010; Sukumaran et al., 2020). In other words, they may be efficient pollinators from a quantity, but not necessarily from a quality perspective. Previous studies have shown that in *N. attenuata*, despite being a self-compatible species, not all pollen donors have the same chance of siring seeds and there are variations in mate selection preferences between accessions (Bhattacharya and Baldwin, 2012; Guo et al., 2019). Given that, some pollinators might transfer pollen loads of high intraspecific diversity and therefore contribute more to a diverse pool of pollen mates available for competition on the stigma. It still remains to be tested to which extent *M. sexta* and the other pollinators really contribute to outcrossing, and not only geitonogamy.

When testing for the capacity for self-pollination, the flowers of Ut1 and Ut2 produce less seeds in the absence of pollen supplementation (**Figure 4B**). One possible explanation for this could be that autogamous selfing flowers are pollen limited. In *Ixiolirion songaricum*, for example, the distance between the anther and stigma has been shown to negatively correlate with autonomous self-pollen deposition on the stigma (Jia and Tan, 2012). Here, Ut2 has a protruding stigma (**Figure 2C**), which may limit the ability to autogamously self-pollinate. An alternative hypothesis for the difference in seed set between autogamous selfing and pollen supplementation could be that flowers of Ut1 and Ut2 only invest in realizing their full reproductive potential when flowers have been visited. For example, flowers of *Heliconia tortuosa* use the capacity of tropical hummingbirds to extract nectar as a cue to turn on their reproduction (Betts et al., 2015).

When using emasculated flowers, subsequently disabling the possibility of flowers to autonomously self-pollinate, Ut2 produces the greatest number of seeds after visitation by a moth carrying pollen on the proboscis (**Figure 2E**). The protruding position of the stigma of Ut2 compared to a location inside the corolla tube (Ut1, Ca, Az; **Figure 1C**) might enhance the possibility of successful pollen deposition by pollinators, particularly with pollinators that do not dive inside the flower. Furthermore, this exposes the stigma to receive outcross pollen instead of self-pollen after the foraging interaction. The fact that flowers of Ut2 open earlier during the day (**Figure 1F**) could increase the chance to receive outcross pollen by both day and night pollinators.

Besides the differences found in floral traits that can indicate certain local adaptations, the variance range of the plant traits can give us information about the degeneration by drift of a particular trait. Higher trait variation will relate to either release from pollinator-mediated selection or a switch to generalist pollinators that do not necessarily impose selective pressure in a particular direction for a plant trait. In our analysis of variance range, we found almost no difference with the exception of the seed output after *M. sexta* interaction. Based on the presented results, we cannot conclude that the floral traits degenerate by drift of pollinator release or multidirectional generalist pollinator pressures.

Spatial (and temporal) variation in plant and pollinator traits as well as abundance can result in a geographic mosaic of coevolution, and previous studies have reported geographic covariation of different flower and pollinator traits (e.g., Thompson and Cunningham, 2002; Anderson and Johnson, 2008; Brown et al., 2011; Gross et al., 2016). In *N. attenuata*, variation in flower angle (Yon et al., 2017b; Haverkamp et al., 2019), flower opening (**Figure 1F**), and the specific volatile compound BA (Haverkamp et al., 2018, **Figure 1E**) might act as a pollinator filter, with the result that certain accessions are more specialized toward *M. sexta* pollination than others as a local adaptation to the pollinator community. Besides BA emission (**Figure 1E**), *M. sexta* foraging success (**Supplementary Figure 2**) and the reproductive success of the plant resulting from *M. sexta* visitation (**Figures 2D,E**) indicate specialization. Despite its interaction with a broad pollinator community and its self-pollination assurance, certain *N. attenuata* accessions (Ut1, Ut2,

and Az) seem to be more specialized by exploiting the sensory abilities of *M. sexta*. Whether a specialization of *N. attenuata* accessions toward *M. sexta* pollination could also entail the possibility of receiving pollen loads of high intraspecific diversity, thus offering the stigma a broader pool of mates to choose from, remains to be tested in further studies.

Taken together, our study highlights how the ability of a plant species to adapt to the sensory bias of the local pollinator community or to resort to self-pollination might be crucial in determining the geographic range of a particular plant species. Gathering more knowledge of population-specific differences in the communication between flowers and pollinators might therefore be of the highest importance for the conservation of both plant and insect species across habitats.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AH, FY, JB, and XL performed the experiments and analyzed the results. JB and FY wrote the first draft of the manuscript. All authors contributed to the revision and experimental design of the study.

FUNDING

The funding was provided by the Max-Planck Society.

ACKNOWLEDGMENTS

We are grateful to Danny Kessler for fruitful discussions. Furthermore, we want to thank Sascha Bucks for his support in maintaining the insect colony and the glasshouse team of the MPICE for maintaining the different plant lines.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.680463/full#supplementary-material

Supplementary Figure 1 | Nectar properties of four *Nicotiana attenuata* accessions. **(A)** Boxplot showing the nectar amount ($p < 0.0001$). **(B)** Boxplot of the amount of glucose in the nectar ($F = 5.9857$, $p = 0.0044$). **(C)** Boxplot of fructose amount in the nectar ($F = 6.4211$, $p = 0.0031$). **(D)** Boxplot showing the amount of sucrose in the nectar ($F = 4.6708$, $p = 0.0139$). Numbers below plot D represent replicate numbers for all nectar property measurements. Boxplots show the median, the 3rd and 4th quartile as well as the interquartile range. All data was analyzed using GLM followed by *post hoc* HSD with Holm adjustment. Letters indicate significant differences ($p < 0.05$).

Supplementary Figure 2 | *Manduca sexta* foraging success on four *N. attenuata* accessions. Plot shows the percentage of flowers on which *M. sexta* foraged successfully. Data was analyzed with GLM with a binomial family logit type for binary data, followed by a pairwise comparison of marginal means. Asterisks indicate statistically significant differences ($p < 0.05$), dot indicates $p < 0.1$.

Supplementary Table 1 | *P*-values after multicomparison of flower morphology and opening, as well as flower and capsule production after *Manduca sexta* visitation and flower contact time. Asterisks indicate statistically significant differences ($* = p<0.05$, $** = p<0.01$, $*** = p<0.001$).

Supplementary Table 2 | *P*-values after multicomparison of phenology data on four *N. attenuata* accessions. Asterisks indicate statistically significant differences ($* = p<0.05$, $** = p<0.01$, $*** = p<0.001$).

REFERENCES

Aizen, M. A. (2003). Down-facing flowers, hummingbirds and rain. *Taxon* 52:675. doi: 10.2307/3647342

Anderson, B., and Johnson, S. D. (2008). The geographical mosaic of coevolution in a plant-pollinator mutualism. *Evolution* 62, 220–225. doi: 10.1111/j.1558-5646.2007.00275.x

Baker, H. G., and Baker, I. (1983). "Floral nectar sugar constituents in relation to pollinator type," in *Handbook of Experimental Pollination Biology*, eds C. E. Jones and R. J. Little (Chicago, IL: University of Chicago Press).

Balamurali, G., Krishna, S., and Somanathan, H. (2015). Senses and signals: evolution of floral signals, pollinator sensory systems and the structure of plant–pollinator interactions. *Curr. Sci.* 108, 1852–1861.

Baldwin, I. T., Preston, C., Euler, M., and Gorham, D. (1997). Patterns and consequences of benzyl acetone floral emissions from *Nicotiana attenuata* plants. *J. Chem. Ecol.* 23, 2327–2343. doi: 10.1023/B:JOEC.0000006677.56380.cd

Betts, M. G., Hadley, A. S., and Kress, W. J. (2015). Pollinator recognition by a keystone tropical plant. *Proc. Natl. Acad. Sci. U.S.A.* 112, 3433–3438. doi: 10.1073/pnas.1419522112

Bhattacharya, S., and Baldwin, I. T. (2012). The post-pollination ethylene burst and the continuation of floral advertisement are harbingers of non-random mate selection in *Nicotiana attenuata*. *Plant J.* 71, 587–601. doi: 10.1111/j.1365-313X.2012.05011.x

Brown, M., Downs, C. T., and Johnson, S. D. (2011). Covariation of flower trait and bird pollinator assamblages among populations of *Kniphofia* linearifolia (Asphodelaceae). *Plant Syst. Evol.* 294, 199–206. doi: 10.1007/s00606-011-0443-1

Carvalheiro, L. G., Biesmeijer, J. C., Benadi, G., Fründ, J., Stang, M., Bartomeus, I., et al. (2014). The potential for indirect effects between co-flowering plants via shared pollinators depends on resource abundance, accessibility and relatedness. *Ecol. Lett.* 17, 1389–1399. doi: 10.1111/ele.12342

Deora, T., Ahmed, M. A., Daniel, T. L., and Brunton, B. W. (2021). Tactile active sensing in an insect plant pollinator. *J. Exp. Biol.* 224, jeb.239442. doi: 10.1242/jeb.239442

Dethier, V. G. (1976). *The Hungry Fly : A Physiological Study of The Behavior Associated With Feeding*. Cambridge, MA: Harvard University Press.

Euler, M., and Baldwin, I. T. (1996). The chemistry of defense and apparency in the corollas of *Nicotiana attenuata*. *Oecologia* 107, 102–112. doi: 10.1007/BF00582240

Galetto, L., Bernardello, G., and Sosa, C. A. (1998). The relationship between floral nectar composition and visitors in *Lycium* (Solanaceae) from Argentina and Chile: what does it reflect? *Flora* 193, 303–314. doi: 10.1016/S0367-2530(17)30851-4

Gómez, J. M., Abdelaziz, M., Camacho, J. P. M., Muñoz-Pajares, A. J., and Perfectti, F. (2009a). Local adaptation and maladaptation to pollinators in a generalist geographic mosaic. *Ecol. Lett.* 12, 672–682. doi: 10.1111/j.1461-0248.2009.01324.x

Gómez, J. M., Perfectti, F., Bosch, J., and Camacho, J. P. M. (2009b). A geographic selection mosaic in a generalized plant – pollinator – herbivore system. *Ecol. Monogr.* 79, 245–263. doi: 10.1890/08-0511.1

Gross, K., Sun, M., and Schiestl, F. P. (2016). Why do floral perfumes become different? Region-specific selection on floral scent in a terrestrial orchid. *PLoS One* 11:e0147975. doi: 10.1371/journal.pone.0147975

Guo, H., Halitschke, R., Wielsch, N., Gase, K., and Baldwin, I. T. (2019). Mate selection in self-compatible wild tobacco results from coordinated variation in homologous self-incompatibility genes. *Curr. Biol.* 29, 2020–2030.e5. doi: 10.1016/j.cub.2019.05.042

Guo, H., Lackus, N. D., Köllner, T. G., Li, R., Bing, J., Wang, Y., et al. (2020). Evolution of a novel and adaptive floral scent in wild tobacco. *Mol. Biol. Evol.* 37, 1090–1099. doi: 10.1093/molbev/msz292

Haverkamp, A., Bing, J., Badeke, E., Hansson, B. S., and Knaden, M. (2016a). Innate olfactory preferences for flowers matching proboscis length ensure optimal energy gain in a hawkmoth. *Nat. Commun.* 7:11644. doi: 10.1038/ncomms11644

Haverkamp, A., Hansson, B. S., Baldwin, I. T., Knaden, M., and Yon, F. (2018). Floral trait variations among wild tobacco populations influence the foraging behavior of hawkmoth pollinators. *Front. Ecol. Evol.* 6:19. doi: 10.3389/fevo.2018.00019

Haverkamp, A., Li, X., Hansson, B. S., Baldwin, I. T., Knaden, M., and Yon, F. (2019). Flower movement balances pollinator needs and pollen protection. *Ecology* 100, 1–11. doi: 10.1002/ecy.2553

Haverkamp, A., Yon, F., Keesey, I. W., Mißbach, C., Koenig, C., Hansson, B. S., et al. (2016b). Hawkmoths evaluate scenting flowers with the tip of their proboscis. *Elife* 5:e15039. doi: 10.7554/eLife.15039.001

Heinrich, B. (1971). Temperature regulation of the sphinx moth, *Manduca sexta*: I. Flight energetics and body temperature during free and tethered fly. *J. Exp. Biol.* 54, 141–151. doi: 10.1242/jeb.54.1.141

Hodges, S. A., Fulton, M., Yang, J. Y., and Whittall, J. B. (2004). Verne grant and evolutionary studies of aquilegia. *New Phytol.* 161, 113–120. doi: 10.1046/j.1469-8137.2003.00950.x

Jia, J., and Tan, D. Y. (2012). Variation in style length and anther–stigma distance in *Ixiolirion songaricum* (Amaryllidaceae). *South Afr. J. Bot.* 81, 19–24. doi: 10.1016/j.sajb.2012.03.011

Kantsa, A., Raguso, R. A., Dyer, A. G., Olesen, J. M., Tscheulin, T., and Petanidou, T. (2018). Disentangling the role of floral sensory stimuli in pollination networks. *Nat. Commun.* 9:1041. doi: 10.1038/s41467-018-03448-w

Kawahara, A. Y., Breinholt, J. W., Ponce, F. V., Haxaire, J., Xiao, L., Lamarre, G. P., et al. (2013). Evolution of *Manduca sexta* hornworms and relatives: biogeographical analysis reveals an ancestral diversification in Central America. *Mol. Phylogenet. Evol.* 68, 381–386. doi: 10.1016/j.ympev.2013.04.017

Kelber, A. (2003). Sugar preferences and feeding strategies in the hawkmoth *Macroglossum stellatarum*. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 189, 661–666. doi: 10.1007/s00359-003-0440-0

Kessler, D. (2012). Context dependency of nectar reward-guided oviposition. *Entomol. Exp. Appl.* 144, 112–122. doi: 10.1111/j.1570-7458.2012.01270.x

Kessler, D., and Baldwin, I. T. (2007). Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of *Nicotiana attenuata*. *Plant J.* 49, 840–854. doi: 10.1111/j.1365-313X.2006.02995.x

Kessler, D., Bhattacharya, S., Diezel, C., Rothe, E., Gase, K., Schöttner, M., et al. (2012). Unpredictability of nectar nicotine promotes outcrossing by hummingbirds in *Nicotiana attenuata*. *Plant J.* 71, 529–538. doi: 10.1111/j.1365-313X.2012.05008.x

Kessler, D., Bing, J., Haverkamp, A., and Baldwin, I. T. (2019). The defensive function of a pollinator−attracting floral volatile. *Funct. Ecol.* 33, 1223–1232. doi: 10.1111/1365-2435.13332

Kessler, D., Diezel, C., and Baldwin, I. T. (2010). Changing pollinators as a means of escaping herbivores. *Curr. Biol.* 20, 237–242. doi: 10.1016/j.cub.2009.11.071

Kessler, D., Gase, K., and Baldwin, I. T. (2008). Field experiments with transformed plants reveal the sense of floral scents. *Science* 321, 1200–1202. doi: 10.1126/science.1160072

Kessler, D., Kallenbach, M., Diezel, C., Rothe, E., Murdock, M., and Baldwin, I. T. (2015). How scent and nectar influence floral antagonists and mutualists. *Elife* 4:e07641. doi: 10.7554/eLife.07641

Koenig, C., Hirsh, A., Bucks, S., Klinner, C., Vogel, H., Shukla, A., et al. (2015). A reference gene set for chemosensory receptor genes of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 66, 51–63. doi: 10.1016/j.ibmb.2015.09.007

Krügel, T., Lim, M., Gase, K., Halitschke, R., and Baldwin, I. T. (2002). *Agrobacterium*-mediated transformation of *Nicotiana attenuata*, a model ecological expression system. *Chemoecology* 12, 177–183. doi: 10.1007/pl00012666

Morgan, M. T., Wilson, W. G., Knight, T. M., Morgan, M. T., Wilson, W. G., and Knight, T. M. (2011). Plant population dynamics, pollinator foraging, and the selection of self-fertilization. *Am. Nat.* 166, 169–183. doi: 10.1086/431317

Perret, M., Chautems, A., Spichiger, R., Peixoto, M., and Savolainen, V. (2001). Nectar sugar composition in relation to pollination syndromes in Sinningieae (Gesneriaceae). *Ann. Bot.* 87, 267–273. doi: 10.1006/anbo.2000.1331

Reisenman, C. E., Riffell, J. A., Bernays, E. A., and Hildebrand, J. G. (2010). Antagonistic effects of floral scent in an insect-plant interaction. *Proc. R. Soc. B Biol. Sci.* 277, 2371–2379. doi: 10.1098/rspb.2010.0163

Riffell, J. A., Abrell, L., and Hildebrand, J. G. (2008). Physical processes and real-time chemical measurement of the insect olfactory environment. *J. Chem. Ecol.* 34, 837–853. doi: 10.1007/s10886-008-9490-7

Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T., et al. (2017). ImageJ2: imageJ for the next generation of scientific image data. *BMC Bioinformatics* 18:529. doi: 10.1186/s12859-017-1934-z

Schäfer, M., Brütting, C., Baldwin, I. T., and Kallenbach, M. (2016). High-throughput quantification of more than 100 primary- and secondary-metabolites, and phytohormones by a single solid-phase extraction based sample preparation with analysis by UHPLC–HESI–MS/MS. *Plant Methods* 12, 1–8. doi: 10.1186/s13007-016-0130-x

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019

Sime, K., and Baldwin, I. T. (2003). Opportunistic out-crossing in *Nicotiana attenuata* (Solanaceae), a predominantly self-fertilizing native tobacco. *BMC Ecol.* 3:6. doi: 10.1186/1472-6785-3-6

Smith, G., Johnson, C., Davidowitz, G., and Bronstein, J. (2017). Linkages between nectaring and oviposition preferences of *Manduca sexta* on two co−blooming *Datura* species in the Sonoran Desert. *Ecol. Entomol.* 43, 85–92. doi: 10.1111/een.12475

Souto-Vilarós, D., Proffit, M., Buatois, B., Rindos, M., Sisol, M., Kuyaiva, T., et al. (2018). Pollination along an elevational gradient mediated both by floral scent and pollinator compatibility in the fig and fig-wasp mutualism. *J. Ecol.* 106, 2256–2273. doi: 10.1111/1365-2745.12995

Sprayberry, J. D. H., and Suver, M. (2011). Hawkmoths' innate flower preferences: a potential selective force on floral biomechanics. *Arthropod. Plant Interact.* 5, 263–268. doi: 10.1007/s11829-011-9150-7

Sukumaran, A., Khanduri, V. P., and Sharma, C. M. (2020). Pollinator-mediated self-pollination and reproductive assurance in an isolated tree of *Magnolia grandiflora* L. *Ecol. Process.* 9:45. doi: 10.1186/s13717-020-00254-5

Thompson, J. N., and Cunningham, B. M. (2002). Geographic structure and dynamics of coevolutionary selection. *Nature* 417, 735–738. doi: 10.1038/nature00810

Thomson, J. N. (2005). *The Geographic Mosaic of Coevolution*. Chicago, IL: University of Chicago Press.

Ushimaru, A., and Hyodo, F. (2005). Why do bilaterally symmetrical flowers orient vertically ? Flower orientation influences pollinator landing behaviour. *Evol. Ecol. Res.* 7, 151–160.

van der Kooi, C. J., Vallejo-Marín, M., and Leonhardt, S. D. (2021). Mutualism and (A)symmetry in plant-pollinator interactions. *Curr. Biol.* 31, R91–R99. doi: 10.1012/j.cub.2020.11.020

van Doorn, W. G., and Van Meeteren, U. (2003). Flower opening and closure: a review. *J. Exp. Bot.* 54, 1801–1812. doi: 10.1093/jxb/erg213

Vaughton, G., and Ramsey, M. (2010). Pollinator-mediated selfing erodes the flexibility of the best-of-both-worlds mating strategy in *Bulbine vagans*. *Funct. Ecol.* 24, 374–382. doi: 10.1111/j.1365-2435.2009.01648.x

Wright, G. A., Choudhary, A. F., and Bentley, M. A. (2009). Reward quality influences the development of learned olfactory biases in honeybees. *Proc. Biol. Sci.* 276, 2597–2604. doi: 10.1098/rspb.2009.0040

Yon, F., Joo, Y., Cortés Llorca, L., Rothe, E., Baldwin, I. T., and Kim, S.-G. (2016). Silencing *Nicotiana attenuata* LHY and ZTL alters circadian rhythms in flowers. *New Phytol.* 209, 1058–1066. doi: 10.1111/nph.13681

Yon, F., Kessler, D., Joo, Y., Cortés Llorca, L., Kim, S. G., and Baldwin, I. T. (2017a). Fitness consequences of altering floral circadian oscillations for *Nicotiana attenuata*. *J. Integr. Plant Biol.* 59, 180–189. doi: 10.1111/jipb.12511

Yon, F., Kessler, D., Joo, Y., Kim, S.-G., and Baldwin, I. T. (2017b). Fitness consequences of a clock pollinator filter in *Nicotiana attenuata* flowers in nature. *J. Integr. Plant Biol.* 59, 805–809. doi: 10.1111/jipb.12579

Zhou, W., Kügler, A., McGale, E., Haverkamp, A., Knaden, M., Guo, H., et al. (2017). Tissue-specific emission of (E)-α-bergamotene helps resolve the dilemma when pollinators are also herbivores. *Curr. Biol.* 27, 1336–1341. doi: 10.1016/j.cub.2017.03.017

Conflict of Interest: XL performed the experiments while working exclusively for the MPI-CE and worked in the manuscript while employed by the Aura Optik GmbH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 20 July 2021
doi: 10.3389/fevo.2021.676743

Floral Volatiles: A Promising Method to Access the Rare Nocturnal and Crepuscular Bees

Carlos A. Martínez-Martínez[1], Guaraci D. Cordeiro[2], Herbeson O. J. Martins[1], Renan O. A. C Kobal[1], Paulo Milet-Pinheiro[3], Mariana A. Stanton[4], Emanuella L. Franco[5], Cristiane Krug[6], Sidnei Mateus[1], Clemens Schlindwein[7], Stefan Dötterl[2] and Isabel Alves-dos-Santos[8]*

[1] Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil, [2] Department of Biosciences, Plant Ecology, Paris-Lodron University of Salzburg, Salzburg, Austria, [3] Laboratório de Ecologia, Universidade de Pernambuco, Campus Petrolina, Petrolina, Brazil, [4] Departamento de Química Fundamental, Instituto de Química, Universidade de São Paulo, São Paulo, Brazil, [5] Universidade Federal do Vale do São Francisco, Colegiado de Ciências da Natureza, Senhor do Bonfim, Brazil, [6] Embrapa Amazônia Ocidental, Empresa Brazileira de Pesquisa Agropecuária, Sete Lagoas, Brazil, [7] Departamento de Botânica, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, [8] Departamento de Ecologia, IBUSP, Universidade de São Paulo, São Paulo, Brazil

OPEN ACCESS

Edited by:
Sara Diana Leonhardt,
Technical University of Munich,
Germany

Reviewed by:
Hannah Burger,
University of Konstanz, Germany
Thomas Eltz,
Ruhr University Bochum, Germany

***Correspondence:**
Guaraci D. Cordeiro
guaradc@gmail.com

Specialty section:
This article was submitted to Chemical Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: 05 March 2021
Accepted: 21 June 2021
Published: 20 July 2021

Citation:
Martínez-Martínez CA, Cordeiro GD, Martins HOJ, Kobal ROAC, Milet-Pinheiro P, Stanton MA, Franco EL, Krug C, Mateus S, Schlindwein C, Dötterl S and Alves-dos-Santos I (2021) Floral Volatiles: A Promising Method to Access the Rare Nocturnal and Crepuscular Bees. Front. Ecol. Evol. 9:676743. doi: 10.3389/fevo.2021.676743

Crepuscular and/or nocturnal bees fly during the dusk, the dawn or part of the night. Due to their short foraging time and sampling bias toward diurnal bees, nocturnal bees are rarely collected and poorly studied. So far, they have been mostly sampled with light and Malaise traps. However, synthetic chemical compounds resembling floral volatiles were recently found to be a promising alternative to attract these bees. By reviewing available literature and collecting original data, we present information on the attraction and sampling of nocturnal bees with scent-baited traps. Bees were actively captured with entomological nets while approaching to filter papers moistened with distinct chemical compound, or passively caught in bottles with scent baits left during the night. So far, all data available are from the Neotropics. Nocturnal bees belonging to three genera, i.e., *Ptiloglossa*, *Megalopta*, and *Megommation* were attracted to at least ten different synthetic compounds and mixtures thereof, identified from bouquets of flowers with nocturnal anthesis. Aromatic compounds, such as 2-phenyletanol, eugenol and methyl salicylate, and the monoterpenoid eucalyptol were the most successful in attracting nocturnal bees. We highlight the effectiveness of olfactory methods to survey crepuscular and nocturnal bees using chemical compounds typically reported as floral scent constituents, and the possibility to record olfactory preferences of each bee species to specific compounds. We suggest to include this method in apifauna surveys in order to improve our current knowledge on the diversity of nocturnal bees in different ecosystems.

Keywords: nocturnal bees inventory, crepuscular bees, apifauna survey, sampling method, floral scents, volatile organic compounds, 2-phenylethanol

INTRODUCTION

The nocturnal and/or crepuscular behavior in bees arose independently in four of the seven bee families: Andrenidae, Apidae, Colletidae, and Halictidae (Wcislo et al., 2004; Warrant, 2007; Danforth et al., 2019). There are about 250 described nocturnal bee species and they fly during the dusk, the dawn or part of the night. These bees can be obligatory nocturnal, such as the giant Indian bee *Xylocopa tranquebarica* (Burgett and Sukumalanand, 2000), or crepuscular, i.e., forage for pollen and nectar at dawn or dusk, such as *Megalopta* and *Ptiloglossa* (Warrant, 2007). Furthermore, under ideal moonlight and cloud-cover conditions, the crepuscular period is extended allowing "crepuscular bees" to search for food also during the night (Kerfoot, 1967; Somanathan et al., 2008; Liporoni et al., 2020). The main anatomical characteristics that indicate nocturnal and/or crepuscular behavior in these bees are the large size of their ocelli and compound eyes, as well as the high number of ommatidia (Kelber et al., 2006; Warrant et al., 2006; Berry et al., 2011), characteristics that improve visual orientation in low light conditions (Wcislo et al., 2004).

Besides visual adaptations for dim light, nocturnal pollinators often heavily depend on floral odors to find their host flowers (Borges et al., 2016). Indeed, nocturnal bees tend to visit and are attracted by flowers releasing a strong perfume at night, so far known to be mainly composed of aromatic (e.g., 2-phenylethanol), aliphatic (e.g., 1-octanol), and terpenoid (e.g., linalool) compounds (Cordeiro et al., 2017, 2019; Krug et al., 2018), all widespread among flower scents (Knudsen et al., 2006). Synthetic compounds that are broadly applied in male orchid bee (Euglossini) surveys were fortuitously found to also attract nocturnal bees (Carvalho et al., 2012; Knoll and Santos, 2012) and, more recently, nocturnal bees were effectively lured with compounds (presented individually or as blends) resembling floral volatiles of some night-blooming host plants of these bees (Cordeiro et al., 2017; Krug et al., 2018). Furthermore, during pollination studies at night, nocturnal bees are recorded on flowers (Hopkins et al., 2000; Somanathan and Borges, 2001; Franco and Gimenes, 2011; Krug et al., 2015; Cordeiro et al., 2017; Soares and Morellato, 2018, Cordeiro et al., 2021).

Nocturnal and/or crepuscular bees (hereafter referred to as nocturnal bees) are usually undersampled, due to their short foraging time and sampling bias toward diurnal bees (Wcislo et al., 2004). As a consequence, representativeness of these bees in insect collections are normally scarce. So far, many of the nocturnal bees collected have been captured with light traps using white light tubes, modified Pennsylvania black light, ultraviolet light (UV), mercury vapor lamps (Chandler, 1961; Wolda and Roubik, 1986) or Malaise traps (Ferrari et al., 2016).

Light traps are efficiently used in the documentation of nocturnal bees, as well as in determining seasonal patterns of other insects (Wolda and Roubik, 1986; Abbas et al., 2019). However, one of their disadvantages is the high cost of batteries or power generators, and lamps with different types of lights being necessary for operating the trap. Likewise, these traps tend to be generalist, attracting various types of insects that are not the object of study, and trapping is strongly affected by abiotic variables such as moon phases and weather (Nowinszky and Puskás, 2017). Furthermore, these traps are fragile and pose danger to the collector, due to UV radiation emitted and toxicity of mercury (Price and Baker, 2016).

Due to the general scarcity of captured specimens of nocturnal bees in insect collections, our current knowledge about their diversity is still underestimated. In this study, we propose a methodological protocol to improve the sampling of nocturnal bees, an approach that might increase information on their diversity and on the olfactory preferences of the different species. In this study, we provide new records and a compilation of literature data on nocturnal bees lured with chemical compounds.

MATERIALS AND METHODS

Nocturnal Bee Sampling

Nocturnal bees were sampled with synthetic chemical compounds from July 2019 to January 2020 in three localities of São Paulo State, southeastern Brazil: Osasco municipality (23°28′ S, 46°46′ W); Neblinas Park (23°44′ S, 46°09′ W); Municipal Reserve Serra do Japi (23°14′ S, 46°58′ W). The method was standardized by offering 1 ml of chemical compounds (or mixtures) added on filter papers (9 cm of diameter) and disposed on tree- or shrub branches at a height between 1.5 and 2 m above the ground. Bees were collected with an entomological handnet while approaching filter papers with chemical compounds (**Figure 1A**), between 04:00 and 6:00 am (before sunrise), and between 05:00 and 07:00 pm (by sunset),

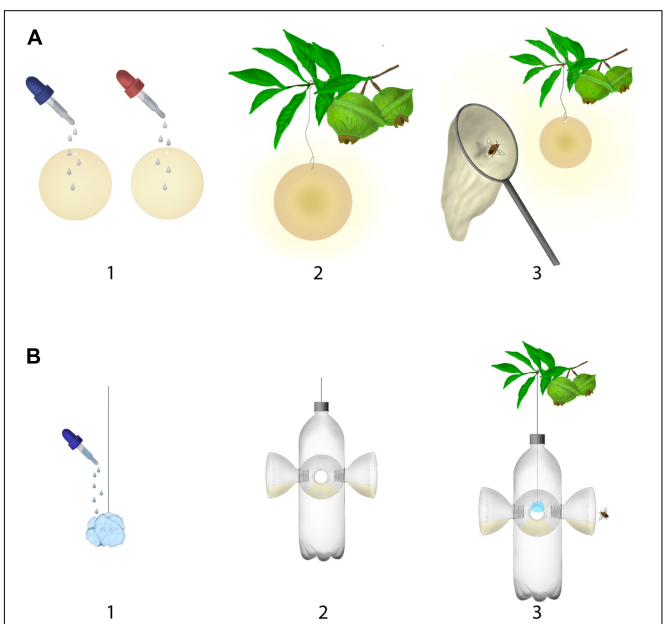

FIGURE 1 | Diagram of the active method **(A)** and passive **(B)** methods used to collect nocturnal bees with chemical compounds. We recommend the application of about 1 ml chemical compounds to filter paper or a cotton ball.

corresponding to the activity time of nocturnal bee species. The bees sampled were transferred to glass vials containing ethyl acetate. We offered compounds resembling floral volatiles previously identified as attractants to nocturnal bees (Cordeiro et al., 2017; Krug et al., 2018), i.e., 1-hexanol (Sigma-Aldrich, ≥99%), 2-phenylethanol (Acros Organics, 99%), 1-octanol (Sigma-Aldrich, ≥99%), and linalool (Sigma-Aldrich, 97%). Our sampling effort summed up to 87 h within 23 days. We are calling this sampling as active method, since the collector remains present during the exposure of the scent and actively collects the approaching bees.

The collected bees were mounted and identified with the taxonomic keys proposed by Moure (1945, 1964), Santos and Silveira (2009),Gonçalves and Santos (2010), Gonzalez et al. (2010) and Santos and Melo (2015), and deposited in the Paulo Nogueira-Neto Entomological Collection at the University of São Paulo (CEPANN), in São Paulo, Brazil.

Literature Review

A systematic review of the literature on nocturnal bees lured with chemical compounds was conducted on Google Scholar, JSTOR, NCBI, Scopus, and Web of science, with the following combinations of keywords: "Compounds" OR "Chemical lures" OR "Floral scent" OR "Floral volatile" OR "Nocturnal bees" OR "Nocturnal and Crepuscular bees" OR "Nocturnal anthesis" OR "Volatile organic compounds." From the selected articles we recovered information about the localities, species, number of individuals attracted, and attractive compounds (**Table 1** and **Supplementary Table 1**). In these studies, the nocturnal bees were mostly sampled with bottles scent traps (similar to those used in Euglossini bees sampling) left during the night. This trap is built with PET bottles, in which a cotton ball impregnated with a synthetic compound is inserted (**Figure 1B**). Chemical compounds used as lures in these previous studies were mostly: eucalyptol, eugenol and methyl salicylate. This sampling protocol is referred hereafter as passive method, since the traps are left in the field without direct supervision and removed in the day after.

RESULTS

In the present and previous studies (literature review), 1115 individuals of 12 species of nocturnal bees were attracted to chemical compounds. The genus with the highest number of individuals and species registered was *Megalopta* (1050 individuals, 8 species) (**Table 1**).

The active sampling method attracted 103 individuals belonging to ten species of nocturnal bees, comprising our sampling (41 individuals, five species) and previous studies (62 individuals, six species). The bees most commonly recorded during the active methods were *Megommation insigne* (33 individuals) and *Megalopta aeneicollis* (25 individuals). Most individuals (39 individuals) were attracted by the aromatic compound 2-phenylethanol (**Table 1**).

The passive method, applied in all previous studies, attracted 1012 individuals, among them *Megalopta amoena* and *M. guimaraesi,* which accounted together for 994 individuals (**Table 1**).

The aromatic compounds eugenol and methyl salicylate trapped 530 individuals from 4 species and 233 individuals from 5 species, respectively (**Table 1**). The only monoterpene tested as single compound, eucalyptol, attracted 152 individuals of three *Megalopta* species. The aliphatic compound 1-octanol, attracted bees of the genera *Megommation* and *Ptiloglossa*. The different mixtures of compounds attracted 57 individuals from seven species, including some unique species, such as *Megalopta cuprea* and *M. piraha* which were only collected with specific mixtures, but not single compounds. Compounds from other chemical classes, such as irregular terpenes and nitrogen-bearing compounds, attracted only few bees (4 individuals) (**Table 1**).

DISCUSSION

The results demonstrate that chemical compounds are appropriate to sample nocturnal bees, and sampling of bees attracted by volatile organic compounds should be incorporated as an additional method to apifauna surveys. It is worthy to offer compounds identified in plants that serve as host for nocturnal bees and use them individually or as blends.

The capture of nocturnal bees has usually been done with black and fluorescent light traps. Chandler (1961) collected 392 individuals of *Sphecodogastra texana* in LaPorte Indiana between 1959 and 1960. However, according to the author there is a possible interference by the killing agent cyanide in the attraction of the bees. Likewise, Wolda and Roubik (1986) collected an astonishing number of individuals of two *Megalopta* species on the island of Barro Colorado, in Panama: 7,713 and 2,487 individuals of *M. ecuadoria* and *M. genalis*, respectively, were sampled. One possible reason that explains the high attraction of these two species of *Megalopta* to light traps on the island was the synchronization with the flowering of the *Tachigalia versicolor* where the light trap was installed (Wolda and Roubik, 1986). The floral volatiles emitted by this plant may have helped attracting the bees to the light trap. Another explanation is that a high abundance of nests of these two species might have been close to the light traps (Roulston, 1997; Wcislo et al., 2004).

Floral synthetic compounds almost exclusively attracted female nocturnal bees (**Table 1**). This differs with the sex of individuals attracted in the Euglossini tribe. Nemésio (2012) mentioned that attracting only males in this tribe has led to taxonomic problems that involve describing species based on male specimens and making it difficult to match males with females. This situation is similar in the nocturnal bees, especially of the genus *Ptiloglossa*, where the taxonomic identification keys were constructed only for male specimens (Moure, 1945). However, the increase in female nocturnal bees in collections, e.g., by using chemical attractants, allows the construction of taxonomic keys that include male and female specimens, as done by Velez-Ruiz (2015).

Our study suggests that chemical compounds sample a higher diversity of species than light traps. Overall 12 species of 3 genera (*Megalopta*, *Megommation*, and *Ptiloglossa*) were

TABLE 1 | Number of individuals of nocturnal bee species lured with synthetic chemical compounds and collected with active and passive methods.

Family/Species	Compound/mixtures (chemical classes)	Active method	Passive method	References
Colletidae				
Ptiloglossa torquata Moure	methyl salicylate (Aro)		1	Almeida et al. (2020)
	vanillin (Aro)		1	Almeida et al. (2020)
Ptiloglossa latecalcarata Moure	Mix 1	10		Cordeiro et al. (2017)
	1-octanol (Ali)	2		Cordeiro et al. (2017)
	2-phenylethanol (Aro)	15		This study
Ptiloglossa pretiosa Friese	2-phenylethanol (Aro)	2♂		This study
	2-phenylethanol (Aro)	1♀		This study
Halictidae				
Megalopta aegis (Vachal)	eugenol (Aro)		1	Knoll and Santos (2012)
	methyl salicylate (Aro)		4	Carvalho et al. (2012)
	eucalyptol (Mon)		2	Knoll and Santos (2012)
	benzyl acetate (Aro)		3	Knoll and Santos (2012)
	benzyl benzoate (Aro)		4	Carvalho et al. (2012)
Megalopta aeneicollis Friese	Mix 2	9		Krug et al. (2018)
	Mix 3	16		Krug et al. (2018)
Megalopta amoena (Spinola)	eugenol (Aro)		238	Almeida et al. (2020); Knoll and Santos (2012)
	methyl salicylate (Aro)		34	Almeida et al. (2020); Carvalho et al. (2012); Knoll and Santos (2012)
	eucalyptol (Mon)		11	Knoll and Santos (2012)
	vanillin (Aro)		13	Knoll and Santos (2012)
	benzyl acetate (Aro)		7	Carvalho et al. (2012); Knoll and Santos (2012)
	benzyl benzoate (Aro)		4	Carvalho et al. (2012)
Megalopta cuprea Friese	Mix 2	1		Krug et al. (2018)
Megalopta guimaraesi Santos and Silveira	benzyl benzoate (Aro)		1	Carvalho et al. (2012)
	methyl salicylate (Aro)		196	Knoll and Santos (2012); Carvalho et al. (2012)
	benzyl acetate (Aro)		12	Carvalho et al. (2012); Knoll and Santos (2012)
	β-ionone (Ite)		1	Carvalho et al. (2012)
	eugenol (Aro)		287	Knoll and Santos (2012)
	eucalyptol (Mon)		139	Knoll and Santos (2012)
	vanillin (Aro)		48	Knoll and Santos (2012)
	skatole (Nbc)		3	Knoll and Santos (2012)
Megalopta piraha Santos and Melo	Mix 3	1		Krug et al. (2018)
Megalopta sodalis (Vachal)	Mix 3	1		Krug et al. (2018)
	eugenol (Aro)		1	Almeida et al. (2020)
	methyl salicylate (Aro)		1	Almeida et al. (2020)
	2-phenylethanol (Aro)	5		This study
	1-octanol (Ali)	2		This study
Megalopta sp. 1	Mix 4	3		Krug et al. (2018)
	Mix 2	2		Krug et al. (2018)
Megommation insigne (Smith)	Mix 1	14		Cordeiro et al. (2017)
	1-octanol (Ali)	3		Cordeiro et al. (2017)
	2-phenylethanol (Aro)	16		This study

All the specimens collected are female, except the two individuals of Ptiloglossa pretiosa.

Chemical classes: *Ali = aliphatic; Aro = aromatic; Mon = monoterpenes; Ite = irregular terpenes; Nbc = nitrogen bearing compounds.*

Mixtures: *Mix 1 (Aro-Ali) = benzyl alcohol (29%), 2-phenylethanol (35%), hexanal (2%), 1-hexanol (13%), (Z)-3-hexen-1-ol (8%), and 1-octanol (13%); Mix 2 (Mon-Aro-Ite-Nbc) = (Z/E)-linalool oxide furanoid (6%), methyl benzoate (19%), linalool (71%), phenylacetonitrile (2%), 4-oxoisophorone (2%), and (Z/E)-linalool oxide pyranoid (0.4%); Mix 3 (Mon-Ses) = (E)-β-ocimene (57%), (Z/E)-linalool oxide furanoid (6%), linalool (30%), and (E)-β-caryophyllene (7%). Mix 4 (Mon-Aro-Ite-Nbc) = (E)-β-ocimene (4%), (Z/E)-linalool oxide furanoid (5%), methyl benzoate (17%), linalool (64%), epoxy-oxoisophorone (5%), phenylacetonitrile (2%), 4-oxoisophorone (2%), and (Z/E)-linalool oxide pyranoid (0.4%).*

collected on chemical lures so far, while the surveys conducted with light traps recorded five species, most of them belonging to the genus *Megalopta* (Chandler, 1961; Kerfoot, 1967; Wolda and Roubik, 1986; Roulston, 1997). Although the sample design is not comparable in terms of time, area, climatic conditions, etc, the superiority in the number of species attracted by the chemical method suggests that this method is more effective in attracting high numbers of species.

The ability of chemical compounds according to the diversity and abundance of attracted nocturnal bees varied among the chemical classes used. However, this may be due to the differences in the design and duration of the sampling carried out by each author in the literary review. Nevertheless, most of the nocturnal bees were attracted to aromatic compounds, such as eugenol, methyl salicylate, 2-phenylethanol, and monoterpenes such as eucalyptol.

The aromatic compounds eugenol and methyl salicylate and the monoterpene eucalyptol, widely used for the attraction of male euglossine bees (Nemésio, 2012), lured 527, 236, and 152 individuals of nocturnal bees, respectively (Carvalho et al., 2012; Knoll and Santos, 2012; Almeida et al., 2020), all from the genus *Megalopta*, with a single exception of one individual of *Ptiloglossa* sampled with methyl salicylate (Almeida et al., 2020). The species most abundantly attracted to the abovementioned three compounds were *M. amoena* and *M. guimaraesi*. Although little is known about the abundance of these compounds in plants with nocturnal anthesis, they are present in a wide variety of plants with diurnal and nocturnal anthesis (Knudsen et al., 2006; El-Sayed, 2021). We believe that the sampling of floral scents in a broader spectrum of plants visited by nocturnal bees might reveal these attractive compounds at least in some representatives.

Another aromatic worth mentioning is 2-phenylethanol. Unlike eugenol, methyl salicylate, and eucalyptol, 2-phenylethanol tends to be less specific and attracts more than one genus and family of nocturnal bees, including rare species such as *P. pretiosa*. Preliminary results also show that this compound is capable of eliciting physiological responses in electroantennography assays (EAG) with *M. insigne* (**Supplementary Figure 1**). This general efficiency of 2-phenylethanol in attracting nocturnal bees may be due to their widespread occurrence among floral scents (Knudsen et al., 2006), sometimes also as major component of bee-pollinated plants (Dobson, 2006), including some with nocturnal anthesis (Shaver et al., 1997; Cordeiro et al., 2017, 2019). This aromatic compound is also a known attractant for diurnal bees (Dötterl and Vereecken, 2010; Rocha-Filho and Garófalo, 2014).

Previous studies demonstrate that not only single compounds but also synthetic mixtures of compounds are capable of attracting nocturnal bee pollinators. The mixtures attracted 41 specimens of nocturnal bees of at least three genera and two families, including some *Megalopta* collected exclusively with these mixtures. Rare species such as *M. cuprea* and *M. piraha* were exclusively attracted to mixtures. Furthermore, unlike the individual compounds, these mixtures have the advantage of resembling the natural aroma emitted by the flowers and attracting potential pollinators (Cordeiro et al., 2017; Krug et al., 2018).

All methods used for apifauna surveys have advantages and disadvantages. The passive method was able to sample a high number of individuals. However, as Euglossini surveys, it can sample hundreds of individuals in one day (Viana et al., 2002; Nemésio and Vasconcelos, 2013), therefore it must be applied with care in fragmented forests with potentially small populations. The active sampling method attracted smaller number of individuals but more species. In addition, in the active sampling, it is possible (for some species) to determine the specimens directly in the field and avoid killing all attracted individuals. Finally, it enables isolating the caught bees in single vials, allowing pollen analyses of each individual. The passive sampling method allows the bottle traps with chemical compound to be left overnight. Thus it is less time consuming than active methods, where collectors spend hours in front of the baits.

To conclude, our study shows that synthetic chemical compounds lure a wide diversity of nocturnal bees. Nocturnal bee species are successfully attracted by aromatic compounds and monoterpenes such as eugenol, methyl salicylate, 2-phenylethanol and eucalyptol. Offering volatile compounds in an active and passive way should be included in nocturnal apifauna surveys, as this approach attracts species otherwise difficult to obtain, and helps clarifying taxonomic issues and the dynamics of their populations of these important pollinators.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of the article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CM-M, HM, IA-d-S, and RK: conceptualization. CM-M, CS, CK, EF, GC, HM, IA-d-S, RK, SD, and SM: investigation, methodology, and carried out collection of data. MS: electroantennographic assay. CM-M, GC, HM, IA-d-S, MS, PM-P, RK, and SD: writing – original draft preparation and review. IA-d-S: Supervision. All authors have read, revised, and agreed the final version of the manuscript.

FUNDING

This research was funded by CNPq (Grant No. PVE 401466/2014-0) to IA-d-S, CS, and SD. CNPq scholarships to HM, IA-d-S, and CS. Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001 accordingto Art. 1°/n° 206 CAPES to CM-M and RK. FAPESP (Grant No. 2014/50316-7) to Massuo Jorge Katon.

ACKNOWLEDGMENTS

We are thankful to the Ronaldo Pereira (Municipal Park Serra do Japi) and Ecofuturo Institute (Neblinas Park) for logistic

support and the permission to work in the biological reserves. We are also grateful to Antônio Rogerio dos Santos for generously allowing collections to be made in his own residence. Likewise, we thank Prof. Massuo Jorge Kato for allowing us to use the infrastructure of the LQPN – Laboratório de Química de Produtos Naturais, University of São Paulo, where the EAG tests were conducted, the Laboratório de Biologia Comparada e Abelhas (LBCA), for the use its facilities for the identification of the collected specimens and Rita Isabel Velez Ruiz for the identification of *Ptiloglossa* species. We thank the Entomological Graduate Program of USP for support. Finally, we are grateful to the two reviewers for their suggestions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.676743/full#supplementary-material

REFERENCES

Abbas, M., Ramzan, M., Hussain, N., Ghaffar, A., Hussain, K., Abbas, S., et al. (2019). Role of light traps in attracting, killing and biodiversity studies of insect pests in Thal. *Pak. J. Agric. Sci.* 32, 684–690.

Almeida, V. M. N., Franco, E. L., Carvalho, M. M., and Pereira, C. V. (2020). "Atração de abelhas crepusculares e diurnas por iscas-odores em duas áreas distintas na Chapada Diamantina-Bahia," *In A Interface Do Conhecimento Sobre Abelhas 2, Org*, eds J. M. Barbosa and L. Batista (Belo Horizonte, Atenas: Editora), 18–33. doi: 10.22533/at.ed.3682001103

Berry, R. P., Wcislo, W. T., and Warrant, E. J. (2011). Ocellar adaptations for dim light vision in a nocturnal bee. *J. Exp. Biol.* 214, 1283–1293. doi: 10.1242/jeb.050427

Borges, R. M., Somanathan, H., and Kelber, A. (2016). Patterns and processes in nocturnal and crepuscular pollination services. *Q. Rev. Biol.* 91, 389–418. doi: 10.1086/689481

Burgett, D. M., and Sukumalanand, P. (2000). Flight activity of *Xylocopa* (*Nyctomelitta*) *tranquebarica*: a night flying carpenter bee (*Hymenoptera*: *apidae*). *J. Apic. Res.* 39, 75–83.

Carvalho, A. T., Maia, A. C. D., Ojima, P. Y., dos Santos, A. A., and Schlindwein, C. (2012). Nocturnal bees are attracted by widespread floral scents. *J. Chem. Ecol.* 38, 315–318. doi: 10.1007/s10886-012-0084-z

Chandler, L. (1961). Light trap collections of the nocturnal bee, *Sphecodogastra texana* (Cresson) (*HymenIoptera*, *Halictidae*). *Proc. Indiana Acad. Sci.* 71, 124–129.

Cordeiro, G. D., dos Santos, I. G. F., da Silva, C. I., Schlindwein, C., Alves-dos-Santos, I., and Dötterl, S. (2019). Nocturnal floral scent profiles of *Myrtaceae* fruit crops. *Phytochemistry* 162, 193–198. doi: 10.1016/j.phytochem.2019.03.011

Cordeiro, G. D., Liporoni, R., Caetano, C. A., Krug, C., Martínez-Martínez, C. A., Martins, H. O. J., et al. (2021). Nocturnal bees as crop pollinators. *Agronomy* 11, 1014.

Cordeiro, G. D., Pinheiro, M., Dötterl, S., and Alves-dos-Santos, I. (2017). Pollination of *Campomanesia phaea* (*Myrtaceae*) by night-active bees: a new nocturnal pollination system mediated by floral scent. *Plant Biol.* 19, 132–139. doi: 10.1111/plb.12520

Danforth, B. N., Minckley, R. L., Neff, J. L., and Fawcett, F. (2019). *The Solitary Bees: Biology, Evolution, Conservation*. Princeton: Princeton University Press.

Dobson, H. E. (2006). "). Relationship between floral fragrance composition and type of pollinator," in *Biology Of Floral Scent*, eds N. Dudareva and E. Pichersky (Boca Raton: CRC Press/Taylor and Francis), 147–198. doi: 10.1201/9781420004007-8

Dötterl, S., and Vereecken, N. J. (2010). The chemical ecology and evolution of bee–flower interactions: a review and perspectives. *Can. J. Zool.* 88, 668–697. doi: 10.1139/z10-031

El-Sayed, A. M. (2021). *The Pherobase: Database of Pheromones and Semiochemicals*. https://www.pherobase.com

Ferrari, R. R., Arantes, R. C., and Silveira, F. A. (2016). A new species of *Mydrosomella* Michener (*Hymenoptera: colletidae*) from the semiarid caatinga domain, northeastern Brazil, with an identification key for the Species in the Genus. *Ann. Zool.* 66, 403–410. doi: 10.3161/00034541anz2016.66.3.006

Franco, E. L., and Gimenes, B. M. (2011). Pollination of *Cambessedesia wurdackii* in Brazilian campo rupestre vegetation, with special reference to crepuscular bees. *J. Insect Sci.* 11, 1–13. doi: 10.1673/031.011.9701

Gonçalves, R. B., and Santos, L. M. (2010). Notes and new species of the halictine genus *Megommation* Moure (*Hymenoptera, Apidae, Augochlorini*). *Zootaxa* 2685, 57–64. doi: 10.11646/zootaxa.2685.1.5

Gonzalez, V. H., Griswold, T., and Ayala, R. (2010). Two new species of nocturnal bees of the genus *Megalopta* (*Hymenoptera: halictidae*) with keys to species. *Rev. Biol. Trop.* 58, 255–263.

Hopkins, M. J. G., Hopkins, H. F., and Sothers, C. A. (2000). Nocturnal pollination of *Parkia velutina* by *Megalopta* bees in Amazonia and its possible significance in the evolution of chiropterophily. *J. Trop. Ecol.* 5, 733–746. doi: 10.1017/s0266467400001681

Kelber, A., Warrant, E. J., Pfaff, M., Wallén, R., Theobald, J. C., Wcislo, W. T., et al. (2006). Light intensity limits foraging activity in nocturnal and crepuscular bees. *Behav. Ecol.* 17, 63–72. doi: 10.1093/beheco/arj001

Kerfoot, W. B. (1967). The lunar periodicity of *Sphecodogastra texana*, a nocturnal bee (*Hymenoptera: halictidae*). *Anim. Behav.* 15, 479–486. doi: 10.1016/0003-3472(67)90047-4

Knoll, F., and Santos, L. M. (2012). Orchid bee baits attracting bees of the genus *Megalopta* (*Hymenoptera*, *Halictidae*) in Bauru region, São Paulo, Brazil: abundance, seasonality, and the importance of odors for dim-light bees. *Rev. Bras. Entomol.* 56, 481–488. doi: 10.1590/s0085-56262012000400013

Knudsen, J. T., Eriksson, R., Gershenzon, J., and Ståhl, B. (2006). Diversity and distribution of floral scent. *Bot. Rev.* 72, 1–120. doi: 10.1663/0006-8101(2006)72[1:dadofs]2.0.co;2

Krug, C., Cordeiro, G. D., Schäffler, I., Silva, C. I., Oliveira, R., Schlindwein, C., et al. (2018). Nocturnal bee pollinators are attracted to *guarana* flowers by their scents. *Front. Plant Sci.* 9:1072. doi: 10.3389/fpls.2018.01072

Krug, C., Garcia, M. V. B., and Gomes, F. B. (2015). A scientific note on new insights in the pollination of guarana *(Paullinia cupana* var. *sorbilis*). *Apidologie* 46, 164–166. doi: 10.1007/s13592-014-0304-3

Liporoni, R., Cordeiro, G. D., Prado, P. I., Schlindwein, C., Warrant, E. J., and Alves-dos-Santos, I. (2020). Light intensity regulates flower visitation in Neotropical nocturnal bees. *Sci. Rep.* 10:15333. doi: 10.1038/s41598-020-72047-x

Moure, J. S. (1945). Contribuição para o conhecimento dos Diphaglossinae, particularmente *Ptiloglossa* (Hym. - *Apoidea*). *Arq. Mus. Paranaense.* 4, 137–178.

Moure, J. S. (1964). As espécies de *Zikanapis*, com a descrição de dois novos subgêneros e duas espécies novas (Hym. *Apoidea*). *Stud. Ent.* 7, 417–458.

Nemésio, A. (2012). Methodological concerns and challenges in ecological studies with orchid bees (*Hymenoptera: Apidae: Euglossina*). *Biosci. J.* 28, 118–135.

Nemésio, A., and Vasconcelos, H. L. (2013). Beta diversity of orchid bees in a tropical biodiversity hotspot. *Biodivers. Conserv.* 22, 1647–1661. doi: 10.1007/s10531-013-0500-x

Nowinszky, L., and Puskás, J. (2017). "Light-trap catch of insects in connection with environmental factors," in *Biological Control of Pest and Vector Insects*, ed. V. D. C. Shields (Croatia, Rijeka: Editorial BoD – Books on Demand), 97–118.

Price, B., and Baker, E. (2016). NightLife: a cheap, robust, LED based light trap for collecting aquatic insects in remote areas. *Biodivers. Data J.* 4:e7648. doi: 10.3897/BDJ.4.e7648

Rocha-Filho, L. C., and Garófalo, C. A. (2014). Phenological patterns and preferences for aromatic compounds by male euglossine bees (*Hymenoptera*, *Apidae*) in two coastal ecosystems of the Brazilian Atlantic Forest. *Neotrop. Entomol.* 43, 9–20. doi: 10.1007/s13744-013-0173-x

Roulston, T. A. H. (1997). Hourly capture of two species of *Megalopta* (*Hymenoptera: Apoidea; Halictidae*) at black lights in Panama with notes on nocturnal foraging by bees. *J. Kans. Entomol. Soc.* 70, 189–196.

Santos, L. M., and Melo, G. A. R. (2015). Updating the taxonomy of the bee genus *Megalopta* (*Hymenoptera*: *Apidae*, *Augochlorini*) including revision of the Brazilian species. *J. Nat. Hist.* 49, 575–674. doi: 10.1080/00222933.2014.946106

Santos, L. M., and Silveira, F. A. (2009). Taxonomic notes on *Megalopta* Smith, 1853 (*Hymenoptera: Halictidae: Augochlorini*) with a synopsis of the species in the state of Minas Gerais, Brazil. *Zootaxa* 2194, 1–20. doi: 10.11646/zootaxa.2194.1.1

Shaver, T. N., Lingren, P. D., and Marshall, H. F. (1997). Nighttime Variation in Volatile Content of Flowers of the Night Blooming Plant *Gaura drummondii*. *J. Chem. Ecol.* 23, 2673–2682. doi: 10.1023/a:1022550607873

Soares, N. C., and Morellato, L. P. C. (2018). Crepuscular pollination and reproductive ecology of *Trembleya laniflora* (*Melastomataceae*), an endemic species in mountain rupestrian grasslands. *Flora* 238, 138–147. doi: 10.1016/j.flora.2016.12.005

Somanathan, H., and Borges, R. M. (2001). Nocturnal Pollination by the Carpenter Bee *Xylocopa tenuiscapa* (*Apidae*) and the Effect of Floral Display on Fruit Set of *Heterophragma quadriloculare* (*Bignoniaceae*) in India 1. *Biotropica* 33, 78–89. doi: 10.1646/0006-3606(2001)033[0078:npbtcb]2.0.co;2

Somanathan, H., Borges, R. M., Warrant, E. J., and Kelber, A. (2008). Visual ecology of Indian carpenter bees I: light intensities and flight activity. *J. Comp. Physiol. A* 194, 97–107. doi: 10.1007/s00359-007-0291-1

Velez-Ruiz, R. I. (2015). *A Revision of the Bee Genus Ptiloglossa Smith (Hymenoptera: Colletidae: Diphaglossinae)* Ph.D. thesis. Brookings SD: South Dakota State University.

Viana, B. F., Kleinert, A. M. P., and Neves, E. L. D. (2002). Comunidade de Euglossini (*Hymenoptera*, *Apidae*) das dunas litorâneas do Abaeté, Salvador, Bahia, Brasil. *Ver. Bras. Entomol.* 46, 539–545. doi: 10.1590/s0085-56262002000400008

Warrant, E. J. (2007). Nocturnal bees. *Curr. Biol.* 17, R991–R992. doi: 10.1016/j.cub.2007.09.009

Warrant, E. J., Kelber, A., Wallén, R., and Wcislo, W. T. (2006). Ocellar optics in nocturnal and diurnal bees and wasps. *Arthropod Struct. Dev.* 35, 293–305. doi: 10.1016/j.asd.2006.08.012

Wcislo, W. T., Arneson, L., Roesch, K., Gonzalez, V., Smith, A., and Fernández, H. (2004). The evolution of nocturnal behaviour in sweat bees, *Megalopta genalis* and *M. ecuadoria* (*Hymenoptera*: *Halictidae*): an escape from competitors and enemies? *Biol. J. Linn. Soc.* 83, 377–373. doi: 10.1111/j.1095-8312.2004.00399.x

Wolda, H., and Roubik, D. W. (1986). Nocturnal bee abundance and seasonal bee activity in a Panamanian forest. *Ecology* 67, 426–433. doi: 10.2307/1938586

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 27 July 2021
doi: 10.3389/fevo.2021.676289

Odour Learning Bees Have Longer Foraging Careers Than Non-learners in a Natural Environment

Lisa J. Evans[1,2*†], *Karen E. Smith*[1] *and Nigel E. Raine*[1,3†]

[1] School of Biological Sciences, Royal Holloway University of London, Egham, United Kingdom, [2] Plant & Food Research Australia, Queensland University of Technology, Brisbane, QLD, Australia, [3] School of Environmental Sciences, University of Guelph, Guelph, ON, Canada

Individual animals allowed the opportunity to learn generally outperform those prevented from learning, yet, within a species the capacity for learning varies markedly. The evolutionary processes that maintain this variation in learning ability are not yet well understood. Several studies demonstrate links between fitness traits and visual learning, but the selection pressures operating on cognitive traits are likely influenced by multiple sensory modalities. In addition to vision, most animals will use a combination of hearing, olfaction (smell), gustation (taste), and touch to gain information about their environment. Some animals demonstrate individual preference for, or enhanced learning performance using certain senses in relation to particular aspects of their behaviour (e.g., foraging), whereas conspecific individuals may show different preferences. By assessing fitness traits in relation to different sensory modalities we will strengthen our understanding of factors driving observed variation in learning ability. We assessed the relationship between the olfactory learning ability of bumble bees (*Bombus terrestris*) and their foraging performance in their natural environment. We found that bees which failed to learn this odour-reward association had shorter foraging careers; foraging for fewer days and thus provisioning their colonies with fewer resources. This was not due to a reduced propensity to forage, but may have been due to a reduced ability to return to their colony. When comparing among only individuals that did learn, we found that the rate at which floral resources were collected was similar, regardless of how they performed in the olfactory learning task. Our results demonstrate that an ability to learn olfactory cues can have a positive impact of the foraging performance of *B. terrestris* in a natural environment, but echo findings of earlier studies on visual learning, which suggest that enhanced learning is not necessarily beneficial for bee foragers provisioning their colony.

Keywords: bumblebee behavior, cognitive ecology, olfaction, pollinator behaviour, resource collection, social insects

OPEN ACCESS

Edited by:
Johannes Spaethe, Julius Maximilian University of Würzburg, Germany

Reviewed by:
Fabio Santos Nascimento, University of São Paulo Ribeirão Preto, Brazil
Amelie Cabirol, University of Trento, Italy

***Correspondence:**
Lisa J. Evans
lisa.evans@plantandfood.co.nz

†ORCID:
Lisa J. Evans
orcid.org/0000-0001-7353-9750
Nigel E. Raine
orcid.org/0000-0001-6343-2829

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *05 March 2021*
Accepted: *25 June 2021*
Published: *27 July 2021*

Citation:
Evans LJ, Smith KE and Raine NE (2021) Odour Learning Bees Have Longer Foraging Careers Than Non-learners in a Natural Environment. Front. Ecol. Evol. 9:676289. doi: 10.3389/fevo.2021.676289

INTRODUCTION

An animal's capacity for learning can influence essentially every aspect of its behaviour, including its ability to find food, attract mates, and avoid predators (Nowicki et al., 2002; Lönnstedt et al., 2012; Sergio et al., 2014). Individuals given the opportunity to learn associations between sensory cues and risk/reward outcomes generally outperform those prevented from learning (e.g., higher mating success, growth, reproductive output; Dukas and Bernays, 2000; Dukas and Duan, 2000;

Lönnstedt et al., 2012; Ward-Fear et al., 2016). Ultimately, learning enables individuals to respond to environmental change within their lifetime (Greenlees et al., 2010; Ward-Fear et al., 2016).

Despite these apparent advantages of learning, considerable variation in learning capacity can often be observed among individuals within the same species (e.g., Chittka et al., 2003; Raine et al., 2006b; van den Berg et al., 2011; White and Brown, 2014). This intraspecific variation exists because being a "good learner" does not always provide an overall fitness benefit. Cognitive function has a metabolic cost (Foley and Lee, 1991) and inherently fast-learning individuals may face trade-offs between learning and other cognitive functions (Hermer et al., 2018) or other energetically demanding processes including longevity, immune function and reproduction (Dukas, 1999; Mallon et al., 2003; Mery and Kawecki, 2003, 2004; Burger et al., 2008; Burns et al., 2011; Jaumann et al., 2013). Learning can also have an opportunity cost; the time taken for a foraging animal to learn its preferred food source and the subsequent commitment to the learned food source can mean it forgoes exploitation of other resources (Eliassen et al., 2007; Evans and Raine, 2014). While it is clear that these trade-offs can affect the learning abilities of individuals, the evolutionary processes that maintain this variation in learning ability within natural populations are not yet well understood (Raine et al., 2006a; Morand-Ferron and Quinn, 2015; Morand-Ferron, 2017; Boogert et al., 2018).

Links between learning performance and fitness traits under natural conditions have, so far, only been investigated in few species and all these studies focus on visual learning (Raine and Chittka, 2008; Evans et al., 2017; Huebner et al., 2018; Madden et al., 2018). The selection pressures operating on cognitive traits are likely influenced by multiple sensory modalities. In addition to visual cues, most animals will use a combination of sound, taste, touch, and/or smell when forming learnt associations (Dukas, 2008; De Agrò et al., 2020; Flanigan et al., 2021). The relative importance of different sensory modalities can sometimes be obvious with regards to species ecology; depending on whether the animal is active at day or night, either vision or olfaction are often a more prominent modality than the other (Balkenius et al., 2006). But the salience of different modalities can also depend on context and/or environment (Maaswinkel and Whishaw, 1999; Andersson and Dobson, 2003; Kaczorowski et al., 2012), and reliance on a particular cue can adaptively shift depending on environmental conditions (Spaethe et al., 2001; Kaczorowski et al., 2012). To add to this complexity, individual animals can favour different sensory cues than their conspecifics (Smith et al., 2004; Raine and Chittka, 2007; Sato et al., 2014), and can also exhibit better learning performance when using a particular sensory modality (Kunze and Gumbert, 2001; Smith and Raine, 2014). The relationship between learning ability and fitness may therefore differ depending on the sensory modality used to assess learning. By assessing fitness traits in relation to different sensory modalities we will strengthen our understanding of factors driving observed differences in learning ability.

Bumble bees are a useful study system for investigating fitness traits and learning though different sensory modalities, because foragers rely on multiple sensory inputs, which serve different functions. For instance, bumble bees rely on both learnt visual and olfactory cues when locating and evaluating their food sources (Chittka and Raine, 2006). Attraction to flowers at a distance is primarily due to the visual cues of the flowers (Manning, 1956; Heinrich, 1976), whereas floral scents provide a localised cue which a bee uses to discriminate between similar flowers and to reject flowers recently depleted of nectar (Manning, 1956; Wright and Schiestl, 2009). The presence of a learned floral scent determines whether a foraging bee alights and/or probes for nectar (Manning, 1956; Kunze and Gumbert, 2001). Foragers use olfactory and tactile cues to communicate with each other, both directly and indirectly (Dornhaus and Chittka, 2001; Saleh et al., 2006). For example, bees in the nest learn the floral scents carried by incoming foragers, which can influence their subsequent foraging choices (Molet et al., 2009). Foragers can also learn to use the scents produced and deposited on flowers by other bees (cuticular hydrocarbon footprints), to avoid recently visited flowers (Goulson et al., 1998; Stout and Goulson, 2001; Pearce et al., 2017).

Using proboscis extension response (PER) conditioning we assessed the olfactory learning performance of foraging naïve *Bombus terrestris* individuals in the lab, then monitored their subsequent foraging performance in a natural environment. In doing so, we gained insight into how odour learning affects foraging success and colony provisioning (both proxy measures of colony fitness). We discuss our results in relation to visual learning in *B. terrestris*, which has previously been assessed in conjunction with foraging performance (see: Raine and Chittka, 2008; Evans et al., 2017).

MATERIALS AND METHODS

Experimental Setup

Five *B. terrestris* colonies (obtained from Biobest—Westerlo, Belgium) were each housed in split colony boxes (**Figure 1A**), which enabled us to assess the olfactory learning performance of foraging naïve bees in the lab and subsequently monitor the foraging performance of the same individuals in a natural environment. Each box was divided in half with mesh (mesh size: 1 × 1 mm), allowing olfactory and/or tactile connections to be maintained between bees and brood on either side of the colony box. One side of the box (internal side) was connected to an enclosed foraging arena (140 × 240 × 120 mm) containing a gravity feeder of sucrose solution (50% v/v) provided *ad libitum,* and 3 g per day of defrosted honey bee-collected pollen (sourced from Koppert Ltd., United Kingdom). The other (external) side was connected to the outside environment through a tube leading to an exit/entrance hole in the laboratory window (**Figures 1D,E**), allowing bees on this side to forage naturally (**Figure 1F**).

At the beginning of the trial, each colony had a queen, brood and an average of 30 workers (range = 23–37), which were divided evenly between the two sides of the colony box. Each queen was moved between sides of the colony box every 24 h to encourage normal queen-right colony behaviour and reduce aggression when tested worker bees were moved between sides

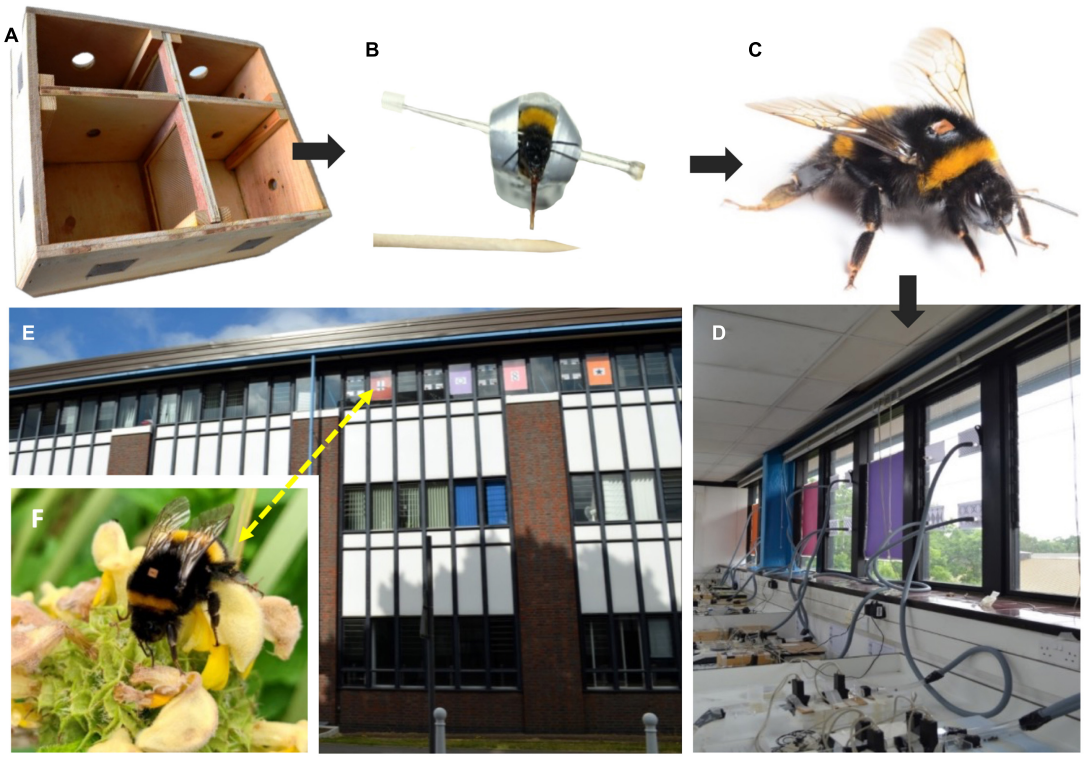

FIGURE 1 | | Diagram showing experimental setup. **(A)** Divided colony box with a mesh partition, which contained the split colonies (colourless, transparent lid removed for clarity). **(B)** Bees identified as foragers, based on frequency of foraging in an indoor foraging arena, were harnessed and tested on their olfactory learning ability using proboscis extension response (PER) conditioning paradigm. **(C)** Assessed individuals were tagged with an RFID tag to enable us to track their subsequent foraging performance. **(D)** After being assessed the bees were transferred to the (external) side of their divided colony box that was connected to the laboratory window. **(E)** Each window exit/entrance was marked with a unique pattern to assist retuning foragers in finding their colony. **(F)** The RFID reader system allowed us to record the foraging behaviour of the bees in the rural/urban landscape surrounding Royal Holloway University of London (Egham Hill, Egham TW20 0EX, United Kingdom). The RFID tag on the bee in image **(F)** has been added digitally for illustrative purposes. Photos **(B,C)** provided by Dylan Smith and Brian Cutting, respectively.

(Schmid-Hempel and Schmid-Hempel, 1998; Evans et al., 2017). For the purpose of identification, newly emerged workers on the internal side were marked daily with uniquely identifiable numbered tags (Opalith tags; Christian Graze KG, Germany). Foraging individuals were identified in the foraging arena, assessed in an olfactory learning paradigm (described below), then re-tagged with an Radio Frequency Identification (RFID) tag (details below; **Figure 1C**) and transferred to the external side of the colony box. Workers emerging in the external side had one of their wings clipped to prevent them from flying and foraging, thus ensuring that only our tested bees (from the internal side) could forage outside. To encourage foraging in our initially small colonies, the external side was provided with no pollen, and only enough sucrose solution to fill three nectar pots—pipetted directly into nectar pots (for a more detailed description of the setup see: Evans et al., 2017).

Assessing Olfactory Learning Performance of Foragers

The five foraging arenas (each connected to the internal side of a different split colony box) were visually checked throughout the day and the identities of any bees on the sucrose or pollen feeders were recorded. Bees were defined as foragers if they had been observed on a feeder on at least three separate occasions, across multiple days. Bees that met these criteria (n = 93 across the five colonies) were assessed on their olfactory learning performance.

Olfactory learning performance was assessed using a PER absolute conditioning paradigm (Riveros and Gronenberg, 2009; Evans et al., 2016). Identified foragers were caught and chilled on ice until they became quiescent and then harnessed within plastic syringe tubes (**Figure 1B**). The bees were fed with sucrose solution (50% v/v) 3 h after being harnessed. When feeding, their antennae were touched with a pipette containing sucrose. If the bee responded by extending its proboscis it was presented with sucrose solution for 2 s. Each bee was given four opportunities to feed. The harnessed bees were left overnight in a ventilated container. The following morning (*ca.* 18 h after harnessing) we again checked their responsiveness to sucrose. If the bee responded it was given a small droplet of sucrose and progressed to the training phase. If a bee failed to respond after four attempts it was removed from the trial (n = 7/93; 7.5%).

A bee was assessed on its ability to learn to associate a fruit odour—lemon (essential oil, Calmer solutions), with a sucrose

reward. Lemon was chosen as our conditioning odour as it is unlikely that these bees would encounter this odour while foraging outside after their learning assessment (as this could potentially influence their foraging decisions). Prior to every training event each harnessed bee was placed individually in an odour extraction hood. The odour stimulus was released from an odour tube, containing 1 μl of the essential oil on filter paper. The volume of air, flow rate, and duration of the odour presentation was controlled by a Programmable Logic Controller computer. During each trial a bee was exposed to 5 s of unscented air then 10 s of odour-containing air. The bee was presented with 0.8 μl of sucrose solution (using a Gilmont syringe) after approximately 6 s of exposure to the odour-containing air (Evans et al., 2016).

Each bee was subjected to 15 trials in which it was exposed to the odour, with a 12 min interval between trials. After each trial we recorded the bees' response; whether it extended its proboscis before being presented with reward (conditioned response or learning event), after it was presented with reward (unconditioned response or non-learning event), or not at all (unresponsive). Bees that did not respond for three consecutive trials were removed from the experiment as they were assumed to be senescing or otherwise no longer responding to foraging cues (n = 6/86; 7%). On completion of the 15 trials all the bees were removed from their harnesses and tagged with an RFID tag (Microsensys GmbH: mic3-Tag; **Figure 1C**), on the back of their thorax over the top of their Opalith tag. Each bee was then placed within the external side of its corresponding colony so that their foraging could be monitored outside the laboratory in a natural environment.

Foraging Performance in a Natural Environment

A pair of RFID readers (Microsensys GmbH: Maja IV reader modules with optimized antenna for mic3-Tag transponders) were attached to the entrance of each colony which recorded when our previously tested and RFID-tagged foragers left and re-entered their nest. This experimental set up yielded data on individual foraging trip frequency and duration. Each colony was also observed for 3 h a day between: 09:00 and 12:00, 12:00 and 15:00 or 15:00 and 18:00, for the duration of the trial (5 days a week for 4 weeks), to estimate the nectar and pollen loads collected by our tagged bees. To control for differences in forager activity levels over the course of a day, the observation period was randomised across colonies. We recorded the mass of any RFID-tagged bee leaving or entering their colony, as they walked over a balance pan (Ohaus Adventurer^{TM} Pro, Ohaus Navigator^{TM}, and Sartorius Practum 213-IS x 2 all accurate to the nearest 0.001 g). Using the balance's dynamic weighing function (designed for weighing moving animals), three mass recordings were taken for each bee and the average of these values used in the analysis. A stopwatch synchronized with the time on the RFID readers was used to record the time of each bee observation, enabling us to identify individuals. The size of pollen loads brought back to the colony were non-invasively estimated, so as not to disrupt normal foraging activity. Each pollen load was classified as being either small, medium, large, or very large, relative to the size of the bee (Gill et al., 2012; Gill and Raine, 2014; Evans et al., 2017).

Analysis

Learning Performance

Learning performance scores were generated for bees tested with the PER paradigm by summing each individual's responses across their 15 conditioning trials. Each correct response (i.e., when a bee extended their proboscis in response to the odour prior to being offered sucrose solution) was given a score of 1, so a learning score of 14 is the maximum a bee could obtain given that no learnt association could have been formed before the first trial. The learning scores were split into four categories: A = 0 (non-learners), B = 1–5, C = 6–10, and D = 11–14 (fastest learners). Learning performance scores were not normally distributed and non-parametric tests were used to assess differences within and among colonies.

Individual Foraging Performance in a Natural Environment

Foraging performance was quantified using the RFID data log of when each tagged bee left and re-entered a colony. These data were manually sorted to determine the number, duration, and timing of the foraging trips made by each bee. As we only tagged foragers, we assumed that all trips away from the nest were foraging bouts (trips), provided that the bee was gone for $\geq$ 8 min and, once they returned, they stayed in the nest (to off-load pollen and/or nectar) for $\geq$ 1 min. These thresholds were based on the duration and sequence of activity of visually confirmed foragers during the observation periods (Evans et al., 2017). Bees were only included in our analyses if they completed at least five foraging bouts.

For each forager we determined the colony that it foraged for the most frequently—their "majority colony," because all foragers visited multiple colonies (mean $\pm$ SE = 4.09 $\pm$ 0.17 colonies). This drifting is typical for closely situated bumble bee colonies (Zanette et al., 2014), and is comparable to the extent of drifting observed by others using a similar setup to assess foraging activity (Gill et al., 2012; Gill and Raine, 2014; Stanley et al., 2016). For 50% of foragers their majority colony was also their natal colony. On average, foragers performed 57.73 $\pm$ 3.92% of their foraging trips for their majority colony, compared to 39.90 $\pm$ 5.39% for their natal colony. For this reason "majority colony" was used in all subsequent analysis.

Foraging Efficiency

All formal analyses were conducted in R v 3.0.2 (R Core Development Team., 2014). Using a series of general linear mixed models (GLMM's, using the lme function in the package nlme: Pinheiro et al., 2014), we determined whether learning ability predicts *nectar collection efficiency* and/or *pollen collection efficiency*. Our basic model contained just majority colony as a random effect. This was compared with four different candidate models that contained the basic model and one of the following possible covariates as a fixed effect: worker size, worker age, foraging experience, and the age of colony when the forager was introduced (see **Table 1** for variable descriptions). We calculated AICc values (Akaike Information Criterion—corrected for small sample size) for each model (selMod function from the pgirmess package: Giraudoux, 2014)

TABLE 1 | Descriptions of the response and predictive variables used in analyses.

Variable used in model(s)	Variable description
Nectar collection efficiency	Estimated amount nectar collected (mg/hr). Calculated by subtracting the mean outgoing mass from the mean incoming mass, the difference was divided by the average time taken to complete observed trips (based on RFID data). Calculated for each bee returning to the colony without pollen.
Pollen collection efficiency	A measure of pollen collected (pollen load/hr). Calculated by assigning a numerical value for pollen load size, i.e., small pollen = 1, medium = 2, large = 3, very large = 4. For each bee, pollen load size was averaged across all bouts in which pollen was collected and divided by the average time taken to complete observed trips (based on RFID data).
Mean daily number of bouts	A measure of the number of foraging trips completed each day. Calculated for each bee by dividing the total number of foraging trips completed (based on RFID data) by the total number of days foraged.
Mean bout duration	A measure of time spent away from colony foraging (mins). Calculated for each bee by dividing the total time spent foraging (based on RFID data) by the number of foraging trips recorded.
Number of days spent foraging	A measure of foraging lifespan. Calculated for each bee by counting the number of days on which the bee foraged (based on RFID data).
Majority colony	The colony for which each bee completed the majority of its foraging trips.
Colony age	The number of days since the colony arrived in the lab at the time each bee was assessed.
Worker size	A mean of all body mass recordings obtained for each bee when they left their colony to forage. Body mass was measured using the dynamic weighing function on a balance. Bees that were not observed when exiting the colony (usually bees that completed very few foraging bouts) were assigned a value based on the mean bee mass for their natal colony (n = 11).
Worker age	Age of worker when odour learning performance was tested. Determined by the number of days since emergence, or if the bee was already present when the colony arrived in the lab (n = 10), its age was estimated by adding 5 days to the colony arrival date.
Foraging experience	The mean number foraging trips completed by a bee prior to (and including) the foraging trip recorded by an observer. For example, if a bee's pollen/nectar load was recorded by an observer on its 5th, 22nd, 35th, and 40th foraging trips, these were averaged to give an experience score of 25.5 (i.e., 5 + 22 + 35 + 40 = 102/4)

and selected the model with the lowest AICc value. Olfactory learning performance was added (as a factor) to the best model to determine whether it significantly lowered (i.e., ΔAICc > 2 units: Burnham et al., 2011) the model's AICc. If it did, we concluded that learning performance was predicting the response variable. This bottom-up model building approach is more conservative than a stepwise deletion, but given our limited sample size it is more appropriate as it avoids over-parameterization inherent in small data sets (Raihani and Bshary, 2012). The fit of the best model was checked by plotting the fitted values against the residual values of the model.

Foraging Activity

Mean daily number of bouts and *mean bout duration* were $\log_{10}$ transformed (to normalise residuals) and analysed with a general mixed model as described above. A generalised linear mixed model (using the glmer function in package lme4: Bates et al., 2014) was used to analyse count data (assumed to have a Poisson error distribution) for *number of days spent foraging*. A basic model was generated and then compared with three additional models that contained either: worker size, worker age, and majority colony age, in addition to the basic model. Learning performance was added to the model with the lowest AICc. The fit of the best model was checked by plotting the fitted values against the residual values of the model.

RESULTS

Learning Performance

We assessed the olfactory learning performance of 80 foragers (mean = 16, range = 13–19 foragers per natal colony) across five colonies. Seventy-five percent of the bees (n = 60) exhibited at least one learnt response. The proportion of correct choices increased with trial number across all five colonies; rising from 0 to 8% in trial 2 to 6–46% in trial 7, and finally 50–77% in trial 15 (**Figure 2A**). Whilst learning performance varied within colonies, there was no significant variation in learning performance among colonies (Kruskal-Wallis, H^4 = 6.064, p = 0.19; **Figure 2B**). Forager learning performance was not predictably affected by factors such as worker age (Spearman's $\rho = -0.021$, p = 0.92; **Supplementary Figure 1A**), worker body mass (Spearman's $\rho = -0.145$, p = 0.46; **Supplementary Figure 1B**) or colony age (Spearman's ρ = 0.037, p = 0.53; **Supplementary Figure 1C**).

Individual Foraging Efficiency in a Natural Environment

Individual patterns of foraging activity were recorded when each RFID-tagged bee left and returned to their nest. We found that 48.8% (n = 39/80) of our tagged bees completed at least five foraging bouts (flights outside the colony lasting at least 8 min). During daily observations, we recorded the efficiency of pollen/nectar collection in 10.27% of the foraging bouts undertaken by 84.6% (n = 33/39) of the tagged foragers. The number of foraging bouts for which pollen/nectar collection was observed per bee ranged between 2 and 22, and was directly proportional to the total number of foraging bouts undertaken by each bee (Spearman's ρ = 0.73, n = 33, $p < 0.001$).

Twenty two tagged bees were further classified as nectar (n = 15) and/or pollen (n = 14) foragers, based on having recorded at least two nectar or pollen-collecting bouts (range = 2–17) during our observations. Thirty two percent (n = 7/22) of these bees foraged for both nectar and pollen, in separate trips. The nectar and pollen collection rates of these bees were positively

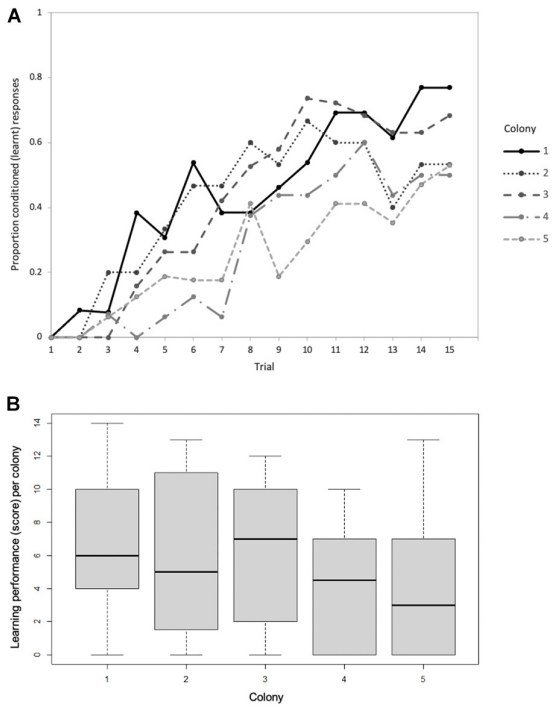

FIGURE 2 | (A) Proportion of conditioned (learnt) responses of *Bombus terrestris* individuals from five (natal) colonies during 15 sequential proboscis extension response (PER) trials. **(B)** Variation in learning performance (learning score) of the five natal colonies based on the number of learnt responses per bee. In each box the thick horizontal bar is the colony median, whilst the lower and upper edges represent the 25 and 75% quartiles, respectively. Whiskers indicate the maximum and minimum values that are not outliers. The numbers of bees tested per colony = 13, 15, 19, 16, and 17, respectively.

TABLE 2 | Candidate models to predict the nectar and pollen collection efficiency.

	Nectar collection		Pollen collection	
	AICc	Δ AICc	AICc	Δ AICc
Basic	**162.62***	**0.00**	**49.59***	**0.57**
Best model + Learning score	162.89	0.27	49.02	0.00
Experience	165.96	3.33	52.44	3.43
Colony age	166.08	3.46	53.38	4.36
Worker mass	166.26	3.64	53.63	4.61
Worker age	166.32	3.69	53.52	4.50

The basic model contained only the intercept and majority colony as a random factor. All other models contained the basic model and the additional factors specified in the model name (Experience, Colony age, Worker mass or Worker age). The model with the lowest AICc value out of the five initial models (indicated with an asterisk) had learning score (LPI) added to it to determine whether this significantly decreased the AICc value (i.e., ΔAICc > 2). The best model (based on the AICc value) is shown in bold. The basic model is considered the best if no model has a significantly lower AICc (i.e., decreased ΔAICc > 2 units).

TABLE 3 | Candidate models to predict the mean number of foraging bouts conducted per day, mean foraging bout duration, and number of days spent foraging by tested foragers.

	Mean bouts per day		Mean bout duration		No. of days foraged	
	AICc	Δ AICc	AICc	Δ AICc	AICc	Δ AICc
Basic	81.58	24.88	54.21	11.00	224.41	1.63
Worker age	83.39	26.59	55.64	12.43	226.54	3.76
Worker mass	83.99	27.29	56.70	13.49	225.48	2.70
Colony age	**56.70***	**0.00**	**43.21***	**0.00**	**222.78***	**0.00**
Best model + Learning score	58.47	1.76	45.23	2.01	223.34	0.56

The basic model contained only the intercept and majority colony as a random factor. All other models contained the basic model and the additional factors specified in the model name (worker age, worker mass or colony age). The model with the lowest AICc value out of the four initial models (indicated with an asterisk) had learning score (LPI) added to it to determine whether this significantly reduced the AICc value (i.e., decreased Δ AICc > 2). The best models (based on the AICc value) are shown in bold.

correlated (Pearson's Correlation, $r = 0.81$, $n = 7$, $p = 0.03$), but this relationship was driven by a single bee that collected both floral resources at a high rate (**Supplementary Figure 2**).

Our best model provides no strong evidence that olfactory learning performance predicts *nectar collection efficiency* or *pollen collection efficiency* (**Table 2**). For nectar collection, the best model was the basic model (which represents the null hypothesis). For pollen collection, although the best model did include learning score, the AICc score of this model was not significantly (>2 AIC units) lower than that of the basic model (ΔAICc 0.57; **Table 2**; raw data presented in **Supplementary Figure 3** for nectar and pollen collection).

Individual Foraging Activity in a Natural Environment

When comparing all bees, a binomial test indicates that there was no difference in the proportion of non-learners (0.50) and learners (0.55) to forage ($p = 0.90$, 95% CI [−0.34, 0.24]). Assessed bees foraged for between 1 and 15 days (mean ± [SE] = 5.64 ± 0.58), completing between 1 and 26 foraging bouts per day (mean ± [SE] = 10.37 ± 0.98), with each bout lasting between 28.67 and 184 min (mean ± [SE] = 67.46 ± 5.57). Once foraging outside of the laboratory, 97% of the bees continued foraging for consecutive days, with the exception of one bee that had two, 1-day breaks during its 15 days of foraging.

Comparing candidate models, learning performance was also not a good predictor of *mean number of bouts per day*, *mean bout duration*, or *number of foraging days* (**Table 3**; raw data presented in **Supplementary Figure 4**). However, visual inspection of the raw data suggested that learning *per se* did appear to affect *Number of foraging days* as bees that showed some learning (learning scores 1–5: mean days foraging = 7.08 ± 0.98 [SE]; learning scores 6–10: mean days foraging = 6.22 ± 0.83 [SE]) foraged for more days than non-learning individuals (mean days foraging = 3.91 ± 1.19 [SE]) **Figure 3A**). Accordingly, when bees were included in the models as non-learners or learners (i.e., a binomial category), learning ability was a good predictor of days foraged. The best model, which contained majority colony, age and learning (model estimate for non-learners: 8.86 ± 4.71[SE] and for learners: 14.66 ± 3.72[SE]) was a significant improvement

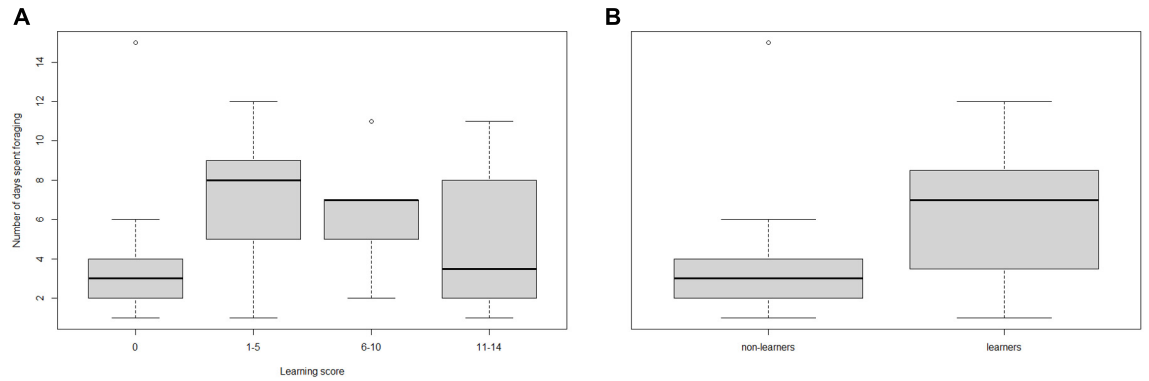

FIGURE 3 | Box and whisker plot showing **(A)** the number of days spent foraging against olfactory learning performance index score, and **(B)** the number of days spent foraging by non-learners vs. learners (raw data). High learning scores are indicative of fast learning individuals, whilst a score of zero means they showed no sign of learning. The number of bees in each learning category: 0 = 11, 1–5 = 13, 6–10 = 9, and 11–14 = 6. In each box the thick horizontal line indicates the median, whilst the lower and upper edges represent the 25 and 75% quartiles, respectively. Whiskers indicate minimum and maximum values that are not outliers. Outliers (± 1.5*IQR) are represented by open circles. **(A)** learning score 0: mean days foraging 3.91 ± 1.19 [SE]; learning score 1–5: 7.08 ± 0.98 [SE]; learning score 6–10: 6.22 ± 0.83 [SE]; learning score 11–15: 4.83 ± 1.62 [SE], **(B)** Non-learners mean days foraging: 3.91 ± 1.19 [SE], learners: 6.32 ± 0.63 [SE].

on the basic model and all tested alternative models (ΔAICc 5.95; **Table 4**; raw data are summarised in **Figure 3B** and presented in full in **Supplementary Figure 4**). Learning was also added as a binomial category in models for *mean number of bouts per day* and *mean bout duration*, however, this did not alter the model's predictions (**Supplementary Table 1**).

DISCUSSION

To determine whether the olfactory learning abilities of *B. terrestris* individuals predict their foraging performance in a natural environment, PER conditioning was used to assess olfactory learning in the laboratory before the foraging performance of the same individuals was monitored in the field. When comparing all individuals that demonstrated odour learning, we found that their learning performance did not predict their foraging efficiency (i.e., nectar or pollen collection rates), daily foraging activity (numbers of bouts completed), or forage-bout duration. The daily rate at which foragers collected floral resources (nectar or pollen) was similar, regardless of how they performed in the olfactory learning task. However, olfactory learning *per se* predicted the duration of their foraging career. Bees that demonstrated some ability to use odour cues as a predictor of sucrose solution reward (learning scores of 1–14) foraged for more days than non-learning individuals (learning score = 0). Consequently, odour learning individuals provided food resources for their colony over a longer period of time.

TABLE 4 | Candidate models to predict the number of days spent foraging by tested foragers.

	No. days foraged	
	AICc	**Δ AICc**
Basic	224.41	7.58
Worker age	226.54	9.72
Worker mass	225.48	8.66
Colony age	222.78*	5.95
Best model + learning category (learner vs. non-learner)	**216.86**	**0.00**

The basic model contained only the intercept and majority colony as a random factor. All other models contained the basic model and the additional factors specified in the model name (worker age, worker mass, or colony age). The model with the lowest AICc value out of the four initial models (indicated with an asterisk) had learning category (learner vs. non-learner) added to it to determine whether this significantly reduced the AICc value (i.e., decreased ΔAICc > 2). The best model (based on the AICc value) is shown in bold.

It is not clear why the non-learning bees foraged for fewer days compared to bees that exhibited some olfactory learning. These bees did not have a lower propensity to forage in their natural environment; they were just as likely to forage as bees demonstrating learning. It is possible that these non-learning individuals were in poor condition and therefore not motivated to learn and more likely to not forage for long or to die early. However, whilst these bees did not learn, they were still responsive/motivated by sucrose throughout the laboratory PER conditioning assessment. It is reasonable to expect that if the bees had been in poor condition they would have been generally less responsive. The non-learning bees were also a similar size and age to their nest mates, meaning it is unlikely that they had a reduced ability to detect the olfactory cues in the learning assessment because they had lower olfactory sensitivity (Spaethe et al., 2007), or because their olfactory systems were less developed (Ray and Ferneyhough, 1997; Laloi et al., 2001). It is possible that the bees demonstrating no olfactory learning were ill equipped for foraging in their natural foraging environment. As well as being important for flower selection, olfactory learning is likely to be important for predator avoidance; enabling bees to detect and avoid potentially lethal encounters with predatory insects (Reader et al., 2006; Bray and Nieh, 2014; Li et al., 2014). Olfactory

learning is also necessary for homing/navigation; olfactory cues near the nest are learned as guides for returning foragers (Foster and Gamboa, 1989; Saleh et al., 2007). Consequently, these bees might have been more likely to succumb to predation or become lost whilst foraging.

Despite the olfactory and visual sensory systems in bees serving some distinct functions (Wright and Schiestl, 2009), and there being differences in the way these cues are learned and retained (Menzel and Greggers, 1985; Kunze and Gumbert, 2001), the relationship between learning ability and foraging performance among *B. terrestris* individuals was similar, regardless of whether learning was assessed using an olfactory (this study) or a visual (colour learning) task (the latter results presented in Evans et al., 2017). Like olfactory learning performance, visual learning performance did not predict floral resource collection rates, daily foraging activity levels, or foraging bout duration (Evans et al., 2017). However, we did find differences in the relationship between olfactory/visual learning performance and the amount of foraging undertaken overall. When comparing only among individuals that learnt the olfactory cues (i.e., non-learners were excluded), we found that the duration of their foraging careers was not predicted by how they performed in the olfactory learning task. In contrast, visual (colour) learning performance did predict foraging career duration, with the fastest visual learners foraging for fewer days overall (Evans et al., 2017).

The shorter foraging careers of faster visual learners (Evans et al., 2017) was thought to have resulted from the energetic cost associated with enhanced cognitive performance, which can negatively impact other energetically demanding processes (Mery and Kawecki, 2003; Mery and Kawecki, 2004; Snell-Rood et al., 2011; Jaumann et al., 2013). Another study provides evidence of a "trade-off" in the opposite direction—increased foraging time lowered olfactory learning performance (reversal learning) among honey bees (Cabirol et al., 2018), further support for an inverse relationship between learning and foraging duration. In the current study, the fastest olfactory learners also had a tendency to forage for fewer days than "average" learners, although this trend was not statistically significant. It is possible that this relationship was less pronounced in the current study because of the smaller number of foragers monitored (compared to Evans et al., 2017). It should be noted that whilst PER is a well-established method of assessing classical conditioning for honey bees and bumble bees (e.g., Takeda, 1961; Riveros and Gronenberg, 2009; Giurfa and Sandoz, 2012), this is the first time the foraging performance of bees has been assessed after completing PER. Our data suggest that the PER assay could have affected the performance of foragers. In comparison with the bees for which colour learning was assessed (by Evans et al., 2017), 10% fewer individuals in the olfactory PER assay foraged, and those that did forage completed 25 percent fewer foraging bouts and foraged for two thirds as long. Reducing the time the bees spend in a harness for PER conditioning may improve results obtained in future studies.

While we have shown that learning is associated with the foraging career duration of *B. terrestris* workers, we have not demonstrated a relationship between olfactory learning performance and rate of resource collection by individual bees. Such a relationship between these variables might be expected because the ability to rapidly learn salient floral cues is thought to enable foragers to better track changes in floral resources that vary across time and space and among plant species (Laverty, 1980; Menzel, 1993; Chittka, 1998). Even a slight decrease in the time spent locating or handling each flower may be an advantage because in a single day individuals will visit thousands of flowers to support themselves and their colony (Raine et al., 2006b). However, it is possible that any benefits in flower-handling efficiency are negligible compared to other time-intensive elements of foraging, including travel between the colony and multiple resource patches (Lihoreau et al., 2010, 2012). It is also possible that we have not used the best measure for assessing foraging efficiency. In addition to needing a sufficient quantity of food, bees require diverse and high quality protein and micronutrients for maintaining healthy workers and to rear their brood (Alaux et al., 2010; Di Pasquale et al., 2013; Vaudo et al., 2016). In future, it may be more useful to consider foraging efficiency in terms of the quality of the floral resource (e.g., the sucrose content of nectar and protein content of pollen), and/or the diversity of pollen sources collected as well as the amount of pollen and nectar collected.

Another possible explanation for our results is that the ability to learn odours more quickly in the rural/residential landscape surrounding the test site (Royal Holloway; Egham TW20 0EX, United Kingdom) simply may not have conferred an advantage in terms of foraging efficiency. Because of the costs associated with learning, its adaptation is expected to be fined-tuned to prevailing ecological (and social) conditions; leading it to be more important in some environments than others (Stephens, 1991; Dunlap and Stephens, 2016; Morand-Ferron et al., 2019). This remains a possible explanation for the apparent lack of relationship between individual learning (either olfactory or visual) and foraging (olfactory assessed in this study and visual in Evans et al., 2017), while (visual) learning and nectar foraging performance of 12 *B. terrestris* colonies were strongly correlated in an urban habitat in central London (Raine and Chittka, 2008). In this urban experiment, bumble bee colonies containing the fastest colour learning individuals also brought in nectar at significantly higher rates in those environmental conditions.

Overall our results suggest that olfactory learning plays a role in foraging success for *B. terrestris*. Individuals that were able to learn the scent-reward association had a longer foraging career and as a consequence collected more floral resources for their colony overall. The reason that non-learners foraged for fewer days remains unclear, it was not due to a reduced propensity to forage, and further work would be needed to determine if it could have been due to a reduced ability of foragers to return to their colony. We did not find statistical support for faster olfactory learners being more efficient or active foragers. Instead our results echo the findings of studies using visual learning (Evans et al., 2017); that suggest a balance exists between the benefits and costs associated with learning.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

LE and KS conceived the project, carried-out the experiment and statistical analyses. LE, KS, and NR designed the research. LE and NR wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The authors gratefully acknowledge the support of the Natural Sciences and Engineering Research Council of Canada in the form of a Discovery Grant (RGPIN-2015-06783) and the Food from Thought: Agricultural Systems for a Healthy Planet Initiative, by the Canada First Research Excellence Fund (grant 000054). LE was supported by The New Zealand Institute for Plant and Food Research and the following scholarships: C. Alma Baker, A.G.K. Overseas Research, and a Queen Elizabeth's Technicians Award. KS was supported by a Crosslands Research Scholarship and the Atkins bequest from Royal Holloway University of London. NR was supported as the Rebanks Family Chair in Pollinator Conservation by the Weston Family Foundation.

ACKNOWLEDGMENTS

We would like to thank Emily Parsons for her help monitoring field foraging bees, Oscar Ramos-Rodriguez for technical assistance, and also Brian Cutting and Jenny Jandt for commenting on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.676289/full#supplementary-material

REFERENCES

Alaux, C., Ducloz, F., Crauser, D., and Le Conte, Y. (2010). Diet effects on honeybee immunocompetence. *Biol. Lett.* 6, 562–565. doi: 10.1098/rsbl.2009.0986

Andersson, S., and Dobson, H. E. M. (2003). Behavioral foraging responses by the butterfly *Heliconius melpomene* to *Lantana camara* floral scent. *J. Chem. Ecol.* 29, 2303–2318. doi: 10.1023/A:1026226514968

Balkenius, A., Rosén, W., and Kelber, A. (2006). The relative importance of olfaction and vision in a diurnal and a nocturnal hawkmoth. *J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol.* 192, 431–437. doi: 10.1007/s00359-005-0081-6

Bates, D., Maechler, M., Bolker, B., and Walker, S. (2014). *lme4: Linear Mixed - Effects Models Using Eigen and s4. R package Version 1.1-6*. Available online at: http://CRAN.R-project.org/package=lme4 (accessed June 22, 2021)

Boogert, N. J., Madden, J. R., Morand-Ferron, J., and Thornton, A. (2018). Measuring and understanding individual differences in cognition. *Philos. Trans. R. Soc. B Biol. Sci.* 373:20170280. doi: 10.1098/rstb.2017.0280

Bray, A., and Nieh, J. (2014). Non-consumptive predator effects shape honey bee foraging and recruitment dancing. *PLoS One* 9:e87459. doi: 10.1371/journal.pone.0087459

Burger, J. M. S., Kolss, M., Pont, J., and Kawecki, T. J. (2008). Learning ability and longevity: a symmetrical evolutionary trade-off in *Drosophila*. *Evolution* 62, 1294–1304. doi: 10.1111/j.1558-5646.2008.00376.x

Burnham, K. P., Anderson, D. R., and Huyvaert, K. P. (2011). AIC model selection and multimodel inference in behavioral ecology: some background, observations, and comparisons. *Behav. Ecol. Sociobiol.* 65, 23–35. doi: 10.1007/s00265-010-1029-6

Burns, J. G., Foucaud, J., and Mery, F. (2011). Costs of memory: lessons from 'mini' brains. *Proc. Biol. Sci.* 278, 923–929. doi: 10.1098/rspb.2010.2488

Cabirol, A., Cope, A. J., Barron, A. B., and Devaud, J. M. (2018). Relationship between brain plasticity, learning and foraging performance in honey bees. *PLoS One* 13:e0196749. doi: 10.1371/journal.pone.0196749

Chittka, L. (1998). Sensorimotor learning in bumblebees: long-term retention and reversal training. *J. Exp. Biol.* 201, 515–524. doi: 10.1242/jeb.201.4.515

Chittka, L., Dyer, A. G., Bock, F., and Dornhaus, A. (2003). Psychophysics – bees trade off foraging speed for accuracy. *Nature* 424, 388–388. doi: 10.1038/424388a

Chittka, L., and Raine, N. E. (2006). Recognition of flowers by pollinators. *Curr. Opin. Plant Biol.* 9, 428–435. doi: 10.1016/j.pbi.2006.05.002

De Agrò, M., Oberhauser, F. B., Loconsole, M., Galli, G., Dal Cin, F., Moretto, E., et al. (2020). Multi-modal cue integration in the black garden ant. *Anim. Cogn.* 23, 1119–1127. doi: 10.1007/s10071-020-01360-9

Di Pasquale, G., Salignon, M., Le Conte, Y., Belzunces, L. P., Decourtye, A., Kretzschmar, A., et al. (2013). Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? *PLoS One* 8:e72016. doi: 10.1371/journal.pone.0072016

Dornhaus, A., and Chittka, L. (2001). Food alert in bumblebees (*Bombus terrestris*): possible mechanisms and evolutionary implications. *Behav. Ecol. Sociobiol.* 50, 570–576. doi: 10.1007/s002650100395

Dukas, R. (1999). Costs of memory: ideas and predictions. *J. Theor. Biol.* 197, 41–50. doi: 10.1006/jtbi.1998.0856

Dukas, R. (2008). Evolutionary biology of insect learning. *Annu. Rev. Entomol.* 53, 145–160. doi: 10.1146/annurev.ento.53.103106.093343

Dukas, R., and Bernays, E. A. (2000). Learning improves growth rate in grasshoppers. *Proc. Natl. Acad. Sci. U. S. A.* 97, 2637–2640. doi: 10.1073/pnas.050461497

Dukas, R., and Duan, J. J. (2000). Potential fitness consequences of associative learning in a parasitoid wasp. *Behav. Ecol.* 11, 536–543. doi: 10.1093/beheco/11.5.536

Dunlap, A. S., and Stephens, D. W. (2016). Reliability, uncertainty, and costs in the evolution of animal learning. *Curr. Opin. Behav. Sci.* 12, 73–79. doi: 10.1016/j.cobeha.2016.09.010

Eliassen, S., Jorgensen, C., Mangel, M., and Giske, J. (2007). Exploration or exploitation: life expectancy changes the value of learning in foraging strategies. *Oikos* 116, 513–523. doi: 10.1111/j.2006.0030-1299.15462.x

Evans, L. J., and Raine, N. E. (2014). Foraging errors play a role in resource exploration by bumble bees (*Bombus terrrestris*). *J. Comp. Physiol. A* 200, 475–484. doi: 10.1007/s00359-014-0905-3

Evans, L. J., Raine, N. E., and Leadbeater, E. (2016). Reproductive environment affects learning performance in bumble bees. *Behav. Ecol. Sociobiol.* 70, 2053–2060. doi: 10.1007/s00265-016-2209-9

Evans, L. J., Smith, K. E., and Raine, N. E. (2017). Fast learning in free-foraging bumble bees is negatively correlated with lifetime resource collection. *Sci. Rep.* 7:496. doi: 10.1038/s41598-017-00389-0

Flanigan, K. A. S., Wiegmann, D. D., Hebets, E. A., and Bingman, V. P. (2021). Multisensory integration supports configural learning of a home refuge in the whip spider *Phrynus marginemaculatus*. *J. Exp. Biol.* 224:jeb238444. doi: 10.1242/jeb.238444

Foley, R. A., and Lee, P. C. (1991). Ecology and energetics of encephalization in hominid evolution. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 334, 223–232. doi: 10.1098/rstb.1991.0111

Foster, R. L., and Gamboa, G. J. (1989). Nest entrance marking with colony specific odors by the bumble bee *Bombus occidentalis* (Hymenoptera: Apidae). *Ethology* 81, 273–278. doi: 10.1111/j.1439-0310.1989.tb00773.x

Gill, R. J., and Raine, N. E. (2014). Chronic impairment of bumblebee natural foraging behaviour induced by sublethal pesticide exposure. *Funct. Ecol.* 28, 1459–1471. doi: 10.1111/1365-2435.12292

Gill, R. J., Ramos-Rodriguez, O., and Raine, N. E. (2012). Combined pesticide exposure severely affects individual- and colony-level traits in bees. *Nature* 491, 105–108. doi: 10.1038/nature11585

Giraudoux, P. (2014). *pgirmess: Data Analysis in Ecology. R Package Version 1.5.9*. Available online at: http://CRAN.R-project.org/package=pgirmess (accessed April 5,2021)

Giurfa, M., and Sandoz, J.-C. (2012). Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn. Mem.* 19, 54–66. doi: 10.1101/lm.024711.111

Goulson, D., Hawson, S. A., and Stout, J. C. (1998). Foraging bumblebees avoid flowers already visited by conspecifics or by other bumblebee species. *Anim. Behav.* 55, 199–206. doi: 10.1006/anbe.1997.0570

Greenlees, M. J., Phillips, B. L., and Shine, R. (2010). Adjusting to a toxic invader: native Australian frogs learn not to prey on cane toads. *Behav. Ecol.* 21, 966–971. doi: 10.1093/beheco/arq095

Heinrich, B. (1976). The foraging specializations of individual bumblebees. *Ecol. Monogr.* 46, 105–128. doi: 10.2307/1942246

Hermer, E., Cauchoix, M., Chaine, A. S., and Morand-Ferron, J. (2018). Elevation-related differences in serial reversal learning ability in a nonscatter hoarding passerine. *Behav. Ecol.* 29, 840–847. doi: 10.1093/beheco/ary067

Huebner, F., Fichtel, C., and Kappeler, P. M. (2018). Linking cognition with fitness in a wild primate: fitness correlates of problem-solving performance and spatial learning ability. *Philos. Trans. R. Soc. B* 373:20170295. doi: 10.1098/rstb.2017.0295

Jaumann, S., Scudelari, R., and Naug, D. (2013). Energetic cost of learning and memory can cause cognitive impairment in honeybees. *Biol. Lett.* 9:20130149. doi: 10.1098/rsbl.2013.0149

Kaczorowski, R. L., Leonard, A. S., Dornhaus, A., and Papaj, D. R. (2012). Floral signal complexity as a possible adaptation to environmental variability: a test using nectar-foraging bumblebees. *Bombus impatiens*. *Anim. Behav.* 83, 905–913. doi: 10.1016/j.anbehav.2012.01.007

Kunze, J., and Gumbert, A. (2001). The combined effect of color and odor on flower choice behavior of bumble bees in flower mimicry systems. *Behav. Ecol.* 12, 447–456. doi: 10.1093/beheco/12.4.447

Laloi, D., Gallois, M., Roger, B., and Pham-Delègue, M.-H. (2001). Changes with age in olfactory conditioning performance of worker honey bees (*Apis mellifera*). *Apidologie* 32, 231–242. doi: 10.1051/apido:2001125

Laverty, T. M. (1980). The flower-visiting behaviour of bumble bees: floral complexity and learning. *Can. J. Zool.* 58, 1324–1335. doi: 10.1139/z80-184

Li, J., Wang, Z., Tan, K., Qu, Y., and Nieh, J. C. (2014). Giant Asian honeybees use olfactory eavesdropping to detect and avoid ant predators. *Anim. Behav.* 97, 69–76. doi: 10.1016/j.anbehav.2014.08.015

Lihoreau, M., Chittka, L., and Raine, N. E. (2010). Travel optimization by foraging bumblebees through readjustments of traplines after discovery of new feeding locations. *Am. Nat.* 176, 744–757. doi: 10.1086/657042

Lihoreau, M., Raine, N. E., Reynolds, A. M., Stelzer, R. J., Lim, K. S., Smith, A. D., et al. (2012). Radar tracking and motion-sensitive cameras on flowers reveal the development of pollinator multi-destination routes over large spatial scales. *PLoS Biol.* 10:e1001392. doi: 10.1371/journal.pbio.1001392

Lönnstedt, O. M., McCormick, M. I., Meekan, M. G., Ferrari, M. C. O., and Chivers, D. P. (2012). Learn and live: predator experience and feeding history determines prey behaviour and survival. *Proc. Biol. Sci.* 279, 2091–2098. doi: 10.1098/rspb.2011.2516

Maaswinkel, H., and Whishaw, I. Q. (1999). Homing with locale, taxon, and dead reckoning strategies by foraging rats: sensory hierarchy in spatial navigation. *Behav. Brain Res.* 99, 143–152. doi: 10.1016/s0166-4328(98)00100-4

Madden, J. R., Langley, E. J. G., Whiteside, M. A., Beardsworth, C. E., and van Horik, J. O. (2018). The quick are the dead: pheasants that are slow to reverse a learned association survive for longer in the wild. *Philos. Trans. R. Soc. B* 373:20170297. doi: 10.1098/rstb.2017.0297

Mallon, E. B., Brockmann, A., and Schmid-Hempel, P. (2003). Immune response inhibits associative learning in insects. *Proc. Biol. Sci.* 270, 2471–2473. doi: 10.1098/rspb.2003.2456

Manning, A. (1956). Some aspects of the foraging behaviour of bumble bees. *Behaviour* 9, 164–201. doi: 10.1163/156853956x00291

Menzel, R. (1993). Associative learning in honey bees. *Apidologie* 24, 157–168. doi: 10.1051/apido:19930301

Menzel, R., and Greggers, U. (1985). Natural phototaxis and its relationship to color-vision in honeybees. *J. Comp. Physiol. A Sens. Neural. Behav. Physiol.* 157, 311–321. doi: 10.1007/bf00618121

Mery, F., and Kawecki, T. J. (2003). A fitness cost of learning ability in *Drosophila melanogaster*. *Proc. Biol. Sci.* 270, 2465–2469. doi: 10.1098/rspb.2003.2548

Mery, F., and Kawecki, T. J. (2004). An operating cost of learning in *Drosophila melanogaster*. *Anim. Behav.* 68, 589–598. doi: 10.1016/j.anbehav.2003.12.005

Molet, M., Chittka, L., and Raine, N. E. (2009). How floral odours are learned inside the bumblebee (*Bombus terrestris*) nest. *Naturwissenschaften* 96, 213–219. doi: 10.1007/s00114-008-0465-x

Morand-Ferron, J. (2017). Why learn? The adaptive value of associative learning in wild populations. *Curr. Opin. Behav. Sci.* 16, 73–79. doi: 10.1016/j.cobeha.2017.03.008

Morand-Ferron, J., Hermer, E., Jones, T. B., and Thompson, M. J. (2019). Environmental variability, the value of information, and learning in winter residents. *Anim. Behav.* 147, 137–145. doi: 10.1016/j.anbehav.2018.09.008

Morand-Ferron, J., and Quinn, J. L. (2015). The evolution of cognition in natural populations. *Trends Cogn. Sci.* 19, 235–237. doi: 10.1016/j.tics.2015.03.005

Nowicki, S., Searcy, W. A., and Peters, S. (2002). Quality of song learning affects female response to male bird song. *Proc. Biol. Sci.* 269, 1949–1954. doi: 10.1098/rspb.2002.2124

Pearce, R. F., Giuggioli, L., and Rands, S. A. (2017). Bumblebees can discriminate between scent-marks deposited by conspecifics. *Sci. Rep.* 7:43872. doi: 10.1038/srep43872

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and R Core Team. (2014). *nlme: Linear and Nonlinear Mixed Effects Models_. R Package Version 3.1-117*. Available online at: http://CRAN.R-project.org/package=nlme (accessed February4, 2021)

R Core Development Team. (2014). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Raihani, N. J., and Bshary, R. (2012). A positive effect of flowers rather than eye images in a large-scale, cross-cultural dictator game. *Proc. Biol. Sci.* 279, 3556–3564. doi: 10.1098/rspb.2012.0758

Raine, N. E., and Chittka, L. (2007). The adaptive significance of sensory bias in a foraging context: floral colour preferences in the bumblebee *Bombus terrestris*. *PLoS One* 2:e556. doi: 10.1371/journal.pone.0000556

Raine, N. E., and Chittka, L. (2008). The correlation of learning speed and natural foraging success in bumble-bees. *Proc. Biol. Sci.* 275, 803–808. doi: 10.1098/rspb.2007.1652

Raine, N. E., Ings, T. C., Dornhaus, A., Saleh, N., and Chittka, L. (2006a). Adaptation, genetic drift, pleiotropy, and history in the evolution of bee foraging behavior. *Adv. Study Behav.* 36, 305–354. doi: 10.1016/s0065-3454(06)36007-x

Raine, N. E., Ings, T. C., Ramos-Rodriguez, O., and Chittka, L. (2006b). Intercolony variation in learning performance of a wild British bumblebee population (Hymenoptera: Apidae: *Bombus terrestris audax*). *Entomol. Gen.* 28, 241–256. doi: 10.1127/entom.gen/28/2006/241

Ray, S., and Ferneyhough, B. (1997). The effects of age on olfactory learning and memory in the honey bee *Apis mellifera*. *Neuroreport* 8, 789–793. doi: 10.1097/00001756-199702100-00042

Reader, T., Higginson, A. D., Barnard, C. J., and Gilbert, F. S. (2006). The effects of predation risk from crab spiders on bee foraging behavior. *Behav. Ecol.* 17, 933–939. doi: 10.1093/beheco/arl027

Riveros, A. J., and Gronenberg, W. (2009). Olfactory learning and memory in the bumblebee *Bombus occidentalis*. *Naturwissenschaften* 96, 851–856. doi: 10.1007/s00114-009-0532-y

Saleh, N., Ohashi, K., Thomson, J. D., and Chittka, L. (2006). Facultative use of the repellent scent mark in foraging bumblebees: complex versus simple flowers. *Anim. Behav.* 71, 847–854. doi: 10.1016/j.anbehav.2005.06.014

Saleh, N., Scott, A. G., Bryning, G. P., and Chittka, L. (2007). Distinguishing signals and cues: bumblebees use general footprints to generate adaptive behaviour at

flowers and nest. *Arthropod Plant Interact.* 1, 119–127. doi: 10.1007/s11829-007-9011-6

Sato, A., Ozawa, N., and Karino, K. (2014). Variation in female guppy preference for male olfactory and visual traits. *J. Ethol.* 32, 137–143. doi: 10.1007/s10164-014-0402-8

Schmid-Hempel, R., and Schmid-Hempel, P. (1998). Colony performance and immunocompetence of a social insect, *Bombus terrestris*, in poor and variable environments. *Funct. Ecol.* 12, 22–30. doi: 10.1046/j.1365-2435.1998.00153.x

Sergio, F., Tanferna, A., De Stephanis, R., Jimenez, L. L., Blas, J., Tavecchia, G., et al. (2014). Individual improvements and selective mortality shape lifelong migratory performance. *Nature* 515, 410–413. doi: 10.1038/nature13696

Smith, C., Barber, I., Wootton, R. J., and Chittka, L. (2004). A receiver bias in the origin of three–spined stickleback mate choice. *Proc. Biol. Sci.* 271, 949–955. doi: 10.1098/rspb.2004.2690

Smith, K. E., and Raine, N. E. (2014). A comparison of visual and olfactory learning performance in the bumblebee *Bombus terrestris*. *Behav. Ecol. Sociobiol.* 68, 1549–1559. doi: 10.1007/s00265-014-1765-0

Snell-Rood, E. C., Davidowitz, G., and Papaj, D. R. (2011). Reproductive tradeoffs of learning in a butterfly. *Behav. Ecol.* 22, 291–302. doi: 10.1093/beheco/arq169

Spaethe, J., Brockmann, A., Halbig, C., and Tautz, J. (2007). Size determines antennal sensitivity and behavioral threshold to odors in bumblebee workers. *Naturwissenschaften* 94, 733–739. doi: 10.1007/s00114-007-0251-1

Spaethe, J., Tautz, J., and Chittka, L. (2001). Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proc. Natl. Acad. Sci. U. S. A.* 98, 3898–3903. doi: 10.1073/pnas.071053098

Stanley, D. A., Russell, A. L., Morrison, S. J., Rogers, C., and Raine, N. E. (2016). Investigating the impacts of field−realistic exposure to a neonicotinoid pesticide on bumblebee foraging, homing ability and colony growth. *J. Appl. Ecol.* 53, 1440–1449. doi: 10.1111/1365-2664.12689

Stephens, D. W. (1991). Change, regularity, and value in the evolution of animal learning. *Behav. Ecol.* 2, 77–89. doi: 10.1093/beheco/2.1.77

Stout, J. C., and Goulson, D. (2001). The use of conspecific and interspecific scent marks by foraging bumblebees and honeybees. *Anim. Behav.* 62, 183–189. doi: 10.1006/anbe.2001.1729

Takeda, K. (1961). Classical conditioned response in the honey bee. *J. Insect Physiol.* 6, 168–179. doi: 10.1016/0022-1910(61)90060-9

van den Berg, M., Duivenvoorde, L., Wang, G. H., Tribuhl, S., Bukovinszky, T., Vet, L. E. M., et al. (2011). Natural variation in learning and memory dynamics studied by artificial selection on learning rate in parasitic wasps. *Anim. Behav.* 81, 325–333. doi: 10.1016/j.anbehav.2010.11.002

Vaudo, A. D., Patch, H. M., Mortensen, D. A., Tooker, J. F., and Grozinger, C. M. (2016). Macronutrient ratios in pollen shape bumble bee (*Bombus impatiens*) foraging strategies and floral preferences. *Proc. Natl. Acad. Sci. U. S. A.* 113, E4035–E4042. doi: 10.1073/pnas.1606101113

Ward-Fear, G., Pearson, D. J., Brown, G. P., Rangers, B., and Shine, R. (2016). Ecological immunization: in situ training of free-ranging predatory lizards reduces their vulnerability to invasive toxic prey. *Biol. Lett.* 12:20150863. doi: 10.1098/rsbl.2015.0863

White, G. E., and Brown, C. (2014). A comparison of spatial learning and memory capabilities in intertidal gobies. *Behav. Ecol. Sociobiol.* 68, 1393–1401. doi: 10.1007/s00265-014-1747-2

Wright, G. A., and Schiestl, F. P. (2009). The evolution of floral scent: the influence of olfactory learning by insect pollinators on the honest signalling of floral rewards. *Funct. Ecol.* 23, 841–851. doi: 10.1111/j.1365-2435.2009.01627.x

Zanette, L. R. S., Miller, S. D. L., Faria, C. M. A., Lopez-Vaamonde, C., and Bourke, A. F. G. (2014). Bumble bee workers drift to conspecific nests at field scales. *Ecol. Entomol.* 39, 347–354. doi: 10.1111/een.12109

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 30 July 2021
doi: 10.3389/fevo.2021.682960

Floral Cues of Non-host Plants Attract Oligolectic *Chelostoma rapunculi* Bees

Hannah Burger, Nadine Joos and Manfred Ayasse*

Institute of Evolutionary Ecology and Conservation Genomics, Ulm University, Ulm, Germany

Oligolectic bees are highly dependent on the availability of the host plants to which they are specialized. Nevertheless, females of *Chelostoma rapunculi* have recently been monitored occasionally to visit *Malva moschata* and *Geranium sanguineum* flowers, in addition to their well-known *Campanula* spp. hosts. The questions therefore arise which floral cues promote visits to non-host plants. As host-specific floral cues are key attractants for oligolectic bees, we have studied the attractiveness of olfactory and visual cues of the established host *Campanula trachelium* in comparison to the non-host plants *G. sanguineum* and *M. moschata* in behavioral experiments. Chemical and electrophysiological analyses of the floral scent and spectral measurements of floral colors were used to compare and contrast host and non-host plants. The behavioral experiments showed that foraging-naïve bees, in particular, were attracted by olfactory cues of the non-host plants, and that they did not favor the *Campanula* host scent in choice experiments. Many electrophysiologically active floral volatiles were present in common in the studied plants, although each species produced an individual scent profile. Spiroacetals, the key components that enable *C. rapunculi* to recognize *Campanula* hosts, were detected in trace amounts in *Geranium* but could not be proved to occur in *Malva*. The visual floral cues of all species were particularly attractive for foraging-experienced bees. The high attractiveness of *G. sanguineum* and *M. moschata* flowers to *C. rapunculi* bees and the floral traits that are similar to the *Campanula* host plants can be a first step to the beginning of a host expansion or change which, however, rarely occurs in oligolectic bees.

Keywords: floral scent, color, spiroacetals, *Campanula*, pollen-specialist bee, *Malva*, *Geranium*

OPEN ACCESS

Edited by:
Sara Diana Leonhardt, Technical University of Munich, Germany

Reviewed by:
Tamar Keasar, University of Haifa - Oranim, Israel
Kelsey Jaye Ruth Paularena Byers, John Innes Centre, United Kingdom

****Correspondence:***
Hannah Burger
info@hannah-burger.de

Specialty section:
This article was submitted to Chemical Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *19 March 2021*
Accepted: *06 July 2021*
Published: *30 July 2021*

Citation:
Burger H, Joos N and Ayasse M (2021) Floral Cues of Non-host Plants Attract Oligolectic Chelostoma rapunculi Bees. Front. Ecol. Evol. 9:682960. doi: 10.3389/fevo.2021.682960

INTRODUCTION

Flowers are essential for bees, as almost all bee species gather their food exclusively from flowers. Bees are also the primary pollen vectors in most ecosystems (Michener, 2007). Nectar and pollen are the main food sources used for the bee's own nourishment and are needed in large amounts to provision the brood (Westrich, 2018). The pollen of up to several hundred flowers is required to rear a single offspring (Müller et al., 2006). Because of the enormous amount of required pollen, flowering plants are thought to be the limiting factor for the abundance of bees in an area (Müller et al., 2006). This is especially the case for specialist (oligolectic) bees that collect pollen from only

a few plants of a given genus or family (Müller and Kuhlmann, 2008) and the larvae normally fail to develop on pollen of non-host flowers (Praz et al., 2008b). The absence of specific host plants in a habitat leads to a lack of the corresponding oligolectic bee species, independently of other living conditions (Westrich, 2018). Generalist (polylectic) bees, in contrast, visit various plant taxa. They are able to switch to more abundant plant species if a preferred pollen host is not available. As floral resources are tending to decrease because of agricultural practices, bee specialists are particularly threatened at present (Müller et al., 2006).

Although strong selection is expected to act on oligolectic bees to reduce their heavy dependence upon a limited number of host plants, a broadening of their diet has occurred only occasionally in evolution (Sedivy et al., 2008). Bees seem to have evolved strong physiological adaptations to deal with only a few plant species that are similar in their pollen properties (Sedivy et al., 2008). For example, the larvae of the *Campanula*-specialist *Chelostoma rapunculi* fail to develop on the pollen of various non-host species which was also demonstrated for other oligoleges (Praz et al., 2008b). In contrast, individuals of the Asteraceae-specialist *Heriades truncorum* successfully developed on experimentally offered *Echium* or *Campanula* pollen (Praz et al., 2008b). Nevertheless, those individuals did not forage on the non-host plants (Praz et al., 2008a). Constraints in recognizing or handling the flowers might have prevented the bees from collecting pollen, but this suggestion awaits to be studied experimentally (Sedivy et al., 2008).

For host plant finding, oligolectic bees rely on visual and olfactory floral cues (Dötterl and Vereecken, 2010). Host-specific olfactory cues are normally used to recognize hosts and to differentiate them from non-hosts (Burger et al., 2010, 2012; Milet-Pinheiro et al., 2012). In these studies, the bees clearly preferred the scent of hosts when offered against non-host scent cues. Color cues are also involved in host finding but they are often not host-specific and do not function alone as a recognition cue (Burger et al., 2010). Foraging-naïve oligolectic *C. rapunculi* bees recognize their host plants, for example, by means of spiroacetals, i.e., the host-specific key components of the floral scent of different *Campanula* species (Milet-Pinheiro et al., 2013; Brandt et al., 2017). As soon as the newly emerged bees are foraging-experienced, they change their preference, visual cues and a bouquet of commonly occurring volatiles become reliable in the foraging behavior of experienced females (Milet-Pinheiro et al., 2012, 2013).

Chelostoma rapunculi bees are known to restrict their pollen collection to plants of the genus *Campanula* (Westrich, 2018). However, females have recently been monitored to occasionally visit *Malva* and *Geranium* species, among them *M. moschata* and *G. sanguineum*, in addition to their well-known *Campanula* hosts (observations from 1996 onward, Southern Germany; Datenbank Wildbienen-Kataster, Entomologischer Verein Stuttgart, and personal communication Hans Schwenninger). Occasional visits do not necessarily lead to a host change but could be a first step toward the incorporation of a new host. Since floral scent has been shown to play the most important role in the location of suitable flowers by *C. rapunculi* bees (Milet-Pinheiro et al., 2012), olfactory cues of *Malva* and *Geranium* flowers might also function as attractants to these non-hosts. Additionally, lilac and blueish colors in the range of different *Campanula* species are also attractants for *C. rapunculi* bees (Milet-Pinheiro et al., 2015).

In this study, we have investigated the attractiveness of olfactory and visual floral cues of *G. sanguineum* and *M. moschata* for foraging-naïve and experienced *C. rapunculi* bees and tested whether the bees show a preference when floral cues of the non-hosts are offered against those of the established host *C. trachelium*. Further, we have compared the floral scent bouquets between *C. trachelium*, and two non-hosts, *M. moschata* and *G. sanguineum* using electrophysiological and chemical analyses. As *C. rapunculi* bees are highly tuned to *Campanula*-specific volatiles (Milet-Pinheiro et al., 2013), we expected that *M. moschata* and *G. sanguineum* show similarities in their scent bouquets with *C. trachelium* if the olfactory cues of the non-hosts are indeed attractive to the bees. Additionally, we have modeled the floral color spectra of these plants, as visual cues are also involved in host plant finding of oligolectic bees.

MATERIALS AND METHODS

Study Organisms

Chelostoma rapunculi (Lepeletier 1841) (Megachilidae) (**Figure 1**) is an oligolectic bee species with a distribution in many parts of Europe including Germany (Westrich, 2018). The bees nest in existing cavities, favoring deadwood and the boreholes of insects, but also accept trap nests such as the tubes of reed plants. The bees are highly specialized on *Campanula* spp. *C. rapunculi* is a univoltine bee species with its highest activity from early June until August.

The flowering times of the study plants overlap with the periods of activity of the bees. *C. trachelium* L. (Campanulaceae) is a European perennial herb that blooms from June to September. It is one of the main hosts of *C. rapunculi* and was already used as study organism in previous investigations on the flower recognition behavior of *C. rapunculi* bees (Milet-Pinheiro et al., 2012, 2013). *G. sanguineum* L. (Geraniaceae) is a persistent plant with a wide-spread distribution in Europe (Nebel et al., 1993). In Germany, the plant blooms from May to September and offer pollen and nectar (Westrich, 2018). *M. moschata* L. (Malvaceae) is an herbaceous perennial plant that is distributed throughout Europe (Nebel et al., 1993). The plants flowers from late June to October and provide nectar and a huge amount of pollen (Westrich, 2018). The taxa *Geranium* and *Malva* are both phylogenetically unrelated to *Campanula* (Asterids) but grouped together in the malvid clade of the Rosids (Angiosperm Phylogeny Website[1]).

Behavioral Experiments

Establishment of the Bee Population in an Experimental Flight Cage

For the behavioral studies, the bees were kept in a flight cage situated in the Botanical Garden of the University of Ulm. The

[1] www.mobot.org

FIGURE 1 | Flowers of the studied plant species *Campanula trachelium*, *Geranium sanguineum*, and *Malva moschata* and flower-visiting *Chelostoma rapunculi* females (left and right photograph).

flight cage consisted of a steel frame (7 m length × 3.5 m width × 2.2 m height) covered with a fine mesh (stitch density of 1 mm × 0.5 mm), the lower edges of which were buried in the soil to a depth of 0.5 m. A roof of UV permeable acrylic glass protected the cage. This setup allowed the simulation of abiotic conditions similar to those of the natural environment.

To obtain foraging-naïve bees, colonized trap nests were placed in a cage at the beginning of May, so that *C. rapunculi* bees emerged directly into the cage. The bees were fed with sugar water, prepared by Apiinvert (Südzucker AG, Ochsenfurt, Germany) and mixed with water to give a 40% solution. Sugar water was offered in saturated sponges placed in Petri dishes. The bees had no contact to the study plants *C. trachelium*, *M. moschata*, or *G. sanguineum*, which were used in the bioassays. Following the behavioral experiments with foraging-naïve bees, the same bees were allowed to forage and feed on their host plant *C. trachelium* to become foraging-experienced. To allow the bees to familiarize themselves with their host plant, bioassays were conducted at least 3 days after *Campanula* flowers were offered to the bees.

Set-Up of Behavioral Experiments

To test the attractiveness of decoupled floral cues of *M. moschata*, *G. sanguineum*, and *C. trachelium* for *C. rapunculi* bees, dual choice bioassays were conducted in the flight cage as previously described in Burger et al. (2010) and Milet-Pinheiro et al. (2012). Foraging-naïve and experienced bees were offered a choice between olfactory or visual cues of inflorescences of each species against an empty control. Furthermore, decoupled floral cues of *M. moschata* and *G. sanguineum* were tested against those of *C. trachelium*. The plant samples consisted of about 30 flowers each and were covered with cylinders to hide either visual or olfactory cues (**Figure 2**).

The behavioral experiments were conducted on sunny days between 9:30 and 15:00 h, when the bees were most active. Two cylinders were offered at a distance of 1 m from each other in each choice experiment. Each test was conducted for a total of 30 min, during which the position of the two cylinders was exchanged after 15 min. As only limited numbers of bees were available for the experiments each season (approx. 50 or 20 individuals in the first or later seasons, respectively, at the beginning of the experimental series), all tests were repeated either 1 or 2 years later, and the responses were summed up within each experiment. Responses were pooled, as individuals of both years responded

FIGURE 2 | Cylinders to test behavioral responses to decoupled floral cues. The set-up for testing olfactory cues **(A)** consisted of a gray plastic cylinder that was connected to a pump and that had small holes allowing scent diffusion. For visual cues **(B)**, transparent plexiglass cylinders were used but without holes.

equally in all bioassays (Fisher's Exact tests[2]: $0.16 < P < 1.00$). The number of bees approaching the set-up at a maximum distance of 10 cm and landing on the cylinder was recorded as the behavioral response. To ensure that an individual bee was counted only once in a specific two-choice test, the approaching or landing bees were caught. An exact binomial test (see text footnote 2) was used to test for difference in total bee response in all dual-choice experiments.

Scent Analysis

Collection of Scent Samples

The volatiles of *M. moschata*, *G. sanguineum*, and *C. trachelium* inflorescences were collected using dynamic headspace methods (Dötterl and Jürgens, 2005). The samples were obtained to compare scent bouquets between species and to identify the components (samples for thermal desorption), as well as for electrophysiological investigations (solvent samples). Floral scents were collected *in situ* from potted plants or from cut inflorescences that were placed in water. Approximately 20 different plant individuals of each species were available for scent collection. For each sample, six inflorescences were enclosed in a polyester oven bag (20 × 30 cm; Toppits®).

To obtain samples for thermal desorption, the scent of enclosed flowers was enriched for half an hour. Volatiles were then trapped for 1 h in an adsorbent tube through which air was drawn at a rate of 150 ml/min by using a membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany). The adsorbent tube consisted of glass tubes (length 2 cm, inner diameter 2 mm) that were filled with a mixture of 1.5 mg Tenax-TA (mesh 60–80; Supelco, Bellefonte, PA, United States) and 1.5 mg Carbotrap B (mesh 20–40, Supelco, Bellefonte, PA, United States), which were fixed with glass wool (Dötterl and Jürgens, 2005). In total, 10 samples of each plant species were collected. As the analyses revealed that the scent concentration was relatively low in the samples, we collected three more samples of *M. moschata* and *G. sanguineum*, each sample containing 30 inflorescences. Additionally, scent from non-flowering plant parts ($N = 3$) and blank controls ($N = 3$) without plant material were collected in order to identify flower-specific compounds.

Additionally, solvent headspace samples for the electrophysiological analyses were collected for 6 h in a larger adsorbent tube (length 9 cm, inner diameter 2 mm) at a flow rate of 100 ml/min^{-1}. The adsorbent tubes were filled with 10 mg of the absorbent Super Q (mesh 80/100, Alltech Associates Inc., United States). Volatiles were eluted with 100 µl dichloromethane (99.9%, Merck KGaA, Darmstadt, Germany). Three adsorbent tubes were pooled to obtain one sample, which was concentrated under a gentle stream of nitrogen and stored at −20°C. In total, six samples of each plant species were collected.

Electrophysiological Analysis

To determine the floral compounds that trigger antennal responses in *C. rapunculi* bees, gas chromatography coupled to electroantennographic detection (GC-EAD) experiments were performed for *C. trachelium* ($N = 3$), *G. sanguineum* ($N = 10$), and *M. moschata* ($N = 7$). The GC-EAD system consisted of an HP 6890 Hewlett-Packard gas chromatograph (Agilent Technologies, United States) equipped with a flame-ionization detector (FID) and an EAD set-up (Syntech, Hilversum, Netherlands). The separation of compounds was performed on a DB-5 column (30 cm long; 0.25 mm i.d.; 0.25 µm film thickness; J&W, United States). Hydrogen was used as the carrier gas at a constant flow of 2 ml/min. Two microliters of the solvent headspace samples were injected in splitless mode into the GC injector at an initial temperature of 40°C. After 1 min, the splitter was opened, and the oven temperature was increased at a rate of 10°C/min to a final temperature of 250°C, which was held for 3 min. In order to record the FID and EAD responses simultaneously, the GC effluent was split (split ratio FID:EAD = 1:1) under a make-up gas supply (nitrogen, 25 ml/min). The effluent was humidified with a filtered airflow of 100 ml/min and directed to the antennal preparation set-up via a glass tube of 95 mm length. Antennae of foraging-naïve *C. rapunculi* females were cut at the base and the tip and mounted between two glass capillaries that were connected to gold electrodes closing an electric circuit. The capillaries were filled with insect Ringer's solution (5 g NaCl; 0.42 g KCl; 0.19 g $CaCl_2$, in 1,000 ml demineralized water). New antennae were prepared for each run. A compound was considered to be EAD-active if a response was detected in at least three replicates. The EAD-active compounds were identified using gas chromatography coupled to mass spectrometry (GC-MS).

Chemical Analysis

To identify and compare the volatiles in the floral scent bouquet of the plants, the headspace samples were analyzed using gas chromatography (7890B GC system, Agilent Technologies, United States) coupled to mass spectrometry (Agilent 5977A mass selective detector). The GC was equipped with a thermal desorption unit (TDU, Gerstel, Mühlheim a. d. Ruhr, Germany) and a cold injection system (CIS 4C, Gerstel). A quartz micro-vial (length: 15 mm; inner diameter: 2 mm; Varian) containing 1 µl of a solvent sample or a thermal desorption sample was inserted into the injection unit by using an autosampler (Gerstel MAS Modular Analytical Systems Controller C506). The analytes were injected in splitless mode onto a non-polar column (DB-5ms, 30 m length, 250 µm inner diameter, 0.25 µm film thickness, J&W, United States). Analytes were thermally desorbed at 300°C for 8 min and refocused with liquid nitrogen. Helium was used as carrier gas at a constant flow of 1.5 ml per min. The oven program started at 40°C (held for 2 min) and was increased at a rate of 6°C per min to 200°C (held for 25 min; total run time 50 min). The MS interface and the ion source had temperatures of 250 and 230°C, respectively. Mass spectra were taken at 70 eV (in EI mode) from *m/z* 30 to 350.

Active compounds were assigned to GC-MS runs of the solvent scent samples that were used for the GC-EAD analyses by comparing the elution sequence and Kovats retention indices. An alkane series was run on all used systems. Compounds were identified based on their mass spectra by using multiple references from the NIST11 library and on published Kovats retention indices. Spiroacetals were identified based on the

[2]https://www.graphpad.com

mass spectra and retention index described in Milet-Pinheiro et al. (2013). Additionally, the identification of individual components was confirmed by comparison of both mass spectrum and GC retention data on all used systems with those of authentic standards if available. The GC-MS runs were analyzed using Amdis 2.71 (Automated Mass Spectral Deconvolution and Identification System). Absolute amounts were calculated based on an alkane standard (dodecane, 0.1 μg) that was added to a clean thermal desorption tube ($N = 6$).

Comparison of Scent Samples

EAD-active floral scent compounds in the solvent headspace samples were considered for the analysis of the same compounds in thermal desorption samples. Volatiles were categorized as floral compounds when they were only found in inflorescence samples or when they occurred in smaller amounts in leaf samples. EAD-active compounds found in blank controls were excluded from further analyses. Inflorescence scent bouquets of the investigated species were compared using a semi-quantitative approach based on the Bray-Curtis similarity index. The relative ratios of the compounds were transformed to their square root. An analysis of similarity (ANOSIM, 9999 permutations) was performed using species as fixed factors. Non-metric multidimensional scaling, based on the similarity matrices generated, were used to display graphically the differences in scent-profiles among species. SIMPER analysis (similarities percentages routine) was used to reveal those components of the scent bouquet that contributed to the differences between or similarities within (with species as nested factor) the species. The software PRIMER 6.1.6 was used for the analyses (Clarke and Gorley, 2006).

Color Analyses

To measure and compare the spectral reflection of the petals of *C. trachelium*, *G. sanguineum*, and *M. moschata*, we used an Ocean Optics JAZ spectrometer (Ocean Optics Inc., Dunedin, FL, United States) equipped with a pulsed xenon light source (JAZ-PX) and attached to a fiber optic cable (UV/VIS 400 μm; World Precision Instruments Inc., Saracota, FL, United States). The optical fiber was fixed onto an attachment, so that the light touched the investigated object at an angle of 45°. A plate with barium sulfate (Merck KGaA, Darmstadt, Germany) and an open black film canister were used as a white and black standard, respectively. The spectral reflection was recorded from 300 to 700 nm, which corresponds to the color spectrum perceived by bees (Peitsch et al., 1992). For each species, three measurements were taken from petals from three freshly collected plant individuals. The R package pavo (Rx64 Version 3.3.1) was used to process the obtained raw data (Maia et al., 2013).

The mean reflections of the petals, based on the three measurements, were used to determine the loci of the measured colors within the hexagon color space according to Chittka (1992). Bee colors were modeled using the spectral sensitivity of the honeybee because bees have in general similar vision (Peitsch et al., 1992). Hexagon distances were calculated as Euclidean distances between the loci of the color stimuli and between the non-colored point, which was the background stimulus with constant reflection (Chittka, 1992). Bumblebees can effectively discriminate colors with distances of at least 0.1 hexagon units

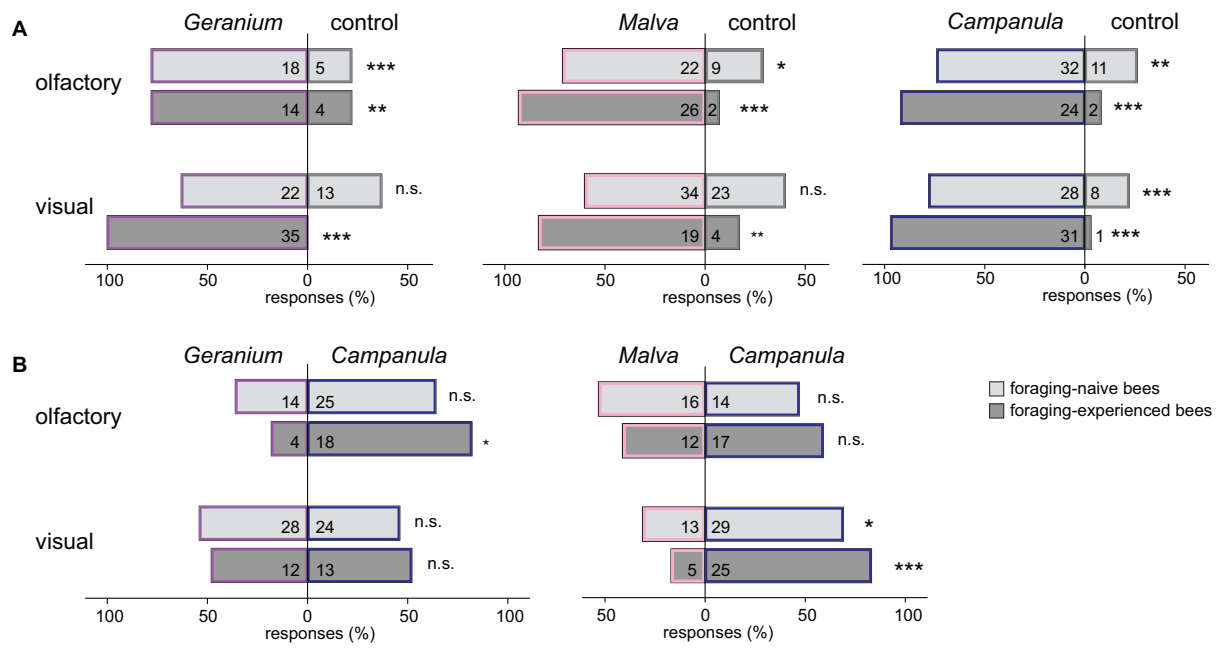

FIGURE 3 | Behavioral responses (in percent) of foraging-naïve (light gray) and foraging-experienced (dark gray) males and females of *C. rapunculi* to olfactory and visual cues of **(A)** *G. sanguineum*, *M. moschata*, and *C. trachelium* inflorescences tested against an empty control and **(B)** *G. sanguineum* and *M. moschata* against *C. trachelium* (exact binomial test: n.s.: $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

(Dyer and Chittka, 2004). The reflectance function of a typical green leaf was used as a background color (Chittka et al., 1994).

RESULTS

Attractiveness of Floral Cues

In the behavioral experiments, the olfactory cues of all plant species were significantly more attractive than an empty control for naïve and experienced bees (for number of responding bees, see **Figure 3A**). Visual cues tested against a control were significantly more attractive for all plants in experienced bees, but for naïve ones, only for *C. trachelium*.

Naïve bees showed no preference for one plant species when olfactory cues of either *G. sanguineum* or *M. moschata* were tested against those of *C. trachelium* (for number of responding bees, see **Figure 3B**). Experienced bees also showed no preference when *M. moschata* was tested against *C. trachelium*, but significantly more experienced bees preferred the olfactory cues of *C. trachelium when* tested against *G. sanguineum*. Regarding visual cues, both naïve and experienced bees did not prefer *G. sanguineum* over *C. trachelium* but significantly preferred *C. trachelium* over *M. moschata*.

Electrophysiologically Active Compounds and Comparison of Floral Scent Bouquets

In the GC-EAD analysis, 21 different antennal responses of *C. rapunculi* were registered in total (**Figure 4**). The responses were assigned to 32 compounds belonging to the following chemical classes: 2 aliphatic compounds, 18 terpenes, 2 spiroacetals, 3 benzenoids and phenylpropanoids, 1 nitrogen-containing compound, and 6 unknowns (**Table 1**). The two spiroacetals 1,6-dioxaspiro[4.5]decane and (*Z*)-7-methyl-1,6-dioxaspiro[4.5]decane (*Z*-conophthorin) were detected in quantifiable amounts in *C. trachelium* and *G. sanguineum* (**Table 1**). Overall, the scent bouquets significantly differed between the study species in the semi-quantitative comparison (ANOSIM $R = 0.65$, $p < 0.001$; **Figure 5**). (*E*)-β-ocimene was the main compound in all three plant species and contributed most to the similarity of samples within each

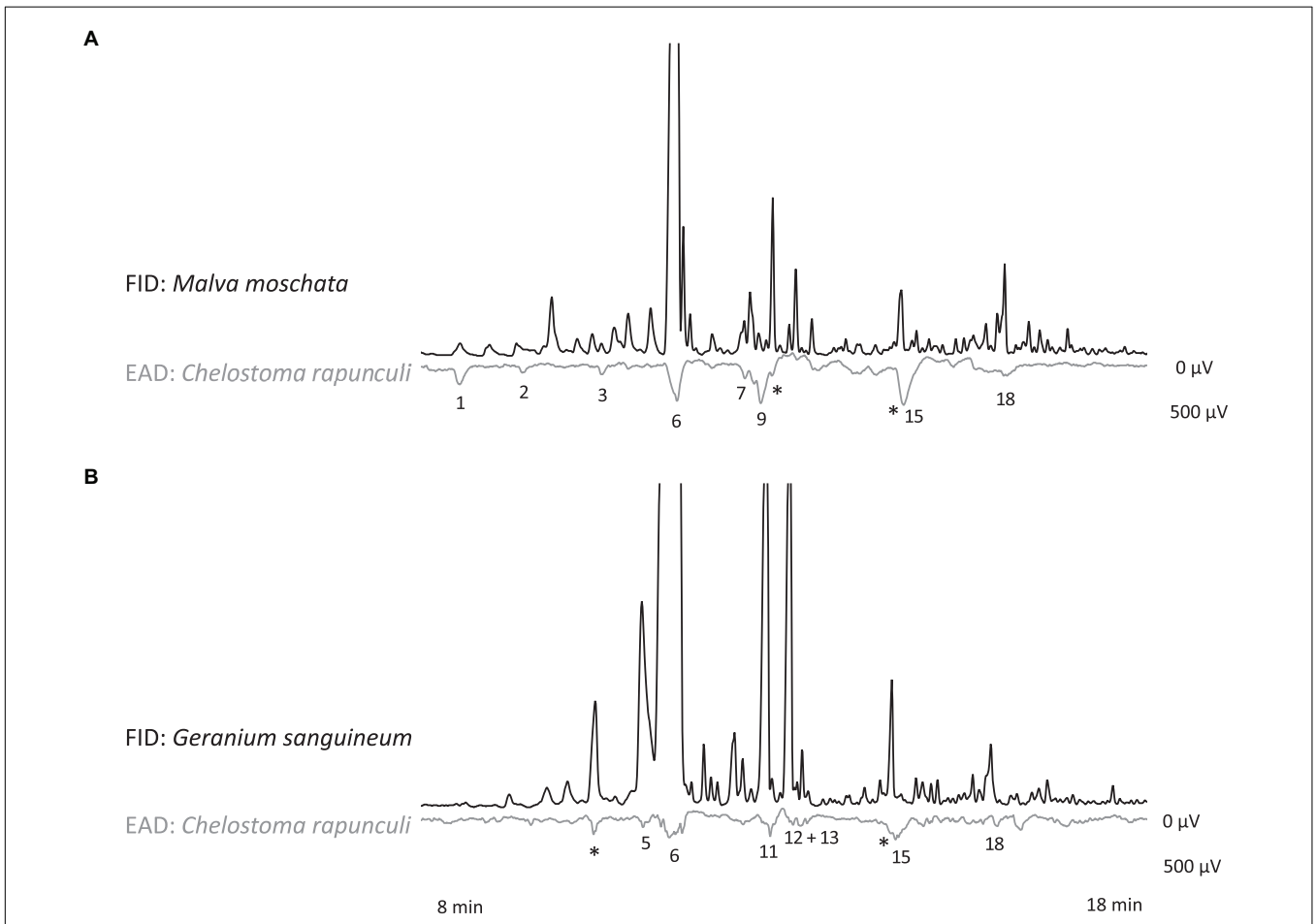

FIGURE 4 | Simultaneous recordings of gas chromatographic (FID) and electroantennographic (EAD) signals for antennae of *C. rapunculi* females and a headspace sample of **(A)** *M. moschata* and **(B)** *G. sanguineum* inflorescences. Responses of the shown individuals are labeled with numbers that correspond to numbers given in **Table 1**. Responses to components of control samples are indicated by an asterisk.

TABLE 1 | Absolute (first line) and relative amounts (mean ± standard error) of GC-EAD active compounds in the headspace samples of inflorescences from *C. trachelium*, *G. sanguineum*, and *M. moschata*.

No.	Chemical compound	RI	*Campanula trachelium* (*N* = 10)	*Geranium sanguineum* (*N* = 13)	*Malva moschata* (*N* = 13)
μg per sample (*N* = 10)			0.95 ± 0.02	190.23 ± 3.19	77.39 ± 0.45
Aliphatic compounds					
8	2-Nonanone	1091	0.33 ± 0.25	–	–
16	(*Z*)-3-Hexenyl isovalerate[a]	1233	–	0.91 ± 0.40	0.66 ± 0.57
Terpenes					
1	Anisole	917	0.01 ± 0.01	0.01 ± 0.01	0.96 ± 0.37
2	Camphene[a]	946	–	0.03 ± 0.02	0.14 ± 0.09
3	β-Myrcene[a]	990	1.51 ± 0.34	1.56 ± 0.30	1.08 ± 0.16
4	Limonene[a]	1028	8.76 ± 2.89	1.04 ± 0.20	0.60 ± 0.06
5	(*Z*)-β-Ocimene[a]	1039	9.09 ± 1.60	8.75 ± 1.38	5.14 ± 0.56
6	(*E*)-β-Ocimene[a]	1054	**36.82 ± 4.07**	**33.18 ± 4.91**	**56.99 ± 4.86**
7	Terpinolene[a]	1088	0.08 ± 0.04	0.11 ± 0.03	0.05 ± 0.02
8	Linalool oxide (furanoid)	1089	0.15 ± 0.07	–	–
8	Guaiacol	1091	0.07 ± 0.05	–	0.03 ± 0.03
9	Linalool[a]	1101	7.18 ± 2.29	1.35 ± 0.21	0.42 ± 0.19
11	(*E*)-4,8-Dimethyl-1,3,7-nonatriene[a]	1117	–	12.90 ± 1.45	4.27 ± 0.73
12	(*E*)-2,6-Dimethyl-1,3,5,7-octatetraene[a]	1131	2.66 ± 0.55	6.19 ± 0.88	8.04 ± 0.98
12	allo-Ocimene[a]	1132	1.90 ± 0.33	3.75 ± 1.18	1.00 ± 0.12
13	neo-allo-Ocimene[a]	1142	0.95 ± 0.27	0.75 ± 0.33	0.41 ± 0.12
15	α-Terpineol[a]	1192	0.33 ± 0.18	0.66 ± 0.25	–
19	α-Copaene[a]	1378	6.66 ± 1.68	0.79 ± 0.26	0.05 ± 0.04
20	Geranylacetone[a]	1453	4.17 ± 1.39	0.26 ± 0.10	0.17 ± 0.06
21	(*E,E*)-α-farnesene[a]	1511	8.08 ± 2.06	11.62 ± 3.23	1.76 ± 0.49
Spiroacetals					
6	1,6-dioxaspiro[4.5]decane	1058	1.50 ± 0.56	0.04 ± 0.02	–
13	(*Z*)-7-methyl-1,6-dioxaspiro[4.5]decane (*Z*-conophthorin)	1140	2.01 ± 0.63	0.01 ± 0.01	–
Benzenoids and phenylpropanoids					
6	Phenylacetaldehyde[a]	1045	3.44 ± 0.90	0.11 ± 0.04	0.31 ± 0.23
10	2-phenylethanol[a]	1115	1.59 ± 1.33	0.02 ± 0.01	0.04 ± 0.03
17	Phenylethyl acetate[a]	1258	0.15 ± 0.11	0.11 ± 0.08	–
Nitrogen-containing compounds					
13	Phenylacetonitrile	1141	–	0.08 ± 0.02	0.04 ± 0.03
Unknowns *m/z*					
4	81, 67, 55[a]	1023	0.16 ± 0.12	5.40 ± 3.48	10.38 ± 4.62
7	91, 119, 134[a]	1080	0.48 ± 0.20	1.52 ± 0.25	1.05 ± 0.15
14	94, 59, 79[a]	1168	0.68 ± 0.18	1.55 ± 0.27	1.25 ± 0.22
14	95, 150, 79[a]	1183	0.08 ± 0.04	0.75 ± 0.13	0.43 ± 0.05
15	109, 43, 91[a]	1209	0.71 ± 0.22	5.24 ± 0.96	3.45 ± 0.69
18	97, 72, 82[a]	1273	0.44 ± 0.31	1.26 ± 0.14	1.20 ± 0.21

Numbers (No.) indicate antennal responses and correspond to the numbers shown in ***Figure 4****. Compounds marked in bold indicate the most abundant compound in each species. Volatiles are listed according to chemical class and Kovats Retention Index (RI). Compounds indicated with* [a] *are also found in green plant parts in lesser amounts.*

plant species. This compound, followed by (*Z*)-β-ocimene and (*E*)-4,8-dimethyl-1,3,7-nonatriene, also contributed most to the similarity between the species. Dissimilarities between *G. sanguineum* and *C. trachelium* were mostly attributable to (*E*)-4,8-dimethyl-1,3,7-nonatriene and one unknown compound (*m/z* 81, 67, 55, RI 1023) between *M. moschata* and *C. trachelium*, followed by α-copaene and limonene in both groups.

Comparison of Floral Colors

The corollas of the investigated plant species were colored UV-blue or blue based on the categories of the bee color

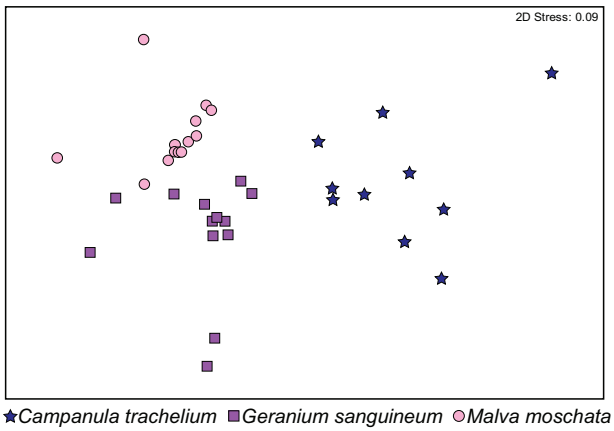

FIGURE 5 | Non-metric multidimensional scaling based on the Bray-Curtis similarities of the semi-quantitative amounts of EAD-active compounds identified in headspace samples from *C. trachelium* ($N = 10$), *G. sanguineum* ($N = 13$), and *M. moschata* ($N = 13$) inflorescences (ANOSIM $R = 0.65$, $P < 0.001$).

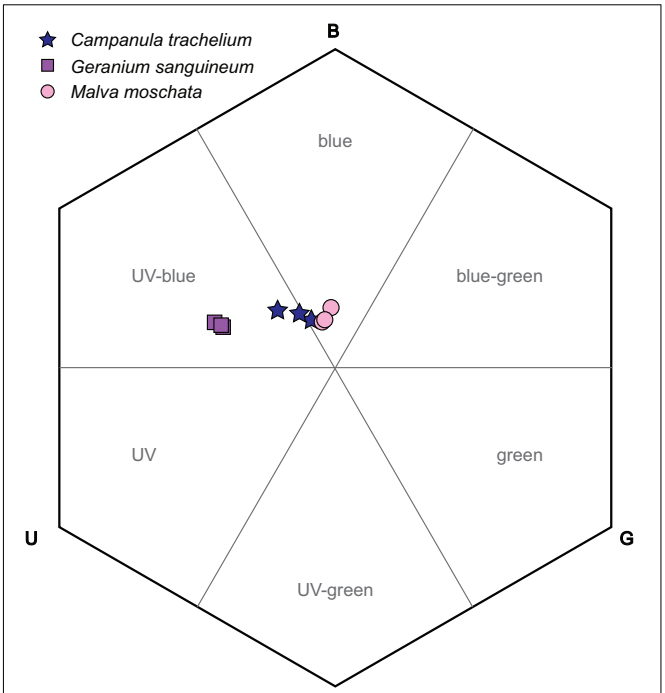

FIGURE 6 | Corolla color loci of *C. trachelium*, *G. sanguineum*, and *M. moschata* in the bee color hexagon based on the excitations of the UV, blue, and green receptors (U, B, G).

hexagon (**Figure 6**). The loci of *C. trachelium* was located at the intersection of both categories, whereas those of *G. sanguineum* and *M. moschata* lay in the UV-blue section and in the blue section, respectively. The smallest distance between species was between *C. trachelium* and *M. moschata* (0.08 units) and largest between *G. sanguineum* and *M. moschata* (0.32 units). The distance to the center of the hexagon (background colors) was smallest for *M. moschata* (0.16 units), intermediate for *C. trachelium* (0.21 units), and largest for *G. sanguineum* (0.38 units).

DISCUSSION

Our experiments show that oligolectic *C. rapunculi* bees are not only attracted by floral cues of the established host plant *C. trachelium*, but also by those of *G. sanguineum* and *M. moschata*. The floral scent and color of the plant species were species-specific but had some of the traits in common.

Pollen specialist bees visit sometimes or even regularly further plant species, besides their pollen hosts to collect nectar, which can explain the attractiveness of non-host floral cues in the absence of host plants. When offered, however, against an attractive host, oligolectic bees clearly prefer the olfactory cues of their host as shown in previous studies (Burger et al., 2010; Milet-Pinheiro et al., 2012). Accordingly, the olfactory cues of *C. trachelium* were more attractive for *C. rapunculi* bees than the ones of *Echium vulgare* and *Potentilla recta* non-hosts (Milet-Pinheiro et al., 2012). The specialized bees seem to be highly tuned in their search image to detect host plants on which they depend for a successful reproduction. Interestingly, in our experiments naïve *C. rapunculi* bees showed no preference for scent cues of its established host *C. trachelium* when it was offered together with either *G. sanguineum* or *M. moschata*.

We hypothesized that the attractiveness to non-host floral cues is explained by similarities in floral traits with *Campanula* hosts. The scent profiles of *C. trachelium*, *G. sanguineum*, and *M. moschata* were species-specific but also showed similarities. Many of the electrophysiologically active compounds, for example (*E*)-β-ocimene, linalool, or 2-phenylethanol, were shared by the analyzed host and non-hosts. However, these compounds are widely distributed floral scent compounds (Knudsen et al., 2006) and probably do not explain why naïve *C. rapunculi* bees were highly attracted by olfactory cues of *G. sanguineum* and *M. moschata* but not by synthetic mixtures or floral scent of *Echium* sp., that contained or emitted these compounds (Milet-Pinheiro et al., 2012, 2013). Instead, naïve bees responded only to samples that contained spiroacetals, which are characteristic scent compounds for *Campanula* (Milet-Pinheiro et al., 2013). Spiroacetals are rarely produced by flowers of other plant species than *Campanula* (Knudsen et al., 2006) and are the key components enabling *C. rapunculi* to find and recognize their hosts (Milet-Pinheiro et al., 2013). In addition, the antennae of *C. rapunculi* are highly sensitive to these compounds, and the bees are capable of detecting tiny amounts (Brandt et al., 2017). Interestingly, we also found small amounts of spiroacetals in the headspace samples of *G. sanguineum*, which might explain the attractiveness of this non-host species to *C. rapunculi* females. In floral scent samples of *M. moschata*, we detected only parts of the characteristic masses for spiroacetals in the chemical analyses, in amounts that were not quantifiable. Therefore, uncertainty remains as to whether this species emits spiroacetals, too. As

we did not identify floral scent attractants that are shared by the studied species, behavioral experiments with synthetic compounds are needed to identify the behaviorally attractive compounds in *M. moschata* and to test whether spiroacetals are indeed involved in the attractiveness of *G. sanguineum* scent cues.

After the *C. rapunculi* bees gained experience in foraging on *Campanula* in the flight cage, they were still attracted by the olfactory cues of *G. sanguineum* and *M. moschata* when presented against an empty control although *G. sanguineum* wasn't that attractive any more than their *Campanula* host. Once the bees have found a reliable pollen source, they are probably not motivated to seek further plant species and prefer the olfactory cues of hosts over non-hosts as observed for *G. sanguineum* when presented against *C. trachelium*. In contrast, the scent of *M. moschata* still had the same attractiveness as that of *C. trachelium*. We cannot explain this behavior based on our results but the bees might have been seeking nectar because we had removed all food sources during the performance of the bioassays.

Behavioral changes with foraging experience were also observed for visual floral traits. As long as the bees were foraging-naïve, visual cues of *Malva* and *Geranium* were not attractive for *C. rapunculi*, but they were later on for foraging-experienced bees. Oligolectic bees are attracted by a range of different wavelengths within a color category, for example blue and UV-blue stimuli attract *C. rapunculi* bees (Milet-Pinheiro et al., 2015), but these colors are not host-specific and are therefore not used to discriminate hosts and non-hosts (Burger et al., 2010). This might explain why the bees showed varying attractiveness toward the tested visual cues depending on the foraging state (naïve vs. experienced) and tested plant species, although all measured color loci were in the range of different *Campanula* species (Milet-Pinheiro et al., 2015). *G. sanguineum* colors could be clearly discriminated from *C. trachelium* by the bees based on the color modeling, but had the same attractiveness as *C. trachelium* when the visual cues were tested against each other. *M. moschata* flowers were similarly colored but the visual cues of *M. moschata* inflorescences were less attractive than *C. trachelium*. It seems that not only the specific color (dominant wavelength) of the petals explains the attractiveness of visual cues but further visual traits such as shape and size. As the strength of the contrast that a floral color makes to its background is correlated with attractiveness in other bees (Lunau et al., 1996), this visual trait might also influence the choice behavior of *C. rapunculi*. The color modeling showed that the spectrum of *M. moschata* is less detectable against background colors compared with that of the other studied plant species, and, accordingly, in the bioassays, *M. moschata* were less attractive when offered in a choice with *C. trachelium*.

Our own observations in the flight cage showed that *C. rapunculi* bees visited *M. moschata* flowers frequently in the absence of *Campanula* although the bees removed the pollen carefully before they returned to their nest. Interestingly, we also observed increasing numbers of individuals continuously visiting *M. moschata* flowers in the years after our experiments have been performed when we established a bee population outside the flight cage, and *M. moschata* plants were still present at a high density in the close surroundings. The bees had pollen attached to their scopa, but if the bees indeed actively collected pollen or were only contaminated while consuming nectar was not examined. Malvaceae pollen is mechanically protected by spines against collection by at least corbiculate bees (Lunau et al., 2015), but some Malvaceae oligoleges are able to transport their pollen (Schlindwein et al., 2009; Gaglianone, 2000). The observed visits do not mean that the bees are incorporating *Malva* and *Geranium* spp. as new hosts, but it can be a first step. As the host choice behavior in *Chelostoma* bees is thought to be restricted by physiological limits to digest different pollen diets (Praz et al., 2008b; Sedivy et al., 2008), an important experiment would be to study the development of *C. rapunculi* larvae that are fed with pure or different ratios of *Malva* and *Geranium* pollen in future studies. Beside pollen properties, other floral traits were also hypothesized to influence the floral preferences in this bee clade (Sedivy et al., 2008). When *Chelostoma* bees incorporated a new host that is phylogenetically unrelated to previous hosts, the new host had a striking high floral similarity to the previous one (Sedivy et al., 2008). Our study also revealed similarities in floral traits between host and each of the non-hosts: The floral colors of *G. sanguineum* and *M. moschata* were within the range of *Campanula* colors and we found an overlap of floral scent components of that, particularly, spiroacetals in *G. sanguineum* might be an important floral signal for *C. rapunculi*. Other floral cues were in contrast taxon-specific. So, it remains unknown whether the identified floral cues explain the attractiveness of the non-host plants. Follow-up studies and monitoring events are needed to fully understand the interaction between *C. rapunculi* bees and *Malva* and *Geranium* flowers. As more than 70% of plant species, including several of *C. rapunculi's* host species, have declined in Germany during the last decades (Eichenberg et al., 2020), a broadening of the host range might reduce the high dependence of *C. rapunculi* on *Campanula* plants.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MA and HB designed the study. NJ and HB performed the experiments and analyzed the data. HB wrote the manuscript with contributions from NJ and MA. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We gratefully acknowledge K. Brandt, K. C. Heuel, and K. Babucke for their help to perform behavioral experiments, P. Milet-Pinheiro and S. Dötterl for advice in chemical analyses, and the people of the Botanical Garden of Ulm to grow plants for the experiments. We also thank Theresa Jones for linguistic advice and K. Brandt and two reviewers for valuable comments. All experiments comply with the current laws of the country in which they were performed: in this case, Germany.

REFERENCES

Brandt, K., Dötterl, S., Francke, W., Ayasse, M., and Milet-Pinheiro, P. (2017). Flower visitors of campanula: are oligoleges more sensitive to host-specific floral scents than polyleges? *J. Chem. Ecol.* 43, 4–12. doi: 10.1007/s10886-016-0802-z

Burger, H., Dötterl, S., and Ayasse, M. (2010). Host-plant finding and recognition by visual and olfactory floral cues in an oligolectic bee. *Funct. Ecol.* 24, 1234–1240. doi: 10.1111/j.1365-2435.2010.01744.x

Burger, H., Dötterl, S., Häberlein, C. M., Schulz, S., and Ayasse, M. (2012). An arthropod deterrent attracts specialised bees to their host plants. *Oecologia* 168, 727–736. doi: 10.1007/s00442-011-2136-4

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Compar. Physiol. A Sens. Neural Behav. Physiol.* 170, 533–543.

Chittka, L., Shmida, A., Troje, N., and Menzel, R. (1994). Ultraviolet as a component of flower reflections, and the colour perception of Hymenoptera. *Vis. Res.* 34, 1489–1508. doi: 10.1016/0042-6989(94)90151-1

Clarke, K., and Gorley, R. (2006). *Primer v6: User Manual/Tutorial*. Plymouth: Primer-E.

Dötterl, S., and Jürgens, A. (2005). Spatial fragrance patterns in flowers of *Silene latifolia*: lilac compounds as olfactory nectar guides? *Plant Syst. Evol.* 255, 99–109. doi: 10.1007/s00606-005-0344-2

Dötterl, S., and Vereecken, N. J. (2010). The chemical ecology and evolution of bee-flower interactions: a review and perspectives. *Can. J. Zool.* 88, 668–697. doi: 10.1139/Z10-031

Dyer, A. G., and Chittka, L. (2004). Biological significance of distinguishing between similar colours in spectrally variable illumination: bumblebees (*Bombus terrestris*) as a case study. *J. Compar. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 190, 105–114. doi: 10.1007/s00359-003-0475-2

Eichenberg, D., Bowler, D. E., Bonn, A., Bruelheide, H., Grescho, V., Harter, D., et al. (2020). Widespread decline in Central European plant diversity across six decades. *Glob. Chang. Biol.* 27, 1097–1110. doi: 10.1111/gcb.15447

Gaglianone, M. C. (2000). Behavior on flowers, structures associated to pollen transport and nesting biology of *Perditomorpha brunerii* and *Cephalurgus anomalus* (Hymenoptera: Colletidae, Andrenidae). *Rev. Biol.Trop.* 48, 89–99.

Knudsen, J. T., Eriksson, R., Gershenzon, J., and Stahl, B. (2006). Diversity and distribution of floral scent. *Bot. Rev.* 72:1. doi: 10.1663/0006-8101(2006)72[1:dadofs]2.0.co;2

Lunau, K., Wacht, S., and Chittka, L. (1996). Colour choices of naive bumble bees and their implications for colour perception. *J. Compar. Physiol. A Sens. Neural Behav. Physiol.* 178, 477–489.

Lunau, K., Piorek, V., Krohn, O., and Pacini, E. (2015). Just spines—mechanical defense of malvaceous pollen against collection by corbiculate bees. *Apidologie* 46, 144–149.

Maia, R., Eliason, C. M., Bitton, P. P., Doucet, S. M., and Shawkey, M. D. (2013). pavo: an R package for the analysis, visualization and organization of spectral data. *Methods Ecol. Evol.* 4, 906–913.

Michener, C. D. (2007). *The Bees of the World*. Baltimore: Johns Hopkins University Press.

Milet-Pinheiro, P., Ayasse, M., Dobson, H., Schlindwein, C., Francke, W., and Dötterl, S. (2013). The chemical basis of host-plant recognition in a specialized bee pollinator. *J. Chem. Ecol.* 39, 1347–1360. doi: 10.1007/s10886-013-0363-3

Milet-Pinheiro, P., Ayasse, M., Schlindwein, C., Dobson, H., and Dötterl, S. (2012). Host location by visual and olfactory floral cues in an oligolectic bee: innate and learned behavior. *Behav. Ecol.* 23, 531–538. doi: 10.1093/beheco/arr219

Milet-Pinheiro, P., Ayasse, M., and Dötterl, S. (2015). Visual and olfactory floral cues of Campanula (Campanulaceae) and their significance for host recognition by an oligolectic bee pollinator. *PLoS One* 10:e0128577. doi: 10.1371/journal.pone.0128577

Müller, A., Diener, S., Schnyder, S., Stutz, K., Sedivy, C., and Dorn, S. (2006). Quantitative pollen requirements of solitary bees: implications for bee conservation and the evolution of bee-flower relationships. *Biol. Conserv.* 130, 604–615. doi: 10.1016/j.biocon.2006.01.023

Müller, A., and Kuhlmann, M. (2008). Pollen hosts of western palaearctic bees of the genus *Colletes* (Hymenoptera: Colletidae): the Asteraceae paradox. *Biol. J. Linnean Soc.* 95, 719–733. doi: 10.1111/j.1095-8312.2008.01113.x

Nebel, M., Philippi, G., Quinger, B., Rösch, M., Schiefer, J., Sebald, O., et al. (1993). *Die Farn-und Blütenpflanzen Baden-Württembergs*. Stuttgart: E. Ulmer.

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of Hymenopteran insects and their receptor-based colour vision. *J. Compar. Physiol. A Sens. Neural Behav. Physiol.* 170, 23–40. doi: 10.1007/BF00190398

Praz, C. J., Müller, A., and Dorn, S. (2008a). Host recognition in a pollen-specialist bee: evidence for a genetic basis. *Apidologie* 39, 547–557. doi: 10.1051/apido:2008034

Praz, C. J., Müller, A., and Dorn, S. (2008b). Specialized bees fail to develop on non-host pollen: do plants chemically protect their pollen? *Ecology* 89, 795–804. doi: 10.1890/07-0751.1

Sedivy, C., Praz, C. J., Müller, A., Widmer, A., and Dorn, S. (2008). Patterns of host-plant choice in bees of the genus *Chelostoma*: the constraint hypothesis of host-range evolution in bees. *Evolution* 62, 2487–2507. doi: 10.1111/j.1558-5646.2008.00465.x

Schlindwein, C., Pick, R. A., and Martins, C. F. (2009). Evaluation of oligolecty in the Brazilian bee *Ptilothrix plumata* (Hymenoptera, Apidae, Emphorini). *Apidologie* 40, 106–116.

Westrich, P. (2018). *Die Wildbienen Deutschlands*. Stuttgart: Eugen Ulmer.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 25 August 2021
doi: 10.3389/fevo.2021.724712

The Size of it: Scant Evidence That Flower Size Variation Affects Deception in Intersexual Floral Mimicry

Avery L. Russell[1†], Stephanie R. Sanders[1†], Liam A. Wilson[1] and Daniel R. Papaj[2]*

[1] Department of Biology, Missouri State University, Springfield, MO, United States, [2] Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, United States

Mutualisms involve cooperation, but also frequently involve conflict. Plant-pollinator mutualisms are no exception. To facilitate animal pollination, flowering plants often offer pollen (their male gametes) as a food reward. Since plants benefit by maximizing pollen export to conspecific flowers, we might expect plants to cheat on pollen rewards. In intersexual floral mimicry, rewarding pollen-bearing male flowers (models) are mimicked by rewardless female flowers (mimics) on the same plant. Pollinators should therefore learn to avoid the unrewarding mimics. Plants might impede such learning by producing phenotypically variable flowers that cause bees to generalize among models and mimics during learning. In this laboratory study, we used partially artificial flowers (artificial petals, live reproductive parts) modeled after *Begonia odorata* to test whether variation in the size of rewarding male flowers (models) and unrewarding female flowers (mimics) affected how quickly bees learned both to recognize models and to reject mimics. Live unrewarding female flowers have 33% longer petals and have 31% greater surface area than live rewarding male flowers, which bees should easily discriminate. Yet while bees rapidly learned to reduce foraging effort on mimics, learning was not significantly affected by the degree to which flower size varied. Additionally, we found scant evidence that this was a result of bees altering response speed to maintain decision accuracy. Our study failed to provide evidence that flower size variation in intersexual floral mimicry systems exploits pollinator cognition, though we cannot rule out that other floral traits that are variable may be important. Furthermore, we propose that contrary to expectation, phenotypic variability in a Batesian mimicry system may not necessarily have significant effects on whether receivers effectively learn to discriminate models and mimics.

Keywords: signal detection, Batesian mimicry, cognition, learning, intersexual mimicry, imperfect mimicry, flower size, generalization

OPEN ACCESS

Edited by:
Casper J. Van Der Kooi, University of Groningen, Netherlands

Reviewed by:
Scarlett Howard, Deakin University, Australia
Jurene Ellen Kemp, University of Stirling, United Kingdom

***Correspondence:**
Avery L. Russell
AveryRussell@missouristate.edu

†These authors share first authorship

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *14 June 2021*
Accepted: *05 August 2021*
Published: *25 August 2021*

Citation:
Russell AL, Sanders SR, Wilson LA and Papaj DR (2021) The Size of it: Scant Evidence That Flower Size Variation Affects Deception in Intersexual Floral Mimicry. Front. Ecol. Evol. 9:724712. doi: 10.3389/fevo.2021.724712

INTRODUCTION

Conflicts of interest are common in plant-pollinator mutualisms (Bronstein, 2001; Thompson et al., 2013; van der Kooi et al., 2021). To facilitate pollination, flowering plants typically offer pollinators resources, such as pollen and nectar ("floral rewards"; Simpson and Neff, 1981). However, when floral rewards are costly to the plant to produce, maintain, and/or give up, the

plant may benefit by exploiting the pollinator. For instance, the plant may benefit by withholding rewards or falsely advertising rewards, if it is still pollinated (Schiestl, 2005; Essenberg, 2021). Likewise, because foraging can be costly, the pollinator may benefit by exploiting the plant. For example, the pollinator might reduce time spent foraging by bypassing the floral sex organs to extract floral rewards ("robbing") (Maloof and Inouye, 2000; Barker et al., 2018). Such reciprocal exploitation between plant and pollinator is common and frequently involves plants deceiving pollinators into pollinating flowers that lack rewards. Batesian mimicry, in which pollinators are deceived into visiting rewardless flowers that mimic rewarding flowers (models) is particularly widespread and is found in more than 32 plant families (Schiestl and Johnson, 2013; Johnson and Schiestl, 2016; de AvilaJr., Oleques et al., 2017). Successful Batesian mimicry is thought to rely on exploiting constraints on pollinator cognition, because pollinators otherwise will learn to avoid less profitable flowers (Smithson and Macnair, 1997; Whitehead and Peakall, 2012; Russell et al., 2020). Yet how constraints on pollinator learning are exploited in Batesian floral mimicry systems is still poorly understood (Dukas, 1987; Gigord et al., 2001; Schiestl and Johnson, 2013; Johnson and Schiestl, 2016; Goodrich and Jurgens, 2017; but see Kunze and Gumbert, 2000; de Jager and Ellis, 2014; Russell et al., 2020).

Naïve pollinators are expected to adjust their behavior with experience to avoid visiting rewardless mimics, because visiting them is costly to the pollinator (e.g., Ayasse et al., 2000; Schiestl, 2005). Thus, Batesian floral mimics might maximize their benefits by reducing how quickly and how well pollinators learn to discriminate mimics from models (Dukas, 1987; Abbott and Sherratt, 2013; de Jager et al., 2016). One way in which Batesian floral mimics might impede learning is by closely matching the phenotype of models (Sherratt, 2002; Kikuchi and Pfennig, 2013; de Jager et al., 2016). Yet accurate ("perfect") mimicry may not always be achievable, as when there are developmental constraints on the precision of the mimicry (Kikuchi and Pfennig, 2013), or may not even be adaptive, as when imperfect mimicry exploits pollinator sensory biases (Schaefer and Ruxton, 2010; Russell et al., 2020). Given constraints on pollinator cognition, perfect mimicry may also be unnecessary for successful Batesian mimicry. For example, even if floral models and mimics vary in phenotype, pollinators might generalize models and mimics (Wright and Smith, 2003; Lynn et al., 2005). In non-pollinator systems, variation is in fact thought to promote generalization (Amézquita et al., 2013; Gamberale-Stille et al., 2018; Arias et al., 2020). This is thought to be a result of variation increasing the width of the signal distribution, which enhances the perceived similarity of model and mimic (**Figure 1**; Lynn et al., 2005). While generalization is thought to be a fundamental property of learning in animals (Kalish, 1969; Mackintosh, 1974; Enquist and Johnstone, 1997; Cheng, 2002), its role in mediating the success of Batesian mimicry has seldom been examined (but see Ham et al., 2006; Gamberale-Stille et al., 2018).

In flowering plant species that exhibit intersexual floral Batesian mimicry, a single plant species produces male flowers that typically offer a pollen reward to pollinators (primarily bees) and female flowers that are deceptive rewardless mimics (Johnson and Schiestl, 2016). Intraspecific phenotypic differences between male and female flowers are common, with differences in size being particularly obvious and well-documented (Ågren and Schemske, 1995; Schemske and Ågren, 1995; Castillo et al., 2012). Likewise, intrasexual flower size variation is also common (Ågren and Schemske, 1995; Schemske and Ågren, 1995; Galen, 1999; Castillo et al., 2012; Hattori et al., 2016). Given that pollinators, such as bees can learn flower size cues in other contexts and may generalize among different sized flowers of a given plant (Yoshioka et al., 2007; Essenberg et al., 2015; Dixit et al., 2020), intrasexual flower size variation in plant species with intersexual floral mimicry may function to promote pollinator generalization while learning.

How then might a pollinator respond to exploitation by a plant that uses variation in models and/or mimics as a strategy? One possibility is that the pollinator may compensate for a more challenging learning task *via* a speed-accuracy tradeoff (Chittka et al., 2003; Ings and Chittka, 2008; Kulahci et al., 2008; Chittka et al., 2009). In other words, when uncertainty is high, such as when model and mimic flower phenotypes are highly variable and overlapping in phenotype, the pollinator may take more time to decide whether to reject or visit a given flower vs. when uncertainty is low, such as when model and mimic flower phenotypes are relatively invariant and have relatively low phenotypic overlap.

In this laboratory study, we tested whether intrasexual flower size variation in a simultaneously monoecious plant species (*Begonia odorata*) exhibiting intersexual Batesian mimicry caused generalization for a generalist bumble bee (*Bombus impatiens*). Here, intersexual mimicry is observed in terms of overall flower color pattern and divided styles resembling anthers in form and color, i.e., pseudanthery (Johnson and Schiestl, 2016; de Jager and Anderson, 2019). We hypothesized that when intrasexual flower size did not vary, bees would learn more quickly to avoid female flowers than when intrasexual flower size varied. We assessed differences in learning by examining how the rate of correct decisions (approaching and landing on models and approaching but not landing on mimics), incorrect decisions (approaching but not landing on models, approaching and landing on mimics), correct detections (approaching and landing on models), and correct rejections (approaching but not landing on mimics, i.e., false alarms) changed with experience (following Russell et al., 2020). We also predicted that bees might avoid exploitation by the plant to some extent *via* a speed-accuracy tradeoff and thus take more time to make decisions when intrasexual flower size varied vs. when it did not. To manipulate flower size precisely we used artificial corollas that closely resembled live corollas, to which we attached live reproductive parts (**Figure 2**).

MATERIALS AND METHODS

Test Subjects

We maintained three colonies (Koppert Biological Systems, Howell, MI, United States) of the common eastern bumble bee *Bombus impatiens* following Russell et al. (2020). In brief, we allowed colonies to forage freely on 2 M sucrose solution

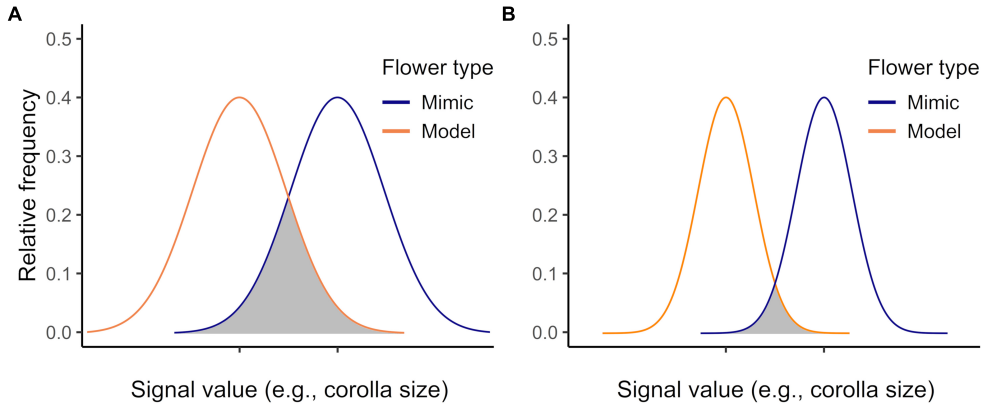

FIGURE 1 | How signal parameters of models and mimics influence receiver behavioral responses. Signal parameters are modeled as Gaussian probability density functions. The more the signal distributions overlap, the greater the uncertainty of signal stimuli for the receiver (grey shading; compare greater overlap in "**A**" to "**B**") (see also Lynn et al., 2005).

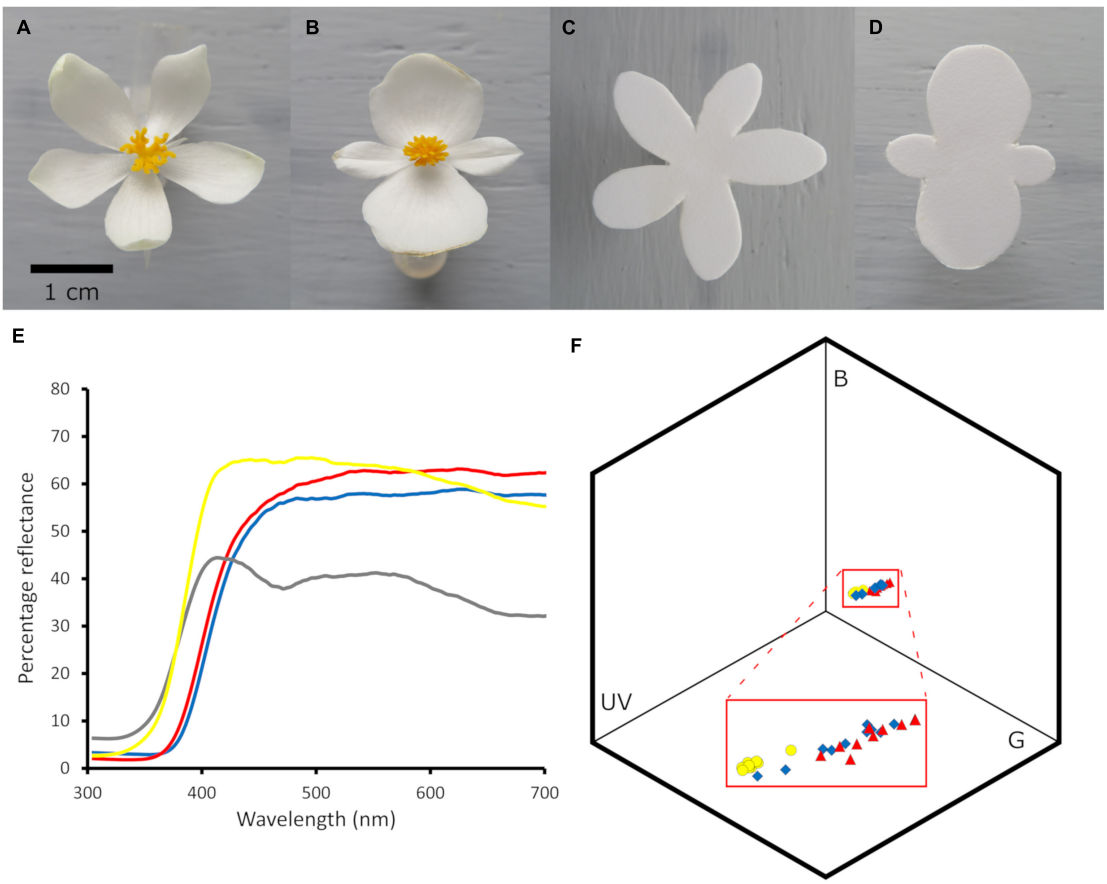

FIGURE 2 | Imperfect mimicry among female (mimics) and male (models) in *Begonia odorata* flowers. **(A)** Female and **(B)** male flowers and artificial corollas of **(C)** female and **(D)** male medium-sized flowers. **(E)** The mean reflectance spectra of the female (red line) and male (blue line) flowers, the artificial corolla (yellow line), and the arena background (grey line) against which flowers were displayed; spectra smoothed using a 100 point moving average in Microsoft Excel. **(F)** The loci in *Bombus impatiens* color space of male petals (blue diamonds), female petals (red triangles), and artificial corollas (yellow circles) against the test arena background: artificial corollas resemble the color of live flower corollas (N = 10, 10, 10 male, female, and artificial flowers, respectively). On average, artificial petals and live petals differed from each other by 0.09 color units and from the background by 0.14 and 0.23 color units, respectively.

and pulverized honeybee-collected pollen (Koppert Biological Systems) from artificial feeders within enclosed foraging arenas (length, width, height: 82 × 60 × 60 cm) set to a 14 h: 10 h light: dark cycle.

We used fresh male and female flowers with mature anthers and styles, respectively, from 10 simultaneously monoecious *Begonia odorata* plants raised in a university greenhouse with supplemental halogen lights to extend day length to a 14: 10 h cycle and with fertilizer applications every other week (Plant Tone, NPK 5: 3: 3). While female *B. odorata* flowers are rewardless and produce neither pollen nor nectar, male *B. odorata* flowers offer pollen, their sole reward to their primary pollinators, bees; bumble bees are among the bee genera known to visit closely related *Begonia* species (Schemske et al., 1996; Pemberton and Wheeler, 2006; Wyatt and Sazima, 2011; de AvilaJr., Oleques et al., 2017).

Female *B. odorata* flowers closely resemble male flowers in bumble bee color vision (**Figure 2**); both flower sexes have creamy white dissected petals, and the female flower's yellow and highly divided styles closely resemble the male flower's numerous yellow stamens (see also Russell et al., 2020). Strikingly, the frontal surface area of female flowers is on average 30.7% greater than that of male flowers (a difference of 141.4 mm^2) and female flowers have on average 33.3% longer petals than male flowers (a difference of 4.8 mm) (N = 84 and 86 female and male flowers measured, respectively; from 7 plants; ∼12 flowers/plant; **Figure 3**). In addition to significant intersexual differences in flower size, intrasexual flower size variation is substantial and flower sexes overlap in size (**Figure 3**). We used ImageJ (National Institutes of Health, Bethesda, MD)[1] to measure flowers that had been photographed at a standard height with their petals gently flattened by glass slides.

Experiment

We tested whether the degree to which corolla size varied influenced how initially flower-naïve bees learned to sample among models (male flowers) and mimics (female flowers). We examined three primary components of sampling behavior ("visits") made by bees visiting arrays of 18 flowers: approaches, landings without sonication (on male flowers such landings typically involved the bee collecting pollen *via* a behavior termed scrabbling; see Russell et al., 2017 for a description), and landings with sonication ("buzzes" or "buzzing") (see flow diagram **Supplementary Figure 1**). An approach was defined as the bee in flight greatly reducing its velocity while facing the flower within 3 cm of the flower. All landings were preceded by an approach (i.e., "correct detections" for models; "false alarms" for mimics) and landings on male flowers (models) nearly exclusively involved collection of pollen. Not all approaches were followed by a landing (i.e., "missed detections" for models; "correct rejections" for mimics). Buzzes, which indicated an attempt at extracting pollen whether or not it was available, were identified by their distinctive sound and occurred only after a bee had landed (see Russell et al., 2016a). Buzzing a male flower constituted a correct behavioral response and buzzing a female flower constituted an incorrect behavioral response.

To precisely manipulate corolla size variation, we constructed artificial plastic corollas (from polypropylene Sterilite container lids) closely matched to the color, size, and shape of live *B. odorata* corollas (**Figures 2**, **4**). The flowers used in behavioral trials were a combination of artificial plastic corollas and the live reproductive parts of freshly clipped male and female flowers, which were hot-glued into the center of the plastic corollas just prior to behavioral trials. Surrogate male flowers had artificial male corollas with male reproductive parts and surrogate female flowers had artificial female corollas with female reproductive parts (**Figures 2**, **4**).

We color matched artificial and live corollas using reflectance spectra of flowers and the arena wall background against which the flowers would be presented in behavioral trials. We measured a variety of plastics and papers and selected the material with qualitatively the least overall deviation from the reflectance spectra of the live flowers' corollas. Each reflectance spectrum consisted of the mean of 10 measurements, taken from different flowers or parts of the arena wall background. All measurements were taken using an UV-VIS spectrometer (Ocean Optics USB2000) with a tungsten-deuterium light source (Ocean Optics DH2000-BAL) and a fluoropolymer white standard (WS-1-SL Spectralon; NH, United States). An RPH reflectance probe (Ocean Optics) was held at constant height and 45° angle above the samples using a holder that shielded the probe from extraneous light. All reflectance measurements were taken using a 5 ms integration time with 500 ms averaging in the same session. Irradiance within the flight arena was measured at the center of the flower array using a Q400-7-SR UV/VIS optical fiber (Ocean Optics), a CC-3-UV-S cosine-corrected (180 degrees) irradiance probe (Ocean Optics), and a tungsten-deuterium calibration light source (Ocean Optics DH-3P-CAL) and a 50 ms integration time and 50 ms averaging.

To characterize what bees perceived, we used our reflectance and irradiance measurements to plot corollas within a color space (e.g., the color hexagon) for *B. impatiens* following Russell et al. (2016b). In brief, the color space diagram was constructed following Chittka (1992), using receptor spectral sensitivities for *B. impatiens* from Skorupski and Chittka (2010) and transformed to spectral sensitivity curves following Stavenga et al. (1993). We used the arena wall on which the flowers were displayed as the background stimulus for the color hexagon and the irradiance of the overhead arena lights in calculations of receptor excitation values.

We split flower-naïve bees approximately equally among two treatments: a no corolla size variation treatment ("control treatment") and a treatment in which corolla size varied ("variation treatment") (**Figure 4**). In the control treatment, we constructed two types of artificial corollas, which were modeled after medium-sized *B. odorata* male and female flowers growing at the time, respectively (**Figures 3**, **4**). In the variation treatment, we constructed six types of artificial corollas that were modeled after small, medium, and large *B. odorata* male and female flowers, respectively (**Figures 3**, **4**). All artificial corollas were thus the size and shape of a sample of live flowers (petal length in

[1] http://imagej.nih.gov/ij/

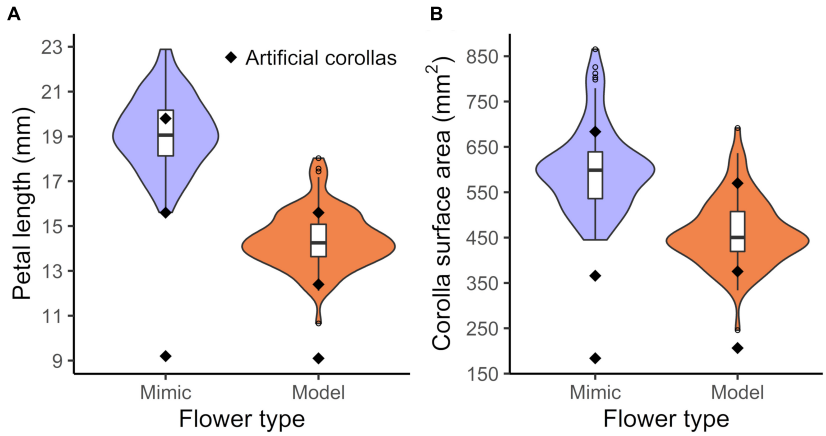

FIGURE 3 | Variation in petal length and corolla surface area of *Begonia odorata* model (male) and mimic (female) flowers and corresponding measurements of artificial corollas. Boxplots of variation in mimic and model **(A)** mean petal length per flower and **(B)** corolla surface area. Artificial corollas (black diamonds) and distribution for the live flowers (violin plots) are also plotted. The artificial corollas were modeled on flowers growing prior to the COVID-19 lockdown—the lockdown delayed measuring a complete distribution of flowers and it appears the distribution of sizes subsequently shifted and narrowed. *N* = 84 and 86 mimic and model flowers, respectively.

mm, male: 9.1, 12.4, 15.6; female: 9.2, 15.6, 19.8; surface area in mm^2, male: 183.5, 366.0, 683.6; female: 206.6, 375.5, 570.0; small, medium, and large artificial corollas, respectively). We visually inspected plants to find small and large flowers for modeling and we used their measurements to then find flowers of intermediate petal length. In both treatments, flowers were spaced 7 cm apart in a 5 × 4 Cartesian grid design on the arena wall and flower sexes (in terms of reproductive organs) were alternated by position and presented in equal frequency to bees (**Figure 4**). In the variation treatment, to avoid any possible position bias, we systematically distributed each flower size class equivalently across the array and changed the pattern of alternation each trial.

To initiate a behavioral trial, flowers were set up and a single flower-naïve worker bee was gently captured from the foraging arena using a 40 dram vial (Bioquip) and immediately released in the center of the test arena following Russell et al. (2017). We terminated the trial after 80 visits (or earlier if the bee stopped visiting flowers for 5 min) to avoid bees depleting models of pollen rewards. To terminate the trial we captured the bee in a 40 dram vial and euthanized it (mean 62 visits; range 7–80 visits; *N* = 37 bees). We tested bees individually and cleaned artificial corollas with 70% ethanol and glued on fresh reproductive parts of flowers for each trial. All trials were video recorded to permit analysis of response speed (see section "Data Analyses").

Data Analyses

All data were analyzed using R v.4.1.0 (R Development Core Team, 2021).

To analyze flower-naïve bees' naïve preference for models (male flowers) vs. mimics (female flowers) on their first flower landing (*N* = 37 bees), we used a G-test (DescTools package; Signorell et al., 2019). From all subsequent analyses we excluded four bees that did not pack pollen into their pollen baskets.

Using two different analyses, we examined how experience and corolla size variation affected the sampling behavior of initially naive bees. In the first analysis we restricted our analysis to bees that had reached a standard learning criterion of 8 of the last 10 visits made to the rewarding models, analyzing only visits up to this learning criterion (*N* = 26 bees). By excluding bees that failed to learn (*N* = 7), we reasoned we would be more likely to find evidence of how learned responses were affected by size variation. We fit a generalized linear mixed model with a binomial distribution (GLMM) using the glmmTMB() function (glmmTMB package; Magnusson et al., 2018), specifying type II Wald chi-square (χ^2) tests *via* the Anova() function (car package; Fox, 2015). We checked model assumptions *via* the DHARMa package (Hartig, 2018). The response variable was sampling behavior ("correct decision," combining correctly rejecting mimics and correctly detecting models) and the explanatory variables were "treatment" (control or variation) and "visit number" (experience). We included "bee" as a random factor, with "visit number" as repeated measures within bee (bee within colony would not converge). To examine whether corolla size variation affected the mean number of flower visits to reach the learning criterion, we fit a *t*-test *via* the t.test() function in R (assumptions of normality and equal variance were met *via* Shapiro-Wilk and F tests, respectively; mgcv package; Wood, 2021).

In the second analysis we fit and checked GLMMs as above to analyze how corolla size variation and experience affected the sampling behavior of all initially naïve bees (including all their visits; *N* = 33 bees). The response variable was sampling behavior (either "correct decision," "correct detection," "correct rejection," "missed detection," "false alarm," "landing," or "sonication given landing") and the explanatory variables were "treatment" and "visit number." We included "bee" and "colony" as random factors, with "visit number" as repeated measures within bee, within colony.

Because response time (flight time between sampling different flowers; i.e., the time between leaving a given flower and

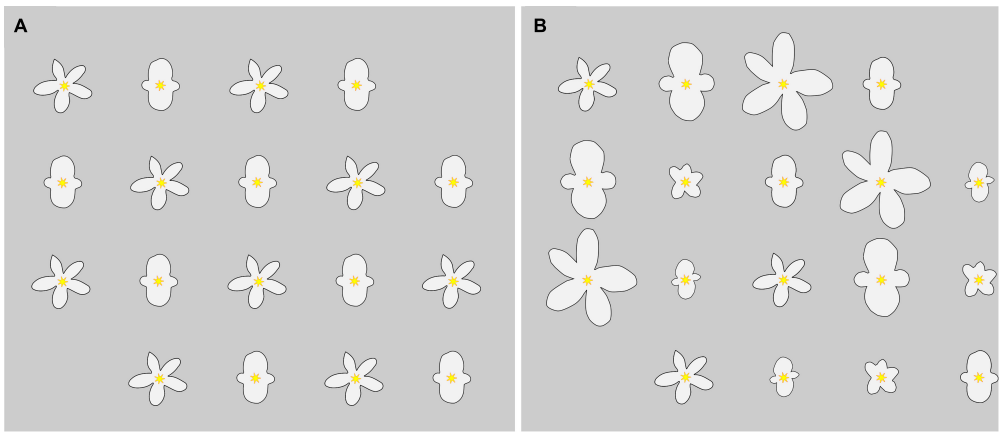

FIGURE 4 | Two possible arrangements of flowers in a 5 × 4 Cartesian grid design for the two treatments. In the **(A)** no corolla size variation treatment ("control treatment"), all artificial corollas were modeled after medium-sized *B. odorata* male and female flowers, alternated by position. In the **(B)** corolla size variation treatment ("variation treatment"), artificial corollas were modeled after small, medium, and large-sized *B. odorata* male and female flowers, alternated by position in terms of reproductive organs and with size classes systematically distributed equivalently across the array.

landing or rejecting the next flower, following Chittka et al., 2003) is known to trade off with discrimination accuracy in bumble bees (Chittka et al., 2003; Kulahci et al., 2008), we analyzed how corolla size variation, flower sex, discrimination accuracy, and experience affected response time by fitting a GLMM (N = 29 bees). The response variable was "response time" and the explanatory variables were "treatment," "flower sex," "accuracy" (whether the decision was correct or incorrect), and "visit number." Random factors were specified as above. We added 0.1 to the response variable and log-transformed it and thereby normalized the residuals. To analyze how response time affected decision accuracy, we used a linear model (LM), with "mean correct decision" (the proportion of visits that involved a correct decision) as the response variable and "treatment" and "mean response time" as the explanatory variables. To characterize response time, we examined digital footage of behavioral trials frame by frame using Avidemux (version 2.7.6) and measured the time between the first 30 visits to the nearest 0.1 s for each behavioral trial. We timed a response starting with the bee ending its visit (i.e., having landed, the bee began beating its wings to leave, or, having approached a flower, the bee turned and accelerated away from the flower) and ending with the bee making its next visit. Of 813 measurements, we deleted 4 identified as outliers by the plot_model() function (sjPlot package; Lüdecke et al., 2021). From this analysis we also excluded 4 bees with corrupted video data or that had completed fewer than 10 approaches.

RESULTS

Corolla Size Variation Had Little Effect on How Bees Learned to Sample

Initially flower-naïve bees strongly and significantly preferred mimics (rewardless female flowers) over models (rewarding male flowers), with 70.3% of first landings being on mimics (G test: G = 6.26, P < 0.013, N = 37 bees). Nonetheless, initially flower-naïve bees rapidly learned to discriminate between mimic and model flowers (**Figure 5**). Bees in both the control and corolla variation treatment that reached the learning criterion made proportionally more correct decisions (combining correctly rejecting mimics and correctly detecting models) across consecutive visits (**Figure 5A**; GLMM: χ^2_1 = 12.71, P < 0.0004). However, these bees did not show any differences in learning between control and corolla variation treatments (**Figure 5A**; GLMM: treatment effect: χ^2_1 = 1.47, P = 0.226; treatment × experience effect: χ^2_1 = 0.43, P = 0.512) and both sets of bees required a similar number of flower visits to reach the learning criterion (t-test: $t_{19.94}$ = 0.942, P = 0.357: mean no. visits ± SE: variation: 25 ± 3.4; control: 21 ± 2.3; N = 26 bees).

For subsequent analyses we assessed learning by all initially flower-naïve bees (including those that did not reach the learning criterion) across all their visits, including visits past the learning criterion. These bees also made proportionally more correct decisions with experience (**Figure 5B**; GLMM: χ^2_1 = 23.53, P < 0.0001; N = 33 bees). This effect of experience was also unaffected by corolla size variation (**Figure 5B**; GLMM: treatment × experience effect: χ^2_1 = 1.47, P = 0.226). Bees greatly improved their ability to correctly reject mimics with experience, but became somewhat worse at correctly detecting models (**Figures 5C,D**; correct rejections: χ^2_1 = 88.96, P < 0.0001; correct detections: χ^2_1 = 17.87, P < 0.0001). These patterns were not affected by corolla size variation (**Figures 5C,D**; GLMMs: correct rejections, treatment effect: χ^2_1 = 2.78, P = 0.095; treatment × experience effect: χ^2_1 = 2.26, P = 0.132; correct detections, treatment effect: χ^2_1 = 0.78, P = 0.377; treatment × experience effect: χ^2_1 = 0.24, P = 0.622).

Although bees missed more detections with experience, they nevertheless improved their proportion of landings on models relative to mimics with successive visits (**Figure 6A**; GLMM: χ^2_1 = 19.51, P < 0.0001). The effect of experience did not depend on the degree of corolla variation (**Figure 6A**; GLMM:

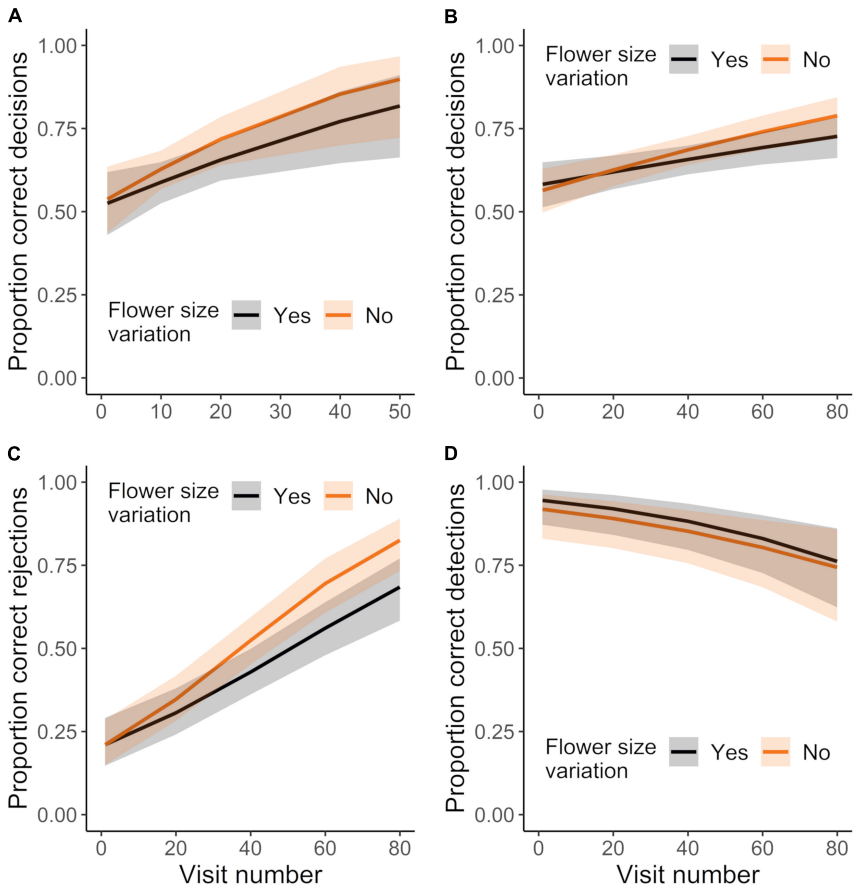

FIGURE 5 | Sampling behavior of initially-naïve bees foraging in treatments that did or did not vary in corolla size. **(A)** Mean proportion of correct decisions for those bees that met the learning criterion, only considering visits up to the point of meeting the criterion. *N* = 14 and 12 bees in the control and corolla size variation treatment, respectively. Mean proportion of **(B)** correct decisions, **(C)** correct rejections, and **(D)** correct detections made by all bees making up to 80 visits. *N* = 15 and 18 bees in the control and variation treatment, respectively (includes those bees in panel "**A**"). Plotted lines indicate estimated means and shaded regions indicate 95% confidence intervals.

treatment effect: $\chi^2_1 = 0.94$, $P = 0.331$; treatment × experience effect: $\chi^2_1 = 1.56$, $P = 0.212$). Additionally, upon landing, bees sonicated mimics significantly less and models significantly more with experience (**Figures 6B,C**; GLMMs: sonicating mimics: $\chi^2_1 = 13.56$, $P < 0.0003$; sonicating models: $\chi^2_1 = 45.15$, $P < 0.0001$). Corolla size variation affected the pattern of bees sonicating models, but not mimics, such that bees in the variation treatment initially buzzed proportionally fewer flowers than bees in the control treatment (**Figures 6B,C**: GLMMs: sonicating mimics, treatment effect: $\chi^2_1 = 0.82$, $P = 0.364$; treatment × experience effect: $\chi^2_1 = 0.64$, $P = 0.426$; sonicating models, treatment effect: $\chi^2_1 = 0.12$, $P = 0.728$; treatment × experience effect: $\chi^2_1 = 3.95$, $P < 0.047$).

Corolla Size Variation Had Little Effect on the Speed of Bee Responses

The general absence of differences between control and variation treatments could have been a result of bees altering their response time to maintain the accuracy of their decisions. While we found that bees responded faster with experience, response time did not significantly differ between control and variation treatments (**Figure 7A**; GLMM: experience effect: $\chi^2_1 = 9.22$, $P < 0.003$; treatment effect: $\chi^2_1 = 0.11$, $P = 0.746$; **Table 1**). However, response time decreased more quickly with experience in the control treatment vs. the variation treatment, with this effect of experience being stronger when visiting mimics (vs. models) or when the decision was incorrect vs. correct (GLMM: treatment × experience × flower sex effect: $\chi^2_1 = 4.04$, $P < 0.045$; treatment × experience × decision type effect: $\chi^2_1 = 6.42$, $P < 0.012$; **Table 1**). We also found that differences in response time on model and mimic flowers depended on whether the decision was correct or incorrect, such that response times to model and mimic were more different when the decision was incorrect vs. when it was correct (**Figure 7B**; GLMM: flower sex × decision type effect: $\chi^2_1 = 27.95$, $P < 0.0001$). Finally we did not find evidence for a speed-accuracy tradeoff: accuracy and response time were in fact negatively correlated across treatments (**Figure 7C**; LM: response time effect: $\chi^2_1 = 7.51$, $P < 0.007$; treatment effect: $\chi^2_1 = 0.04$, $P = 0.839$; treatment × response time effect: $\chi^2_1 = 0.42$, $P = 0.518$; $R^2 = 0.22$).

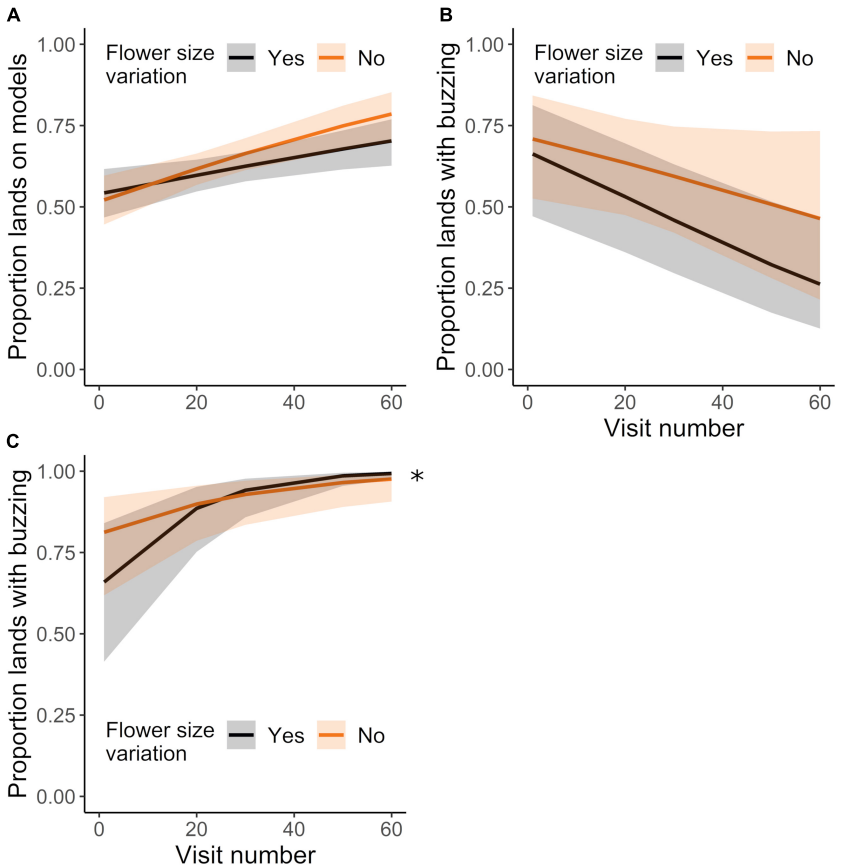

FIGURE 6 | Landing behavior of initially-naïve bees (same dataset as in **Figures 5B–D**, analyzed for different sampling behavior) foraging in the control or corolla size variation treatments. Mean proportion of lands made on **(A)** models (vs. mimics), **(B)** mimics during which the bee buzzed, and on **(C)** models during which the bee buzzed, over the course of up to 60 landings. $N = 15$ and 18 bees in the control and variation treatment, respectively. Plotted lines indicate estimated means and shaded regions indicate 95% confidence intervals. Asterisk indicates a significant difference among treatments at $P < 0.05$.

DISCUSSION

Generalization is thought to be a key mechanism sustaining Batesian mimicry, because when mimics resemble models well enough, receivers are expected to generalize their learned responses to models, to mimics (Ham et al., 2006; Ruxton et al., 2008; Speed and Ruxton, 2010; Rönkä et al., 2018). Signal detection theory predicts that receivers perceive signals as more similar when the signal distribution is broader, which can be a result of phenotypic variation among models and mimics (**Figure 1**; Lynn et al., 2005). Thus variation in model and mimic appearance should promote generalization (Amézquita et al., 2013; Gamberale-Stille et al., 2018; Arias et al., 2020), making it more difficult for bees to learn to avoid mimics (e.g., Gaskett, 2012; Paulus, 2019). We were therefore surprised that when we manipulated variation in model and mimic phenotype in an intersexual floral mimicry system, we found only modest evidence that bumble bees generalized learned responses among model and mimic flowers. Bees tended to reject mimics less when corolla size varied, consistent with variation promoting generalization. However, phenotypic variation in the mimicry did not affect learning to make more correct decisions overall or learning to make more landings on models. Variation also did not affect bees becoming worse at detecting models with experience (potentially a tradeoff with increasing avoidance of mimics, consistent with signal detection theory; Lynn et al., 2005, see also Russell et al., 2020). We also did not find much evidence that bees took longer to learn responses when corolla size varied. Only in terms of the pollen collection motor routine did bees take longer to respond appropriately. Specifically, when corolla size varied, bees buzzed models significantly less frequently, relying more on scrabbling, a less effective pollen extraction behavior (Russell et al., 2017). Because scrabbling is presumed to be less energetically expensive than buzzing (Russell et al., 2017), this response is also potentially consistent with bees "playing it safe" in response to increased uncertainty of model/mimic identity. Assuming our results are broadly representative and generalization is typically weak, intersexual floral mimicry can still be maintained when pollinators do not perfectly discriminate models and mimics and when there is a tradeoff between learning to avoid mimics and missing models, as we find here (see Russell et al., 2020 for an in-depth discussion).

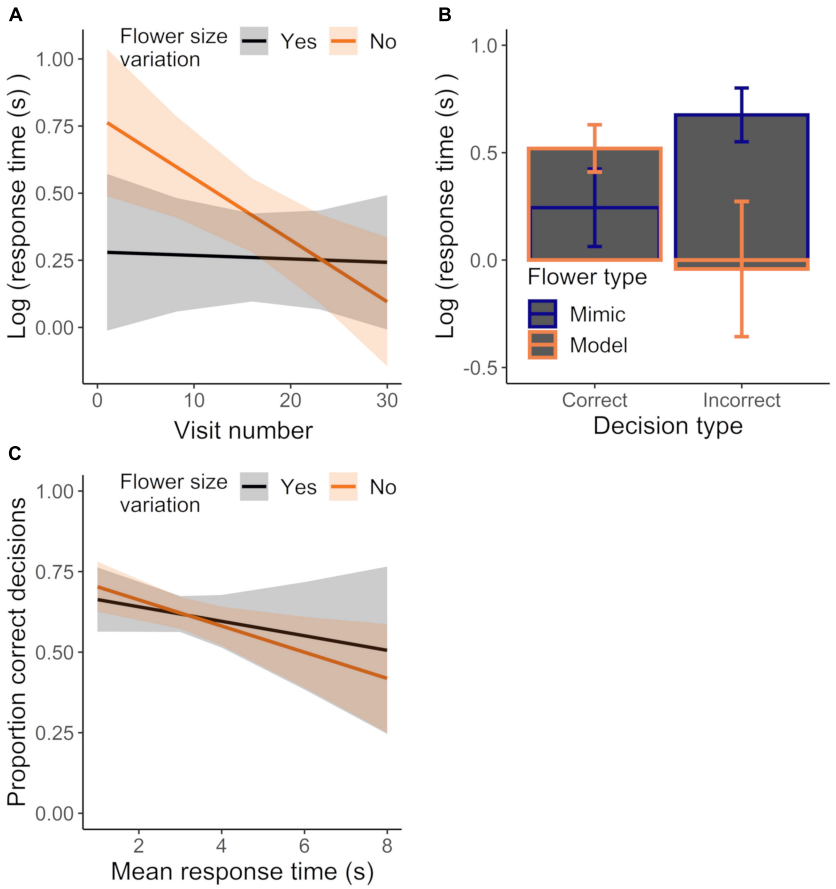

FIGURE 7 | Effect of **(A)** experience on mean response time for initially-naive bees (same dataset as in **Figures 5B–D**) foraging in the control or corolla size variation treatments or **(B)** sampling behavior (incorrect vs. correct decision) and flower sex on response time, regardless of treatment. Data were analyzed in a single model, but effects of different explanatory variables are shown in separate panels for ease of presentation. **(C)** Mean response time plotted against the mean proportion of correct decisions for bees foraging in the control or variation treatment. $N = 16$ and 13 bees in the control and variation treatment, respectively. Plotted lines indicate estimated means and shaded regions indicate 95% confidence intervals.

Flower size in general is thought to be a salient signal for bees (Blarer et al., 2002; Armbruster et al., 2005; Gómez et al., 2008; Essenberg et al., 2015), is used by bees to discriminate among male or female flowers of other *Begonia* species (Schemske and Ågren, 1995; Schemske et al., 1996; Castillo et al., 2012), and differs significantly between model and mimic flowers of our study species, at least in the greenhouse (**Figure 3**). Why then were bee cognitive constraints only modestly affected by variation in flower size? One possibility is that experimental conditions insufficiently replicated conditions under which bees generalize. At least in terms of intrasexual difference in corolla size, surrogate models resembled live flowers. However, we manipulated corolla size using just three discrete size classes in experiments. Assuming greenhouse conditions approximated natural variation, model and mimic corolla size variation is continuous and approximately normally distributed (**Figure 3**). Evidence from other systems suggests that the greater the variation in the signal, the greater the generalization (e.g., Finch et al., 2016; Arias et al., 2020; Miller et al., 2020). Perhaps corolla size variation in our behavioral experiment then was too low, or too categorical, to observe generalization. Additionally, the ratio of models and mimics is thought to influence successful mimicry and could affect generalization if encounter rate influenced learning (Abbott and Sherratt, 2013; de Jager et al., 2016). However, recent work has demonstrated that *Begonia* sex ratio has only a marginal influence on learning (see Russell et al., 2020).

Another possibility is that intrasexual differences other than flower size might have been more salient, thus precluding generalization on the basis of corolla size. For example, corolla dissectedness (number of petals and degree of petal overlap) differs between *B. odorata* model and mimic, and while color (**Figure 1**) and scent (unpublished data; A. Mosher, T. Eltz, and A. Russell) of model and mimic reproductive parts are likely not discriminable by bees, shape may be. For instance, bees readily discriminate flowers with wide vs. narrow petals and prefer more dissected flowers (e.g., Yoshioka et al., 2007; Gómez et al., 2008). Indeed, preference for more dissected flowers might at least partly explain why bees in our study made more than 70% of their first landings on the mimics, an example of exploitation of pollinator sensory bias (consistent with Russell et al., 2020).

TABLE 1 | Type II Wald Chi-square tests for log(response time) as the response variable.

Explanatory variable	χ^2	Degrees of freedom	*P*-value
Treatment (control vs. variation)	0.11	1	0.746
Flower sex (mimic vs. model)	0.07	1	0.795
Decision type (correct vs. incorrect)	2.30	1	0.129
Experience	**9.22**	**1**	**<0.003**
Treatment × Flower sex	1.27	1	0.259
Treatment × Decision type	0.18	1	0.675
Flower sex × Decision type	**27.95**	**1**	**<0.0001**
Treatment × Experience	1.38	1	0.240
Flower sex × Experience	0.87	1	0.350
Decision type × Experience	0.008	1	0.930
Treatment × Flower sex × Decision type	2.61	1	0.106
Treatment × Flower sex × Experience	**4.04**	**1**	**<0.045**
Treatment × Decision type × Experience	**6.42**	**1**	**<0.012**
Flower sex × Decision type × Experience	0.13	1	0.723
Treatment × Flower sex × Decision type × Experience	2.51	1	0.123

Bolded lines indicate significant differences at P < 0.05.

Similarly, when flower size is a less reliable cue of flower sex (as it might be for the variation treatment), bees might have relied on other more reliable cues. Yet then bees in different treatments should probably have shown differences in their learning rate and learned responses, which they largely did not. Future work will be required to determine whether variation in traits potentially more salient than corolla size (see Essenberg et al., 2015) functions to promote generalization of model and mimic.

Alternatively, variation in flower size may in fact exploit constraints on learning, but bees in our study might have compensated by altering how they made decisions. For instance, by taking more time to respond when the learning task is more difficult, bees might have gathered enough information to make a more accurate response (i.e., a speed-accuracy tradeoff; see Abbott and Sherratt, 2013). We found that accuracy and response time were negatively correlated while bees were learning, not positively related as expected. However, this does not rule out the occurrence of a speed-accuracy tradeoff. Rather, learning may be both improving accuracy and reducing response time, and this dual effect of experience may have more than offset the expected accuracy-response time tradeoff. Accordingly, by assessing speed-accuracy tradeoffs after much of the learning has occurred (see Chittka et al., 2003; Ings and Chittka, 2008; Kulahci et al., 2008; Chittka et al., 2009), future work may reveal whether bees alter response speed to compensate for exploitation by intersexual mimicry. Of note, the change in response speed with experience did differ between corolla variation treatments, such that response time decreased more quickly in the control treatment. This result suggests learning was more difficult when corollas varied in size. Consistent with negative reinforcement driving learning, the decrease in response time was also affected more when visiting mimics and by making incorrect decisions.

In summary, we found scant evidence that flower size variation among models and mimics influenced the effectiveness of an intersexual floral mimicry. We corroborate previous work demonstrating that mimicry need not be perfect to be effective and that learning is a key mechanism by which pollinators can reduce exploitation by mimics (see Russell et al., 2020). While it appears unlikely that flower size variation is an evolved strategy on the part of the plant to exploit pollinator cognition, a fuller understanding will require disentangling effects of variation on cognition from speed-accuracy tradeoffs. Batesian models and mimics, including, but not limited to intersexual mimicry, often exhibit striking phenotypic variation, including in color, pattern, and size, and this variation is thought to be important in driving the evolution of mimicry (e.g., Heal, 1982; Joron and Mallet, 1998; Lynn et al., 2005; Penney et al., 2012; Kikuchi and Pfennig, 2013). Yet how phenotypic variation interacts with receiver cognition has only rarely been considered (but see Lynn et al., 2005; Abbott and Sherratt, 2013). Thus, while our study provides rare experimental evidence that phenotypic variation may not necessarily affect receiver cognition, it also indicates that additional investigation of this potential interaction is warranted.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SS, LW, AR, and DP designed the study. SS, LW, and AR performed the research. AR performed the analyses and wrote the first draft of the manuscript. All authors revised the manuscript.

FUNDING

This work was supported by the Missouri State University and a Sigma Xi Grant in Aid of Research, awarded to SS.

ACKNOWLEDGMENTS

We are grateful to the Koppert Biologicals for the bee colonies, the Chicago Botanic Garden for plants, and Rachel Wilkins for assistance with flower measurements. We acknowledge this work was performed on unceded traditional territory of the Osage.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.724712/full#supplementary-material

REFERENCES

Abbott, K. R., and Sherratt, T. N. (2013). Optimal sampling and signal detection: unifying models of attention and speed– accuracy trade-offs. *Behav. Ecol.* 24, 605–616. doi: 10.1093/beheco/art001

Ågren, J., and Schemske, D. W. (1995). Sex allocation in the monoecious herb *Begonia semiovata*. *Evolution* 49, 121–130. doi: 10.1111/j.1558-5646.1995.tb05964.x

Amézquita, A., Castro, L., Arias, M., González, M., and Esquivel, C. (2013). Field but not lab paradigms support generalisation by predators of aposematic polymorphic prey: the *Oophaga histrionica* complex. *Evol. Ecol.* 27, 769–782. doi: 10.1007/s10682-013-9635-1

Arias, M., Davey, J. W., Martin, S., Jiggins, C., Nadeau, N., Joron, M., et al. (2020). How do predators generalize warning signals in simple and complex prey communities? Insights from a videogame. *Proc. Roy. Soc. B Biol. Sci.* 287:20200014. doi: 10.1098/rspb.2020.0014

Armbruster, W. S., Antonsen, L., and Pélabon, C. (2005). Phenotypic selection on *Dalechampia* blossoms: honest signaling affects pollination success. *Ecology* 86, 3323–3333. doi: 10.1890/04-1873

Ayasse, M., Schiestl, F. P., Paulus, H. F., Löfstedt, C., Hansson, B., Ibarra, F., et al. (2000). Evolution of reproductive strategies in the sexually deceptive orchid *Ophrys sphegodes*: how does flower-specific variation of odor signals influence reproductive success? *Evolution* 54, 1995–2006. doi: 10.1111/j.0014-3820.2000.tb01243.x

Barker, J. L., Dornhaus, A., Bronstein, J. L., and Muth, F. (2018). Learning about larceny: experience can bias bumble bees to rob nectar. *Behav. Ecol. Sociobiol.* 72:68.

Blarer, A., Keasar, T., and Shmida, A. (2002). Possible mechanisms for the formation of flower size preferences by foraging bumblebees. *Ethology* 108, 341–351. doi: 10.1046/j.1439-0310.2002.00778.x

Bronstein, J. L. (2001). The exploitation of mutualisms. *Ecol. Lett.* 4, 277–287. doi: 10.1046/j.1461-0248.2001.00218.x

Castillo, R. A., Caballero, H., Karina, B., Fornoni, J., and Domínguez, C. A. (2012). How to cheat when you cannot lie? Deceit pollination in *Begonia gracilis*. *Oecologia* 169, 773–782. doi: 10.1007/s00442-012-2250-y

Cheng, K. (2002). Generalization: mechanistic and functional explanations. *Anim. Cogn.* 5, 33–40. doi: 10.1007/s10071-001-0122-7

Chittka, L. (1992). The color hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of color opponency. *J. Comp. Physiol. A* 170, 533–543.

Chittka, L., Dyer, A. G., Bock, F., and Dornhaus, A. (2003). Bees trade off foraging speed for accuracy. *Nature* 424:388. doi: 10.1038/424388a

Chittka, L., Skorupski, P., and Raine, N. E. (2009). Speed–accuracy tradeoffs in animal decision making. *Trends Ecol. Evol.* 24, 400–407. doi: 10.1016/j.tree.2009.02.010

de Avila, R. Jr., Oleques, S. S., Marciniak, B., and Ribeiro, J. R. I. (2017). Effects of model-mimic frequency on insect visitation and plant reproduction in a self-mimicry pollination system. *Ann. Bot.* 9:lx044. doi: 10.1093/aobpla/plx044

de Jager, M., Newman, E., Theron, G., Botha, P., Barton, M., and Anderson, B. (2016). Pollinators can prefer rewarding models to mimics: consequences for the assumptions of *Batesian* floral mimicry. *Plant Syst. Evol.* 302, 409–418. doi: 10.1007/s00606-015-1276-0

de Jager, M. L., and Anderson, B. (2019). When is resemblance mimicry? *Funct. Ecol.* 39, 1586–1596. doi: 10.1111/1365-2435.13346

de Jager, M. L., and Ellis, A. G. (2014). Costs of deception and learned resistance in deceptive interactions. *Proc. R. Soc. B* 281:20132861. doi: 10.1098/rspb.2013.2861

Dixit, T., Riederer, J. M., Quek, Q., Belford, K., de Wand, T. T., Sicat, R., et al. (2020). Plasticity in flower size as an adaptation to variation in pollinator specificity. *Ecol. Entom.* 45, 1367–1372. doi: 10.1111/een.12921

Dukas, R. (1987). Foraging behavior of three bee species in a natural mimicry system: female flowers which mimic male flowers in *Ecballium elaterium* (Cucurbitaceae). *Oecologia* 74, 256–263. doi: 10.1007/bf00379368

Enquist, M., and Johnstone, R. A. (1997). Generalization and the evolution of symmetry preferences. *Proc. Roy. Soc. B* 264, 1345–1348. doi: 10.1098/rspb.1997.0186

Essenberg, C. J. (2021). Intraspecific relationships between floral signals and rewards with implications for plant fitness. *Ann. Bot.* 13:lab006. doi: 10.1093/aobpla/plab006

Essenberg, C. J., Easter, R. A., Simmons, R. A., and Papaj, D. R. (2015). The value of information in floral cues: bumblebee learning of floral size cues. *Behav. Ecol.* 26, 1335–1344. doi: 10.1093/beheco/arv061

Finch, D. D., Carvalho, P. F., and Goldstone, R. L. (2016). "Variability in category learning: the effect of context change and item variation on knowledge generalization," in *Paper Presented at the Cognitive Science 2016 Database*, (Bloomington, IN).

Fox, J. (2015). *Applied Regression Analysis and Generalized Linear Models*, 3rd Edn. London: Sage Publications, Inc.

Galen, C. (1999). The functional ecology of variation in flower size and form within natural plant populations. *BioScience* 49, 631–640.

Gamberale-Stille, G., Kazemi, B., Balogh, A., and Leimar, O. (2018). Biased generalization of salient traits drives the evolution of warning signals. *Proc. R. Soc B. Biol. Sci.* 285:20180283. doi: 10.1098/rspb.2018.0283

Gaskett, A. C. (2012). Floral shape mimicry and variation in sexually deceptive orchids with a shared pollinator. *Biol. J. Linn. Soc.* 106, 469–481. doi: 10.1111/j.1095-8312.2012.01902.x

Gigord, L. D. B., Macnair, M. R., and Smithson, A. (2001). Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Soó. *Proc. Nat. Acad. Sci. U.S.A.* 98, 6253–6255. doi: 10.1073/pnas.111162598

Gómez, J. M., Bosch, J., Perfectti, F., Fernandez, J. D., Abdelaziz, M., and Camacho, J. P. M. (2008). Association between floral traits and rewards in *Erysimum mediohispanicum* (Brassicaceae). *Ann. Bot.* 101, 1413–1420. doi: 10.1093/aob/mcn053

Goodrich, K. R., and Jurgens, A. (2017). Pollination systems involving floral mimicry of fruit: aspects of their ecology and evolution. *New Phytologist* 217, 74–81. doi: 10.1111/nph.14821

Ham, A. D., Ihalainen, E., Lindstrom, L., and Mappes, J. (2006). Does colour matter? The importance of colour in avoidance learning, memorability and generalisation. *Behav. Ecol. Sociobiol.* 60, 482–491. doi: 10.1007/s00265-006-0190-4

Hartig, F. (2018). *DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. R package version 0.1.6*. Available online at: https://cran.r-project.org/web/packages/DHARMa/index.html (accessed May, 2021).

Hattori, M., Nagano, Y., Shinohara, Y., and Itino, T. (2016). Pattern of flower size variation along an altitudinal gradient differs between Impatiens textori and *Impatiensnoli-tangere*. *J. Plant Interact.* 11, 152–157. doi: 10.1080/17429145.2016.1226437

Heal, J. R. (1982). Colour patterns of *Syprhidae*: IV. mimicry and variation in natural populations of *Eristalis tenax*. *Heredity* 49, 95–109. doi: 10.1038/hdy.1982.68

Ings, T. C., and Chittka, L. (2008). Speed-accuracy tradeoffs and false alarms in bee responses to cryptic predators. *Curr. Biol.* 18, 1520–1524. doi: 10.1016/j.cub.2008.07.074

Johnson, S. D., and Schiestl, F. P. (2016). *Floral Mimicry*. Oxford: Oxford University Press.

Joron, M., and Mallet, J. L. B. (1998). Diversity in mimicry: paradox or paradigm? *Tree* 13, 461–467. doi: 10.1016/s0169-5347(98)01483-9

Kalish, H. I. (1969). "Generalization as an epiphenomenon," in *Learning Processes*, ed. M. H. Marx (New York, NY: Macmillan), 207–297.

Kikuchi, D. W., and Pfennig, D. W. (2013). Imperfect mimicry and the limits of natural selection. *Q. Rev. Biol.* 88, 297–315. doi: 10.1086/673758

Kulahci, I. G., Dornhaus, A., and Papaj, D. R. (2008). Multimodal signals enhance decision making in foraging bumble-bees. *Proc. Biol. Sci.* 275, 797–802. doi: 10.1098/rspb.2007.1176

Kunze, J., and Gumbert, A. (2000). The combined effect of color and odor on flower choice behavior of bumble bees in flower mimicry systems. *Behav. Ecol.* 12, 447–456. doi: 10.1093/beheco/12.4.447

Lüdecke, D., Bartel, A., Schwemmer, C., Powell, C., Kjalovski, A., and Titz, J. (2021). *sjPlot: Data Visualization for Statistics in Social Science. R Package Version 2.8.8*. Available online at: https://cran.r-project.org/web/packages/sjPlot/index.html (accessed May, 2021).

Lynn, S. K., Cnaani, J., and Papaj, D. R. (2005). Peak shift discrimination learning as a mechanism of signal evolution. *Evolution*. 1300–1305.

Mackintosh, N. J. (1974). *The Psychology of Animal Learning*. Cambridge, MA: Academic Press.

Magnusson, A., Skaug, H., Nielsen, A., Berg, C., Kristensen, K., Maechler, M., et al. (2018). *glmmTMB: Generalized Linear Mixed Models using Template Model Builder. R Package Version 1.0.2.1*. Available online at: https://cran.r-project.org/web/packages/glmmTMB/index.html (accessed May, 2021).

Maloof, J. E., and Inouye, D. W. (2000). Are nectar robbers cheaters or mutualists? *Ecology* 81, 2651–2661. doi: 10.1890/0012-9658(2000)081[2651:anrcom]2.0.co;2

Miller, I. D., Dumay, N., Pitt, M., Lam, B., and Armstrong, B. C. (2020). "Context variability promotes generalization in reading aloud: insight from a neural network simulation," in *Proceedings of the 42nd Annual Meeting of the Cognitive Science 2020 database*, (Armstrong, BC: Cognitive Science Society).

Paulus, H. F. (2019). Speciation, pattern recognition and the maximization of pollination: general questions and answers given by the reproductive biology of the orchid genus Ophrys. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav Physiol.* 205, 285–300. doi: 10.1007/s00359-019-01350-4

Pemberton, R. W., and Wheeler, G. S. (2006). Orchid bees don't need orchids: evidence from the naturalization of an orchid bee in Florida. *Ecology* 87, 1995–2001. doi: 10.1890/0012-9658(2006)87[1995:obdnoe]2.0.co;2

Penney, H. D., Hassall, C., Skevington, J. H., Abbott, K. R., and Sherratt, T. N. (2012). A comparative analysis of the evolution of imperfect mimicry. *Nature* 483, 461–464. doi: 10.1038/nature10961

R Development Core Team. (2021). *R: A Language and Environment for Statistical Computing.: R Foundation for Statistical Computing*. Vienna: R Development Core Team.

Rönkä, K., De Pasqual, C., Mappes, J., Gordon, S., and Rojas, B. (2018). Colour alone matters: no predator generalization among morphs of an aposematic moth. *Anim. Behav.* 135, 153–163. doi: 10.1016/j.anbehav.2017.11.015

Russell, A. L., Buchmann, S. L., and Papaj, D. R. (2017). How a generalist bee achieves high efficiency of pollen collection on diverse floral resources. *Behav. Ecol.* 28, 991–1003. doi: 10.1093/beheco/arx058

Russell, A. L., Kikuchi, D. W., Giebink, N. W., and Papaj, D. R. (2020). Sensory bias and signal detection tradeoffs maintain intersexual floral mimicry. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 375:20190469. doi: 10.1098/rstb.2019.0469

Russell, A. L., Leonard, A. S., Gillette, H. D., and Papaj, D. R. (2016a). Concealed floral rewards and the role of experience in floral sonication by bees. *Anim. Behav.* 120, 83–91. doi: 10.1016/j.anbehav.2016.07.024

Russell, A. L., Newman, C. R., and Papaj, D. R. (2016b). White flowers finish last: pollen-foraging bumble bees show biased learning in a floral color polymorphism. *Evol. Ecol.* 31, 173–191. doi: 10.1007/s10682-016-9848-1

Ruxton, G. D., Franks, D. W., Balogh, A. C., and Leimar, O. (2008). Evolutionary implications of the form of predator generalization for aposematic signals and mimicry in prey. *Evolution* 62, 2913–2921. doi: 10.1111/j.1558-5646.2008.00485.x

Schaefer, H. M., and Ruxton, G. D. (2010). Deception in plants: mimicry or perceptual exploitation? *Trends Ecol. Evol.* 24, 676–685. doi: 10.1016/j.tree.2009.06.006

Schemske, D. W., and Ågren, J. (1995). Deceit pollination and selection on female flower size in *Begonia involucrata*: an experimental approach. *Evolution* 49, 207–214. doi: 10.2307/2410306

Schemske, D. W., Ågren, J., and Le Corff, J. (1996). "Deceit Pollination in the monoecious, neotropical herb *Begonia oaxacana* (Begoniaceae)," in *Floral Biology: Studies on Floral Evolution in Animal-Pollinated Plants*, eds D. G. Lloyd and S. C. H. Barrett (Boston, MA: Chapman and Hall), 292–318. doi: 10.1007/978-1-4613-1165-2_11

Schiestl, F. P. (2005). On the success of a swindle: pollination by deception in orchids. *Naturwissenschaften* 92, 255–264. doi: 10.1007/s00114-005-0636-y

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends. Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Sherratt, T. N. (2002). The evolution of imperfect mimicry. *Behav. Ecol.* 6, 821–826. doi: 10.1093/beheco/13.6.821

Signorell, A., Aho, K., Alfons, A., Anderegg, N., Aragon, T., Arachchige, C., et al. (2019). *DescTools: Tools for Descriptive Statistics. R Package Version 0.99.30*. Available online at: https://cran.r-project.org/web/packages/DescTools/index.html (accessed May, 2021).

Simpson, B. B., and Neff, J. L. (1981). Floral rewards: alternatives to pollen and nectar. *Ann. Miss. Bot. Garden* 68, 301–322. doi: 10.2307/2398800

Skorupski, P., and Chittka, L. (2010). Photoreceptor spectral sensitivity in the bumblebee, *Bombus impatiens* (*Hymenoptera: Apidae*). *PLoS One* 5:e12049. doi: 10.1371/journal.pone.0012049

Smithson, A., and Macnair, M. R. (1997). Negative frequency-dependent selection by pollinators on artificial flowers without rewards. *Evolution* 51, 715–723. doi: 10.2307/2411148

Speed, M. P., and Ruxton, G. D. (2010). Imperfect *Batesian mimicry* and the conspicuousness costs of mimetic resemblance. *Am. Nat.* 176, E1–E14.

Stavenga, D. G., Smiths, R. P., and Hoenders, B. J. (1993). Simple exponential functions describing the absorbance bands of visual pigment spectra. *Vis. Res.* 33, 1011–1017. doi: 10.1016/0042-6989(93)90237-q

Thompson, J. N., Schwind, C., Guimaraes, P. R. Jr., and Friberg, M. (2013). Diversification through multitrait evolution in a coevolving interaction. *Proc. Natl. Acad. Sci. U.S.A.* 110, 11487–11492. doi: 10.1073/pnas.1307451110

van der Kooi, C. J., Vallejo-Marin, M., and Leonhardt, S. D. (2021). Mutualisms and (a)symmetry in plant–pollinator interactions. *Curr. Biol.* 31, R91–R99. doi: 10.1016/j.cub.2020.11.020

Whitehead, M. R., and Peakall, R. (2012). Short-term but not long-term patch avoidance in an orchid-pollinating solitary wasp. *Behav. Ecol.* 24, 162–168. doi: 10.1093/beheco/ars149

Wood, S. (2021). *mgcv: Mixed GAM Computation Vehicle with Automatic Smoothness Estimation. R Package Version 1.8-36*. Available online at: https://cran.r-project.org/web/packages/mgcv/index.html (accessed May, 2021).

Wright, G. A., and Smith, B. H. (2003). Variation in complex olfactory stimuli and its influence on odour recognition. *Proc. R. Soc. Lond. B Biol. Sci.* 271, 147–152. doi: 10.1098/rspb.2003.2590

Wyatt, G. E., and Sazima, M. (2011). Pollination and reproductive biology of thirteen species of Begonia in the Serra do Mar state park. São Paulo, Brazil. *J. Pollinat. Ecol.* 6, 95–107.

Yoshioka, Y., Ohashi, K., Konuma, A., Iwata, H., Ohsawa, R., and Ninomiya, S. (2007). Ability of bumblebees to discriminate differences in the shape of artificial flowers of *Primula sieboldii* (*Primulaceae*). *Ann. Bot.* 99, 1175–1182. doi: 10.1093/aob/mcm059

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 03 September 2021
doi: 10.3389/fevo.2021.697355

Negative Effects of the Neonicotinoid Clothianidin on Foraging Behavior and Antennal Sensitivity in Two Common Pollinator Species, *Osmia bicornis* and *Bombus terrestris*

*Florian Straub, Ihotu Joy Orih, Judith Kimmich and Manfred Ayasse**

Department of Biology, Institute of Evolutionary Ecology and Conservation Genomics, Ulm University, Ulm, Germany

Insect species richness and abundance has declined rapidly over the last few decades. Various stressors, such as the conversion of natural habitats, climate change, land-use intensification, agrochemicals and pathogens, are thought to be major factors in this decline. We treated female bees of two common pollinator species in Europe, *Osmia bicornis* and *Bombus terrestris*, with a field-realistic dose of the neonicotinoid clothianidin. We tested its effects on the foraging behavior of *O. bicornis* under semi-natural conditions and on the antennal sensitivity of both bee species to common floral volatiles by using electroantennography. Clothianidin negatively affected the foraging behavior in *O. bicornis* by decreasing the number of flowers visited per foraging flight and by increasing the time per flower visit and the searching time between two flowers. It also decreased the antennal sensitivity to 2-phenylethanol in the two bee species. Thus, clothianidin is clearly a threat for bees via its effects on their foraging behavior and antennal sensitivity and is hence probably detrimental for pollination and the reproductive success of bees.

Keywords: ***Osmia bicornis, Bombus terrestris*, neonicotinoid, clothianidin, foraging flight, antennal sensitivity**

OPEN ACCESS

Edited by:
Sara Diana Leonhardt, Technical University of Munich, Germany

Reviewed by:
Denis Thiery, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), France
Jeremy N. McNeil, Western University, Canada

****Correspondence:***
Manfred Ayasse
manfred.ayasse@uni-ulm.de

Specialty section:
This article was submitted to Chemical Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *19 April 2021*
Accepted: *16 August 2021*
Published: *03 September 2021*

Citation:
Straub F, Orih IJ, Kimmich J and Ayasse M (2021) Negative Effects of the Neonicotinoid Clothianidin on Foraging Behavior and Antennal Sensitivity in Two Common Pollinator Species, Osmia bicornis and Bombus terrestris. Front. Ecol. Evol. 9:697355. doi: 10.3389/fevo.2021.697355

INTRODUCTION

Biodiversity, especially species richness, abundance and the distribution of pollinators is globally declining (Potts et al., 2010; van der Sluijs et al., 2013; Godfray et al., 2014; Goulson et al., 2015; Hallmann et al., 2017). The limited availability of food and nesting resources and the occurrence of parasites and pathogens, climate change, and pesticides are considered to be the main drivers of this decline (Goulson et al., 2015). With regard to pesticides, the use of neonicotinoids is a major threat for the most important agents in pollination, namely honeybees, bumblebees, and solitary bees (Elbert et al., 2008; Godfray et al., 2014).

The intensification of agriculture has transformed the agrochemical landscape and resulted in a massive overuse of pesticides in recent years (Casida and Durkin, 2013; Gross, 2013; van der Sluijs et al., 2013). Among the neonicotinoids, imidacloprid, thiamethoxam, and clothianidin (a breakdown product of thiamethoxam) are the most toxic (Scott-Dupree et al., 2009; Stokstad, 2013; Botías et al., 2015). They have been used as a seed coating or have been applied via foliar or soil treatment until their ban in Germany by the end of 2020 (Elbert et al., 2008; Fent, 2013).

However, the EU Pesticides Database has revealed that some of these banned neonicotinoids are still authorized for use at national level in a few European countries. Because of their long persistence in soil, neonicotinoids can be detected even in untreated plants and soil over years (Hopwood et al., 2012; Botías et al., 2015). Neonicotinoids target the central nervous system of insects in which they act as an agonist of insect nicotinic acetylcholine receptors (nAChR) at the postsynaptic membrane in the nervous system; however, they are not degraded by acetylcholine esterase as is the natural transmitter acetylcholine (Tomizawa and Casida, 2005; Elbert et al., 2008; Fent, 2013; Fischer et al., 2014). Acetylcholine is a highly important transmitter and is suggested to play an important role during transmission from olfactory receptor neurons via the antennal lobe to the mushroom bodies (Fischer et al., 2014). Because of their higher affinity and higher selectivity for insect nAChR over vertebrate nAChR, neonicotinoids are more toxic to insects, as has been clearly shown by the much higher lethal doses (LD50) recorded for vertebrates (Jeschke and Nauen, 2008; Matsuda et al., 2011; Uneme, 2011). In addition to their direct lethal effects, they also exhibit sublethal effects that do not directly cause death in animals (Artz and Pitts-Singer, 2015).

Many of the adverse effects of neonicotinoids have been demonstrated in honeybees. Treatment with clothianidin significantly reduces the life span of *Apis mellifera* workers (Sgolastra et al., 2015; Tsvetkov et al., 2017). Furthermore, Tomé et al. (2012) and Williamson et al. (2014) have found a sublethal effect and confirmed that low doses of clothianidin affect the motor function and the walking behavior in adult neotropical stingless bees and honeybees. Interestingly, two other studies in honeybees and in *Osmia cornuta* have revealed no effects of neonicotinoids on locomotion or even increased locomotive activity (El Hassani et al., 2008; Jin et al., 2015). Neonicotinoids also seem to affect memory and learning by damage to the central nervous system in bees (Tomé et al., 2012; van der Sluijs et al., 2013). In a cognition experiment with *O. cornuta*, Jin et al. (2015) have found a blockage of memory retrieval for learned cues guiding to a food source after neonicotinoid treatment. Further, neonicotinoids affect foraging success, with the treatment of various bee taxa resulting in less directed flights and a lower pollen and nectar foraging efficiency (Desneux et al., 2007; Hopwood et al., 2012; van der Sluijs et al., 2013; Feltham et al., 2014; Fischer et al., 2014; Tan et al., 2014; Tison et al., 2016). In contrast, in the bumblebee *Bombus terrestris*, thiamethoxam does not appear to affect the total length of foraging flights or searching time between two flowers (Stanley and Raine, 2016). However, field experiments and even experiments carried out under semi-field conditions to determine the effects of neonicotinoids on wild bees under natural conditions are scarce. Rundlöf et al. (2015) have performed a huge field study and found a reduced density of wild bees, reduced nesting activity near treated fields and negatively affected colony growth in *B. terrestris*. Since all of the bee species have a function as pollinators, we need to understand the effects of neonicotinoids on foraging behavior and pollination. Moreover, the amount of pollen that females collect affects larval fitness and reproductive success (Radmacher and Strohm, 2010; Seidelmann, 2014; Stanley et al., 2015).

In social insects, semiochemicals are crucial for maintaining the colony (Ayasse and Jarau, 2014). Insect pheromones such as cuticular hydrocarbons (CHCs) or cuticular lipids play a key function and are vital for sustaining the intra-colonial network; pheromones regulate and control worker reproduction and underpin behavioral patterns such as mating or worker reproduction in social insects (Ayasse and Jarau, 2014). In social insects, scent not only has an intraspecific function in communication, but also plays an important role in foragers finding their host plants. In solitary and social insects, floral scent is thought to be a major cue for discriminating and identifying different flowers (Schiestl, 2015). It also serves as a cue enabling bees to distinguish between rewarding and non-rewarding flowers and even the amount of reward that is present within a flower (Dötterl and Vereecken, 2010; Schiestl, 2015). Bees perceive a multitude of semiochemicals via chemical receptors that are located on their antennae (Kaib, 2003). The semiochemical signal is then transmitted via the antennal lobe to the mushroom bodies (Heisenberg, 1998). Here, acetylcholine is intimately involved in transmission (Fischer et al., 2014). However, the effects of neonicotinoids on antennal sensitivity, and especially on receptor level, are poorly investigated and only a few studies are available (i.e., Tappert et al., 2017).

Although wild bees and bumblebees are clearly as important as honeybees in terms of pollination, most studies on the effects of neonicotinoids have focused on various honeybee species and have been performed under laboratory conditions (Michener, 2000; van der Sluijs et al., 2013; Godfray et al., 2014). Only a few studies have focused on solitary bee species but most have shown negative effects after insecticide treatment (Artz and Pitts-Singer, 2015; Jin et al., 2015; Sgolastra et al., 2015). In order to increase our knowledge concerning the effects of neonicotinoids on wild bees, we have studied the effects of clothianidin on the foraging behavior and antennal sensitivity in the red mason bee *Osmia bicornis* and the buff-tailed bumblebee *B. terrestris*. Both *O. bicornis*, the most abundant solitary bee of the genus *Osmia* in Central Europe, and *B. terrestris* are important pollinators in orchards and plantations (Westrich and Dathe, 1997; Gruber et al., 2011). We have treated female bees of both species with field-realistic and sub-lethal doses of clothianidin and looked for differences in their foraging behavior and antennal sensitivity to various floral volatiles. We have also tested floral volatiles in both species and a pheromone component in *B. terrestris*, because both play important roles in colony maintenance and the finding of host plants.

We hypothesized that clothianidin would negatively affect the foraging behavior of female *O. bicornis*. We expected that the number of flowers per foraging flight would decrease and the time per flower and the time between two flowers would increase after clothianidin treatment. Because flower morphology might influence flower handling time, we chose two plant species that differed in their floral morphology, namely one Asteraceae (*Crepis biennis*) and a Ranunculaceae (*Ranunculus* spp.). We further hypothesized a negative effect of clothianidin on the sensitivity of antennal scent receptors in both *O. bicornis*

and *B. terrestris* and expected that clothianidin would decrease antennal sensitivity in both species.

MATERIALS AND METHODS

Study Species

The female *O. bicornis* LINNAEUS 1778 that were used in both experiments were reared in trap nests in the Botanical Garden at Ulm University. Cocoons that had overwintered in a cardboard box at 6°C in a refrigerator were placed into small rearing cages or flight cages (24.5 × 24.5 × 24.5 cm). After hatching and mating, female bees were able to start their own brood in wooden nesting blocks (49.5 × 20 × 17.5 cm) that had holes (diameter 7 mm) drilled into them. The bees were allowed to feed *ad libitum* on a 50% sugar solution, namely a dilution of two-thirds of a 73% sugar solution of API-Invert® (Südzucker AG, Mannheim, Germany) and one-third water. We added 3 g potassium sorbate and 1 g citric acid per 1 l of sugar solution as a preservative. We offered the sugar solution on small pieces of foam in a Petri dish, which was replaced every second or third day. During recordings of the foraging flights of the bees in the flight cages, we removed the Petri dish with the sugar solution. For the antennal sensitivity experiments, bees that had hatched in small flight cages in the laboratory were allowed to mate before they were used for electrophysiological recordings.

For the antennal response experiment, we also used female *B. terrestris* LINNAEUS 1758 reared in the laboratory at Ulm University. After mating and hibernation at 6°C for 10–12 weeks, queens were allowed to found new colonies (for details, see Rottler-Hoermann et al., 2016). As for *O. bicornis*, the bumble bees were fed *ad libitum* on a 50% sugar solution and additionally on fresh pollen (Koppert Biological Systems, Germany), which was replaced every 2 days. The new colonies were kept in wooden boxes (39 × 16.5 × 16 cm) with two separated compartments at a temperature of 27 ± 2°C and a relative humidity of 60–70% under constant darkness. The founding queens of all colonies were originally derived from commercial colonies (Koppert Biological Systems, Germany).

Clothianidin Treatment

To ensure that all bees were treated with a field-realistic amount of clothianidin [(E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine], we chose a concentration of 0.75 ng (1 *Osmia* Equivalent OE) clothianidin per bee for *O. bicornis* (Jin et al., 2015) and 2.55 ng (1 *Bombus* Equivalent BE) for *B. terrestris*. We diluted clothianidin (>98.0%, Sigma-Aldrich, Hamburg, Germany) in the respective amount in acetone (>99.8%, Sigma-Aldrich, Hamburg, Germany). For the experiment we used the pure compound instead of a formulation to avoid any potential effects of additives. Test solutions were stored in brown screw-cap micro-vials (CZT, Kriftel, Germany) at 6°C in a refrigerator to prevent photolysis ([EPA] U.S. Environmental Protection Agency., 2003).

Because the controlled uptake of clothianidin was important to ensure that the same conditions were experienced by all bees, we did not feed them with clothianidin via the sugar solution (see Tan et al., 2014; Jin et al., 2015). Instead, we applied 1 OE/1 BE clothianidin solution or acetone to the soft skin between the last sternite and tergite of the abdomen of each individual (Tappert et al., 2017). This topical application was used as it was comparable with foliar spray treatment in the field, spray experiments with clothianidin having been shown to be the most toxic for *O. lignaria* (Scott-Dupree et al., 2009). We conducted the experiments 1 day after treatment.

Measuring the Effects of Clothianidin

Foraging Behavior of *Osmia bicornis*

Foraging flights of *O. bicornis* (N = 22) were recorded simultaneously in two flight cages (3.00 × 2.00 × 2.20 m) in the Botanical Garden of Ulm University with one flight cage for each treatment. We conducted the experiments between May and June 2017 at comparable ambient air temperatures in the morning since the activity of bees rapidly decreased at high air temperatures. *Ranunculus* spp. and *C. biennis*, which were derived from a wild meadow in the Botanical Garden, served as a pollen and nectar source and were randomly distributed within the flight cage. Because flower morphology influences handling time, we tested two different plant species, namely *Ranunculus* spp. (Ranuculaceae) and *C. biennis* (Asteraceae). The two plant species differ clearly in their flower morphology and are within the broad food spectra of *O. bicornis* and *B. terrestris*. Plants remained in the same position within the flight cages until they wilted (maximum 2 days). For each bee, we recorded the time that they interacted with a flower. In addition, we registered the number of visited flowers, their species identity, and the time between visits from one flower to the next (searching time) per 3-min period. We chose this constant time, because completed foraging flights representing the period that the bees were absent from the nest, with no resting phase but under constant foraging, were rare. To facilitate the recordings, bees were labeled with an individual color code (Revell, Bünde, Germany) on the mesonotum. Foraging behavior after neonicotinoid treatment was only performed with *O. bicornis* because similar studies with *B. terrestris* have previously been performed (see Stanley and Raine, 2016) and have not revealed any effects of neonicotinoid treatment on their foraging behavior.

Antennal Sensitivity of *Osmia bicornis* and *Bombus terrestris*

We performed Electroantennographic analysis (EAG) at Ulm University. EAG is a good method to show the summed receptor potential and thus the response to an odorant at the periphery of the olfactory system (Schiestl and Poll, 2002). We diluted two floral semiochemicals, namely 2-phenylethanol (99%, Sigma-Aldrich, St. Louis, MO, United States) and linalool (racemic mixture, 97%, Sigma-Aldrich, St. Louis, MO, United States), which are common occurring volatile organic compounds (VOCs) of flowers (Knudsen et al., 1993, 2006), to various concentrations in hexane. One µl of the respective compound was diluted in 999 µl hexane (98%, Merck, Darmstadt, Germany) to produce the first test solution (dilution of 10^{-3}). For the following dilution stages, the first test solution (dilution of 10^{-3}) served as a stock solution and was diluted, respectively, to obtain

dilutions of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. This series of five different dilution stages was only applied to *O. bicornis*. For *B. terrestris*, we only used three dilutions (10^{-3}, 10^{-5}, and 10^{-7}) because preliminary studies had shown that differences in the antennal response were only found at a dilution of 10^{-3}. Furthermore, we also tested ethyl palmitate (ethyl hexadecanoate, 99%; Sigma-Aldrich, St. Louis, MO, United States), which is a pheromone component in bumblebees (Rottler-Hoermann et al., 2016), at three concentrations (dilutions of 10^{-3}, 10^{-5}, and 10^{-7}) as a third volatile. In social insects, in particular, pheromones play an important role and are indispensable in maintaining intra-colonial communication and the regulation and control of reproduction (Ayasse and Jarau, 2014). Hexane served as a control in all the experiments. All test solutions were stored in screw-cap micro-tubes (CZT, Kriftel, Germany) at –20°C.

For EAG analysis, we cut off the right antenna of a female bee ($N = 40$ for *O. bicornis* and *B. terrestris*, respectively) at the scapus with spring-scissors. Detached antennas were cut at the first and last segment of the flagellum with a razorblade. Using two micromanipulators (Märzhäuser Wetzlar GmbH & Co. KG, Wetzlar, Germany), we mounted each antenna between two borosilicate glass capillaries (GC150TF-10, Harvard Apparatus Ltd., Edenbridge, United Kingdom) filled with insect Ringer's solution (5 g NaCl, 0.42 g KCl, and 0.19 g $CaCl_2 \cdot 2H_2O$ dissolved in 1 l demineralized water) and connected to gold electrodes. The electrode at the base of the antenna was grounded, while the electrode at the tip was connected to a signal acquisition controller (Intelligent Data Acquisition Controller IDAC 2, Ockenfels SYNTECH GmbH, Kirchzarten, Germany) to record differences in receptor potential. The antenna was placed in front of a glass tube that directed a humidified air stream (volume 30 ml/min) toward the antenna and prevented it from rapidly drying out. Scents were applied to the antenna in a constant order (2-phenylethanol, linalool, ethyl palmitate for *B. terrestris*) with increasing concentration, which means decreasing dilution, and starting with 2-phenylethanol. At the beginning, between the first and second scent compound being presented to *O. bicornis*, between the second and third scent compound being presented to *B. terrestris* and at the end of each test series, we applied hexane and air individually to the antenna in order to normalize data and to correct for possible losses in sensitivity over time. For the stimulus, 10 µl of the respective solution was added to a filter paper strip (VWR International, Leuven, Belgium) and, after evaporation of the solvent for 1 min, the filter paper was inserted into a Pasteur pipette (150 mm, Soda Lime Glass, VWR International, Darmstadt, Germany). For each stimulus measurement, the Pasteur pipette was connected via a silicone tube to a stimulus controller (Syntech Stimulus Controller CS-05, Ockenfels SYNTECH GmbH, Kirchzarten, Germany) that delivered air puffs (30 ms, 25 ml/s) onto the antenna. Antennal responses were analyzed by Syntech EAG software EAGPro (v 2.0, Syntech, Hilversum, Netherlands).

Statistical Analysis

All statistical analyses were conducted with R (version 3.5.2, R Core Team, 2018). We compared recorded data of the neonicotinoid group and the control group of foraging flights (number of flowers per foraging flight, time between two flowers, time per flower visit and time per *C. biennis* flower and *Ranunculus* spp. flower) using a Mann–Whitney *U* test, since the data were not normally distributed. For a comparison of antennal responses, we first normalized the responses by using EAGPro software to correct for possible changes in the sensitivity of an antenna. Response to hexane was set as a response of 100%, whereas all other responses were calculated as values relative to hexane and were log-transformed. We calculated linear mixed-effect models (LME) for each compound by using the *lme* function from the *nlme* package (version 3.1-137, Pinheiro et al., 2014). Treatment and concentration were set as fixed factors, individual as a random factor. We ran a *post hoc* test by using the function *glht* (General Linear Hypotheses) from the multcomp package (version 1.4-16, Hothorn et al., 2008). All model assumptions were validated and were sufficient. A *t*-test followed by a Benjamini–Hochberg correction was used to analyse the response to a certain compound at a certain concentration compared with hexane (100%). If one of the tested scent compounds at a certain concentration showed a significantly higher response than hexane, we assumed that the bees were able to detect that substance at that concentration (Brandt et al., 2017).

RESULTS

Foraging Behavior

Clothianidin altered the foraging behavior in *O. bicornis* females (**Figure 1** and **Table 1**). During a time period of 3 min, females treated with clothianidin visited fewer flowers than untreated bees (**Figure 1A**, Mann–Whitney *U* test: $W_{1,21} = 97.5$, $p = 0.016$) and the searching time was significantly longer (**Figure 1B**, Mann–Whitney *U* test: $W_{1,21} = 9$, $p < 0.001$). Clothianidin had no effect on the average handling time per flower (Mann–Whitney *U* test: $W_{1,16} = 18$, $p = 0.093$). Bees treated with clothianidin exhibited a significantly longer flower visiting time for *Ranunculus* spp. (**Figure 1C**, Mann–Whitney *U* test: $W_{1,16} = 7$, $p = 0.006$) as compared with *C. biennis*. For *C. biennis* flowers, flower handling time was the same for untreated and treated bees (**Figure 1C**, Mann–Whitney *U* test: $W_{1,16} = 36$, $p = 1$).

Antennal Sensitivity

A comparison of antennal responses of *O. bicornis* to 2-phenylethanol and linalool revealed no treatment-dependent differences (**Table 2**). Antennal responses to 2-phenylethanol (LME: $F_{1,4} = 434.448$, $p < 0.001$) and linalool (LME: $F_{1,4} = 467.743$, $p < 0.001$) were significantly different for concentration (**Table 2**). Further, a combined effect of treatment and concentration was detected for 2-phenylethanol (LME: $F_{1,4} = 3.861$, $p < 0.01$). To validate whether a bee can detect a given compound at its respective concentration, we compared the antennal responses with the response to hexane, which was set 100% (**Supplementary Table 2**). For 2-phenylethanol, the responses to the dilutions 10^{-4} and 10^{-3}

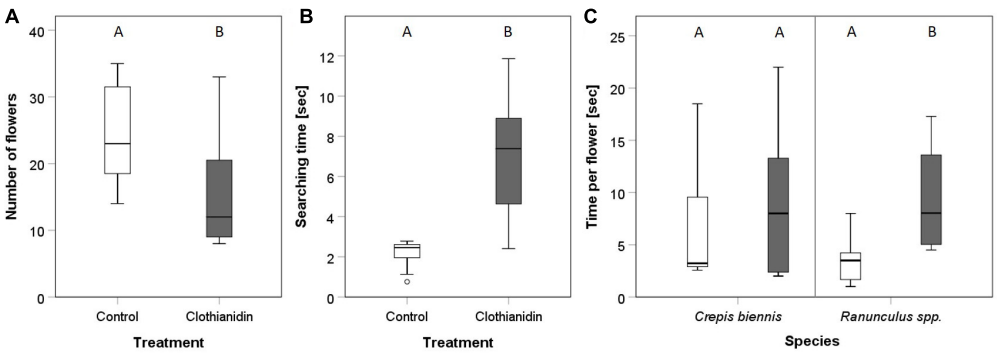

FIGURE 1 | Comparison of the effect of clothianidin treatment on various parts of the foraging flights of *Osmia bicornis*. **(A)** Average number of visited flowers per bee. **(B)** Average time between two flowers (searching time) per bee. **(C)** Average time per flower separated for both plant species *Crepis biennis* and *Ranunculus* spp. Boxes represent the 1st and 3rd quartiles, the median is shown as a solid line. Whiskers show the confidence interval of 95%. Outliers are plotted as individual dots. Different capital letters indicate significant differences between the control (n = 11) and the clothianidin (n = 11) treatment groups.

TABLE 1 | Comparison of various parts of the foraging flights of clothianidin-treated and untreated *Osmia bicornis* females by means of *t*-tests.

	Control		Clothianidin		*N*	*W*	*P*
	Mean	**SD**	**Mean**	**SD**			
Number of flowers	24.8	7.6	16.2	9.8	22	97.5	**0.016**
Searching time [sec]	2.5	1.4	7.1	3.2	22	9	**<0.001**
Time per flower [sec]	5.3	3.0	9.0	4.8	22	45	0.332
Time per *Ranunculus* spp. flower [sec]	3.4	2.3	9.4	5.2	17	7	**0.006**
Time per *Crepis biennis* flower [sec]	7.1	5.8	8.9	7.1	17	36	1

For each part of the foraging flights, the mean and standard deviation are given for the control group and clothianidin treatment group. Significant differences between the two groups are given in bold.

were significantly higher in both treatment groups compared with hexane (**Figure 2**). For the concentration 10^{-5}, only the antennal response of the bees in the control group was higher than that for hexane (*t*-test: $t_{19} = 3.3057$, $p < 0.01$). Thus, bees without clothianidin treatment were able to detect these higher concentrations, whereas clothianidin-treated bees could not. Responses to the two lowest concentrations did not differ from hexane, either in the control group or in the treatment group. For linalool, the responses to the concentration 10^{-4} and 10^{-3} were also significantly higher in both treatment groups compared with hexane and thus were detectable by the bees, whereas the remaining concentrations were not (**Figure 2**).

We found similar results for *B. terrestris*. Antennal responses revealed no treatment-dependent differences (**Table 3**). Antennal response differed significantly by concentration for 2-phenylethanol (LME: $F_{1,2} = 71.895$, $p < 0.001$), linalool (LME: $F_{1,2} = 85.003$, $p < 0.001$) and ethyl palmitate (LME: $F_{1,2} = 11.851$, $p < 0.001$). As for *O. bicornis*, an interactive effect of treatment and concentration on the antennal response was present for 2-phenylethanol (LME: $F_{1,2} = 3.578$, $p < 0.05$) and ethyl palmitate (LME: $F_{1,2} = 4.32$, $p < 0.05$). With regard to 2-phenylethanol and ethyl palmitate, the antennal response in the control group was significantly higher than that for hexane at all concentrations, whereas it was only significantly higher for the concentration 10^{-3} in bees of the treatment group (**Figure 3** and **Supplementary Table 4**). Thus, *B. terrestris* without clothianidin treatment were able to detect 2-phenylethanol and ethyl palmitate at lower concentrations than clothianidin-treated bees. Linalool was only detectable in the highest concentration 10^{-3} in both groups, with and without clothianidin treatment.

DISCUSSION

The results of our behavioral experiments showed that treatment with a field-realistic dose of clothianidin negatively affected the foraging behavior of *O. bicornis*. Treated bees visited significantly fewer flowers than the control group and exhibited a significantly longer searching time between two flowers and a longer visiting time on *Ranunculus* spp. flowers. The EAG analyses showed a decreased sensitivity of antennal scent receptors for 2-phenylethanol in *O. bicornis* and *B. terrestris* and in the sensitivity for ethyl palmitate in *B. terrestris*. Thus, bees treated with clothianidin on average were not able to detect 2-phenylethanol and ethyl palmitate at small concentrations, unlike bees that were not exposed to clothianidin.

Foraging Behavior

In our study, treated females spent a longer time on a flower and needed more time to reach the next flower resulting in lower visitation rates. Bees may need more time per flower, if

TABLE 2 | Results of linear mixed-effect models (LME) for the antennal responses of clothianidin-treated *O. bicornis* females to two different scent compounds, namely 2-phenylethanol and linalool, in comparison with an untreated control.

	2-Phenylethanol			Linalool		
	d.f.	***F***	***P***	**d.f.**	***F***	***P***
Treatment (T)	1	1.179	0.285	1	0.668	0.419
Concentration (C)	4	434.448	**<0.001**	4	467.743	**<0.001**
T × C	4	3.861	**<0.01**	4	0.360	0.837

Significant effects are given in bold.

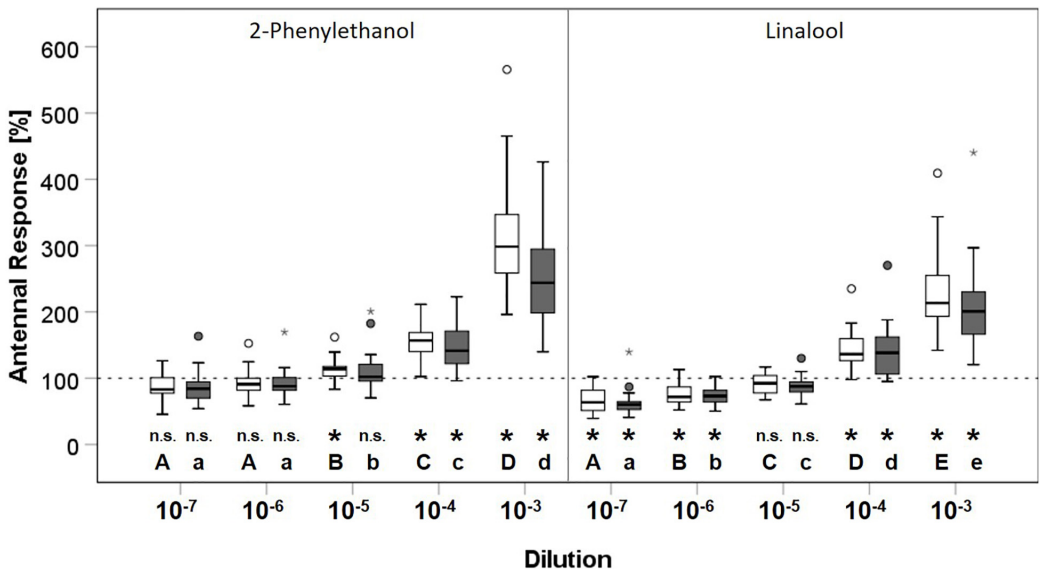

FIGURE 2 | Comparison of the effect of clothianidin treatment on antennal responses of *O. bicornis* (*N* = 40) to 2-phenylethanol and linalool. White boxes represent the control group treated with acetone; the clothianidin treatment group is shown by gray boxes. Boxes represent the 1st and 3rd quartiles, the median is shown as a solid line. Whiskers show the minimum and maximum range of values no further than the 1.5-fold inter-quartile range from the respective hinge. Outliers are plotted as individual dots, extreme outliers as stars. Hexane (response 100%) is shown as a broken line. Different letters indicate significant differences between concentrations for each odor compound within the control (capital letters) or clothianidin (small letters) group. Asterisks ($p < 0.05$) indicate significant differences compared with hexane (n.s.: $p > 0.05$).

TABLE 3 | Results of LME for the antennal responses of clothianidin-treated *Bombus terrestris* females to three different scent compounds, namely 2-phenylethanol, linalool, and ethyl palmitate, in comparison with an untreated control.

	2-Phenylethanol			Linalool			Ethyl palmitate		
	d.f.	***F***	***P***	**d.f.**	***F***	***P***	**d.f.**	***F***	***P***
Treatment (T)	1	1.321	0.258	1	0.072	0.79	1	1.477	0.232
Concentration (C)	2	71.895	**<0.001**	2	85.003	**<0.001**	2	11.851	**<0.001**
T × C	2	3.578	**<0.05**	2	0.582	0.561	2	4.326	**<0.05**

Significant effects are given in bold.

they have problems with handling flowers, particularly while collecting pollen and nectar. A possible explanation is that bees have problems with learning how to manipulate flowers (Stanley and Raine, 2016). They face a blockage of memory retrieval (Jin et al., 2015) for learned handling strategies, thereby increasing their handling time for flowers. The lower flower visitation rates that we found in clothianidin-treated *O. bicornis* females in our study were also observed in a former investigation after the treatment of bees with thiamethoxam (Stanley et al., 2015; Stanley and Raine, 2016). Furthermore, if females try to gain the same amount of pollen per flower from a certain plant species, they may need more time to exploit a flower if they experience problems manipulating it. To test this, future studies should focus on pollen foraging efficiency after neonicotinoid treatment by weighing bees before and after pollen-collecting flights.

In our experiments, we used two different plant families, Asteraceae (*C. biennis*) and Ranunculaceae (*Ranunculus* spp.) in

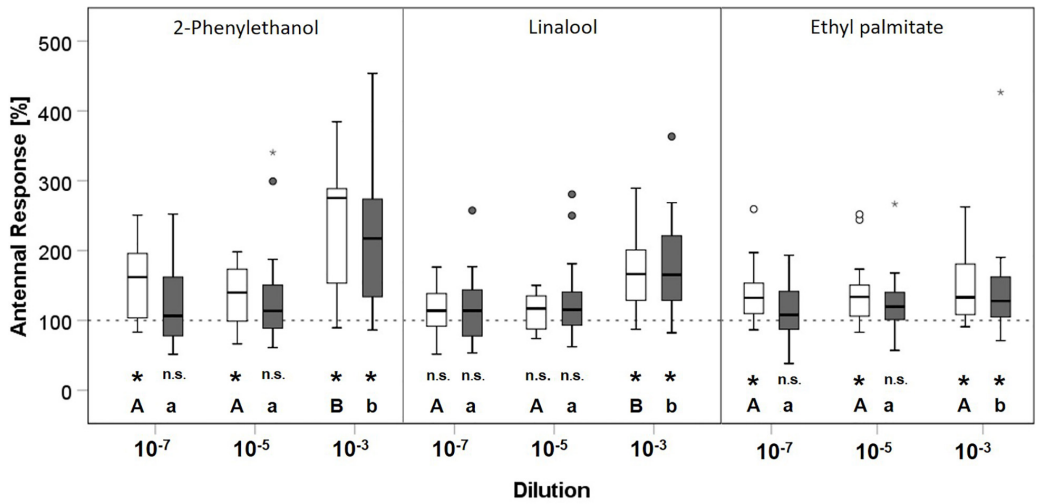

FIGURE 3 | Comparison of the effect of clothianidin treatment on antennal responses of *Bombus terrestris* ($N = 40$) to 2-phenylethanol, linalool and ethyl palmitate. White boxes represent the control group treated with acetone; the clothianidin treatment group is shown by gray boxes. Boxes represent the 1st and 3rd quartiles, the median is shown as a solid line. Whiskers show the minimum and maximum range of values no further than the 1.5-fold inter-quartile range from the respective hinge. Outliers are plotted as individual dots, extreme outliers as stars. Hexane (response 100%) is shown as a broken line. Different letters indicate significant differences between concentrations for each odor compound within the control (capital letters) or clothianidin (small letters) group. Asterisks ($p < 0.05$) indicate significant differences compared to hexane (n.s.: $p > 0.05$).

order to test whether the effect of clothianidin treatment is different depending on flower morphology. Bees treated with clothianidin spent more time handling *Ranunculus* flowers than bees in the control group. For *C. biennis*, which has composite flowers, handling time did not differ in our experiments. In *Ranunculus* spp. flowers, bees have to find the pollen in the center of each flower, whereas in the composite flowers of *C. biennis*, they can pick pollen from the whole flower head. This shows that bees have problems in handling flowers, depending on the complexity of a flower. A more than two-fold increase in the flower visiting time of *B. terrestris* after neonicotinoid treatment was observed in a study using the complex flowers of *Lotus corniculatus* (Stanley and Raine, 2016). Further, the authors mentioned that bumble bees experimentally exposed to a chronic dose of thiamethoxam learnt how to manipulate these complex flowers much more slowly than untreated bees.

In addition to the longer handling time of flowers by treated *O. bicornis* females, our study clearly showed that the time for searching for a new flower was also increased. To visit a further food source, bees have to fly from one flower to another, if these are not arranged in inflorescences. One reason for the increasing searching time is a disruption of the physical ability to fly. Tosi et al. (2017) have described an alteration in flight ability in honeybees after thiamethoxam treatment, which leads to decreased flight duration, distance and velocity. In further studies, honeybees treated with neonicotinoids also showed less well directed flights; this is a possible explanation for an increase in searching time and thus a decrease in the number of visited flowers (van der Sluijs et al., 2013; Fischer et al., 2014; Tison et al., 2016). In particular, the homing flights of honeybees and thus their navigation were affected in these studies. The longer searching times shown by our treated bees also suggest that their navigation skills are reduced within their three-dimensional surroundings. Disorientation after treatment with neonicotinoids might be the result of disturbed memory and learning behavior as shown in former studies (Desneux et al., 2007; Fent, 2013; van der Sluijs et al., 2013). Because flowers with nectar and pollen are an unreliable food source, bees rely on their memory to find good resources (Gross, 2013). Bees also clearly use floral traits such as color, shape and scent plus landmarks to find valuable food sources such as flowers (Gross, 2013; Knauer and Schiestl, 2015). Thus, a blockage of memory retrieval for learned cues that guide the bee to a food source might increase searching time (Jin et al., 2015). The effects of the increasing handling time and increasing searching time result in an increase of the total time of a foraging flight and, thus, the risk of pollinators being confronted with potential predators also increases. The reason for disturbed memory and olfactory learning might be a disturbance of the mushroom bodies, which are important in olfactory learning and which have been shown to be reduced in volume after neonicotinoid treatment (Heisenberg, 1998; Rybak and Menzel, 2010; Tomé et al., 2012; Fent, 2013). However, we have not investigated this aspect, because we have focused on the antennal receptors and not on higher brain structures in our study. Bees use floral scents to find flowers as a nectar and pollen source. Problems in finding new flowers, leading to increased searching times between two flowers, probably arise because of the effect of decreased antennal sensitivity, as we have found for certain of the tested chemical volatile compounds.

Antennal Sensitivity

Our result showed an effect of clothianidin on the antennal sensitivity in *O. bicornis* and *B. terrestris* for certain of the tested compounds that included not only typical floral volatiles

(Knudsen et al., 1993, 2006) but also pheromone components (Rottler-Hoermann et al., 2016). Bees treated with clothianidin were unable to detect 2-phenylethanol and ethyl palmitate in small concentrations, unlike bees that had not been exposed to clothianidin. Scent plays an important role in the finding of host plants and also serves as a cue in long-range attraction (Dötterl and Schäffler, 2007; Burger et al., 2010). Thus, it is crucial for bees to be able to detect low concentrations of floral scent compounds. A decreased sensitivity towards floral volatiles might lead to disturbances in the finding of host plants in both our studied species. If bees cannot find flowers or at least have problems locating them, they will not find appropriate amounts of pollen, a resource that plays and important role affecting the fitness of bees (Radmacher and Strohm, 2010). With regard to bumble bees, we should also mention disturbances in pheromone perception, since pheromones are crucial for intracolonial communication (Ayasse and Jarau, 2014), the disruption of which can lead to severe changes in colony maintenance and the stability of the colony. To the best of our knowledge, this is the first study that has shown an effect of neonicotinoids on antennal sensitivity in bees. In contrast to our findings, Artz and Pitts-Singer (2015) detected no changes in antennal sensitivity after fungicide treatment in *O. lignaria*. However, they used a different treatment mode involving nocturnal spray applications of their plants. In their approach, bees do not come into direct contact with the pesticide but take it up via nectar or pollen. This shows clearly that treatment modes can differ from each other in their effects on pollinators.

However, a study by Hesselbach and Scheiner (2018) has revealed a loss in taste sensitivity in honeybees after treatment with flupyradifurone, which binds nAChR similarly to neonicotinoids. Because neonicotinoids target receptors in the insect nervous system, receptors at the antennal level might also be affected and, thus, antennal sensitivity might be reduced. Comparing the substance classes of 2-phenylethanol (a benzenoid) and linalool (a monoterpene), clothianidin only reduced antennal sensitivity for 2-phenylethanol indicating that it might affect different scent receptor classes differently. To test this possibility, a broader range of scent compounds from various common substance classes should be tested in further approaches. As our EAG investigations compare the summed receptor potential per antenna they offer good evidence for the strength of total neurological activity within the antenna. Although we cannot identify single neuron activity, it is clearly seen that there is a difference in comparison to the control. In order to investigate the effects of clothianidin on the olfactory-receptor-neuron processing system at higher brain levels (e.g., antennal lobe and mushroom bodies), as suggested by Artz and Pitts-Singer (2015), it would be necessary to perform single cell recordings of peripheral olfactory neurons or calcium imaging.

CONCLUSION

Our study has clearly shown that clothianidin impairs the foraging behavior of *O. bicornis* and the antennal sensitivity of *O. bicornis* and *B. terrestris*. Since we have used field-realistic doses of clothianidin, we can expect similar effects in field populations of pollinating wild bees. The effect of neonicotinoids and other insecticides is probably twofold. On one hand, the ecosystem service of pollination provided by bees is negatively affected; in particular, the chances of flowers being pollinated decrease and, consequently, the number of fruits or seeds produced also decreases. On the other hand, in the longer term, a decrease in the biodiversity and abundance of wild bee populations can be expected, since disturbed foraging behavior will also affect the number of progeny and therefore the reproductive success of bees. However, our results in two pollinator species cannot necessarily be extrapolated to other pollinator groups, which might show different responses to insecticides. Thus, we need urgently to study of a variety of pollinators and pollinator groups (such as solitary bees, bumblebees, or even hoverflies), and not only honeybees.

In nature, bees collect pollen and nectar from several plants and fields, all possibly treated with a variety of pesticides over several days or weeks. Bees are therefore exposed to mixtures of agrochemicals over long periods of time. Thus, the effects of pesticides under real life conditions might be much more drastic than those determined in our study.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

FS, IJO, JK, and MA designed the experiments. FS, IJO, and JK performed the experiments. FS and MA wrote the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We are grateful to the staff of the Botanical Garden Ulm for their help during the flight cage experiments and to Elisabeth Arnold and Elke Stamm for their support in the laboratory. Further, we thank Theresa Jones for linguistic advice and Jonas Kuppler for statistical advice and helpful comments that improved this manuscript. We also thank two independent reviewers for their helpful comments that greatly improved this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.697355/full#supplementary-material

REFERENCES

Artz, D. R., and Pitts-Singer, T. L. (2015). Effects of fungicide and adjuvant sprays on nesting behavior in two managed solitary bees, osmia lignaria and megachile rotundata. *PloS One* 10:1371. doi: 10.1371/journal.pone.0135688

Ayasse, M., and Jarau, S. (2014). Chemical ecology of bumble bees. *Annu. Rev. Entomol.* 59, 299–319. doi: 10.1146/annurev-ento-011613-161949

Botías, C., David, A., Horwood, J., Abdul-Sada, A., Nicholls, E., Hill, E., et al. (2015). Neonicotinoid residues in wildflowers, a potential route of chronic exposure for bees. *Environ. Sci. Technol.* 49, 12731–12740. doi: 10.1021/acs.est.5b03459

Brandt, K., Dötterl, S., Francke, W., Ayasse, M., and Milet-Pinheiro, P. (2017). Flower visitors of campanula: are oligoleges more sensitive to host-specific floral scents than polyleges? *J. Chem. Ecol.* 43, 4–12. doi: 10.1007/s10886-016-0802-z

Burger, H., Dötterl, S., and Ayasse, M. (2010). Host-plant finding and recognition by visual and olfactory floral cues in an oligolectic bee. *Funct. Ecol.* 24, 1234–1240. doi: 10.1111/j.1365-2435.2010.01744.x

Casida, J. E., and Durkin, K. A. (2013). Neuroactive insecticides: Targets, selectivity, resistance, and secondary effects. *Annu. Rev. Entomol.* 58, 99–117. doi: 10.1146/annurev-ento-120811-153645

Desneux, N., Decourtye, A., and Delpuech, J. M. (2007). The sublethal effects of pesticides on beneficial arthropods. *Annu. Rev. Entomol.* 52, 81–106. doi: 10.1146/annurev.ento.52.110405.091440

Dötterl, S., and Schäffler, I. (2007). Flower scent of floral oil-producing *lysimachia punctata* as attractant for the oil-bee *macropis fulvipes*. *J. Chem. Ecol.* 33, 441–445. doi: 10.1007/s10886-006-9237-2

Dötterl, S., and Vereecken, N. J. (2010). The chemical ecology and evolution of bee-flower interactions: a review and perspectives. *Can. J. Zool.* 88, 668–697. doi: 10.1139/Z10-031

El Hassani, A. K., Dacher, M., Gary, V., Lambin, M., Gauthier, M., and Armengaud, C. (2008). Effects of sublethal doses of acetamiprid and thiamethoxam on the behavior of the honeybee (*Apis mellifera*). *Arch. Environ. Contam. Toxicol.* 54, 653–661. doi: 10.1007/s00244-007-9071-8

Elbert, A., Haas, M., Springer, B., Thielert, W., and Nauen, R. (2008). Applied aspects of neonicotinoid uses in crop protection. *Pest. Manage. Sci.* 64, 1099–1105. doi: 10.1002/ps.1616

Feltham, H., Park, K., and Goulson, D. (2014). Field realistic doses of pesticide imidacloprid reduce bumblebee pollen foraging efficiency. *Ecotoxicology*. 23, 317–323. doi: 10.1007/s10646-014-1189-7

Fent, K. (2013). *Ökotoxikologie: Umweltchemie-Toxikologie-Ökologie*. New York, NY: Georg Thieme.

Fischer, J., Müller, T., Spatz, A.-K., Greggers, U., Grünewald, B., and Menzel, R. (2014). Neonicotinoids interfere with specific components of navigation in honeybees. *PloS One* 9:91364. doi: 10.1371/journal.pone.0091364

Godfray, H. C. J., Blacquière, T., Field, L. M., Hails, R. S., Petrokofsky, G., Potts, S. G., et al. (2014). A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. *Proc. Biol. Sci.* 281:558. doi: 10.1098/rspb.2014.0558

Goulson, D., Nicholls, E., Botías, C., and Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* 347:1255957. doi: 10.1126/science.1255957

Gross, M. (2013). EU ban puts spotlight on complex effects of neonicotinoids. *Curr. Biol.* 23, 462–464. doi: 10.1016/j.cub.2013.05.030

Gruber, B., Eckel, K., Everaars, J., and Dormann, C. F. (2011). On managing the red mason bee (*Osmia bicornis*) in apple orchards. *Apidologie*. 42, 564–576. doi: 10.1007/s13592-011-0059-z

Hallmann, C. A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., et al. (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PloS One* 12:1371. doi: 10.1371/journal.pone.0185809

Heisenberg, M. (1998). What do the mushroom bodies do for the insect brain? An introduction. *Learn Mem.* 5, 1–10. doi: 10.1101/lm.5.1.1

Hesselbach, H., and Scheiner, R. (2018). Effects of the novel pesticide flupyradifurone (Sivanto) on honeybee taste and cognition. *Sci. Rep.* 28, 354–366. doi: 10.1038/s41598-018-23200-0

Hopwood, J., Vaughan, M., Shepherd, M., Biddinger, D., Mader, E., Black, S. H., et al. (2012). *Are neonicotinoids killing bees: a review of research into the effects of neonicotinoid insecticides on bees, with recommendations for action*. Portland: The Xerces Society for Invertebrate Conservation.

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical J.* 50, 346–363. doi: 10.1002/bimj.200810425

Jeschke, P., and Nauen, R. (2008). Neonicotinoids-from zero to hero in insecticide chemistry. *Pest. Manage. Sci.* 64, 1084–1098. doi: 10.1002/ps.1631

Jin, N., Klein, S., Leimig, F., Bischoff, G., and Menzel, R. (2015). The neonicotinoid clothianidin interferes with navigation of the solitary bee *Osmia cornuta* in a laboratory test. *J. Exp. Biol.* 218, 2821–2825. doi: 10.1242/jeb.123612

Kaib, M. (2003). "Chemorezeption," in *Lehrbuch der Entomologie: Teil 1*, eds K. Dettner and W. Peters (Berlin: Spektrum Akademischer Verlag), 302–320.

Knauer, A. C., and Schiestl, F. P. (2015). Bees use honest floral signals as indicators of reward when visiting flowers. *Ecol. Lett.* 18, 135–143. doi: 10.1111/ele.12386

Knudsen, J. T., Eriksson, R., Gershenzon, J., and Ståhl, B. (2006). Diversity and distribution of floral scent. *Bot. Rev.* 72, 1–120. doi: 10.1663/0006-8101200672

Knudsen, J. T., Tollsten, L., and Bergström, L. (1993). Floral scents—a checklist of volatile compounds isolated by head-space techniques. *Phytochemistry*. 33, 253–280. doi: 10.1016/0031-9422(93)85502-I

Matsuda, K., Buckingham, S. D., Kleier, D., Rauh, J. J., Grauso, M., and Sattelle, D. B. (2011). Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends Pharmacol. Sci.* 22, 573–580. doi: 10.1016/S0165-6147(00)01820-4

Michener, C. D. (2000). *The bees of the world*. Baltimore: JHU press.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and Team, R. C. (2014). *nlme: Linear and nonlinear mixed effects models. R package version 3.1-117*. http://CRAN.R-project.org/package=nlme.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., and Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends Ecol. Evol.* 25, 345–353. doi: 10.1016/j.tree.2010.01.007

R Core Team. (2018). *R: A language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Radmacher, S., and Strohm, E. (2010). Factors affecting offspring body size in the solitary bee *Osmia bicornis* (Hymenoptera, Megachilidae). *Apidologie* 41, 169–177. doi: 10.1051/apido/2009064

Rottler-Hoermann, A.-M., Schulz, S., and Ayasse, M. (2016). Nest wax triggers worker reproduction in the bumblebee *Bombus terrestris*. *R. Soc. Open Sci.* 3:599. doi: 10.1098/rsos.150599

Rundlöf, M., Andersson, G. K. S., Bommarco, R., Fries, I., Hederström, V., Herbertsson, L., et al. (2015). Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature* 521, 77–80. doi: 10.1038/nature14420

Rybak, J., and Menzel, R. (2010). "Mushroom Body of the Honeybee," in *Handbook of Brain Microcircuits*, eds G. M. Shepherd and S. Grillner (Oxford: Oxford University Press), 433–438. doi: 10.1093/med/9780195389883.003.0044

Schiestl, F. P. (2015). Ecology and evolution of floral volatile-mediated information transfer in plants. *New Phytol.* 206, 571–577. doi: 10.1111/nph.13243

Schiestl, F. P., and Poll, F. M. (2002). "Detection of physiologically active flower volatiles using gas chromatography coupled with electroantennography," in *Molecular methods of plant analysis, volume 21: analysis of taste and aroma*, eds J. F. Jackso, H. F. Linskens, and R. Inman (Berlin: Springer), 173–198. doi: 10.1007/978-3-662-04857-3_9

Scott-Dupree, C. D., Conroy, L., and Harris, C. R. (2009). Impact of Currently Used or Potentially Useful Insecticides for Canola Agroecosystems on *Bombus impatiens* (Hymenoptera: Apidae), *Megachile rotundata* (Hymentoptera: Megachilidae), and *Osmia lignaria* (Hymenoptera: Megachilidae. *J. Econ. Entomol.* 102, 177–182. doi: 10.1603/029.102.0125

Seidelmann, K. (2014). Optimal progeny body size in a solitary bee, *Osmia bicornis* (Apoidea: Megachilidae. *Ecol. Entomol.* 39, 656–663. doi: 10.1111/een.12145

Sgolastra, F., Tosi, S., Medrzycki, P., Porrini, C., and Burgio, G. (2015). Toxicity of Spirotetramat on Solitary Bee Larvae, *Osmia cornuta* (Hymenoptera: Megachilidae), in Laboratory Conditions. *J. Apic. Sci.* 59:24. doi: 10.1515/jas-2015-0024

Stanley, D. A., Garratt, M. P. D., Wickens, J. B., Wickens, V. J., Potts, S. G., and Raine, N. E. (2015). Neonicotinoid pesticide exposure impairs crop pollination services provided by bumblebees. *Nature* 528, 548–550. doi: 10.1038/nature16167

Stanley, D. A., and Raine, N. E. (2016). Chronic exposure to a neonicotinoid pesticide alters the interactions between bumblebees and wild plants. *Funct. Ecol.* 30, 1132–1139. doi: 10.1111/1365-2435.12644

Stokstad, E. (2013). Pesticides under fire for risks to pollinators. *Science* 340, 674–676. doi: 10.1126/science.340.6133.674

Tan, K., Chen, W., Dong, S., Liu, X., Wang, Y., and Nieh, J. C. (2014). Imidacloprid alters foraging and decreases bee avoidance of predators. *PloS One* 9:2725. doi: 10.1371/journal.pone.0102725

Tappert, L., Pokorny, T., Hofferberth, J., and Ruther, J. (2017). Sublethal doses of imidacloprid disrupt sexual communication and host finding in a parasitoid wasp. *Sci. Rep*. 7:42756. doi: 10.1038/srep42756

Tison, L., Hahn, M.-L., Holtz, S., Rößner, A., Greggers, U., Bischoff, G., et al. (2016). Honey Bees' behavior is impaired by chronic exposure to the neonicotinoid thiacloprid in the field. *Environ. Sci. Technol.* 50, 7218–7227. doi: 10.1021/acs.est.6b02658

Tomé, H. V. V., Martins, G. F., Lima, M. A. P., Campos, L. A. O., and Guedes, R. N. C. (2012). Imidacloprid-induced impairment of mushroom bodies and behavior of the native stingless bee *Melipona quadrifasciata anthidioides*. *PloS One* 7:6. doi: 10.1371/journal.pone.0038406

Tomizawa, M., and Casida, J. E. (2005). Neonicotinoid insecticide toxicology: Mechanisms of selective action. *Annu. Rev. Pharmacol. Toxicol.* 45, 247–268. doi: 10.1146/annurev.pharmtox.45.120403.095930

Tosi, S., Burgio, G., and Nieh, J. C. (2017). A common neonicotinoid pesticide, thiamethoxam, impairs honey bee flight ability. *Sci. Rep*. 7:1361-8. doi: 10.1038/s41598-017-01361-8

Tsvetkov, N., Samson-Robert, O., Sood, K., Patel, H. S., Malena, D. A., Gajiwala, P. H., et al. (2017). Chronic exposure to neonicotinoids reduces honey bee health near corn crops. *Science* 356, 1395–1397. doi: 10.1126/science.aam7470

Uneme, H. (2011). Chemistry of clothianidin and related compounds. *J. Agric. Food Chem.* 59, 2932–2937. doi: 10.1021/jf1024938

van der Sluijs, J. P., Simon-Delso, N., Goulson, D., Maxim, L., Bonmatin, J.-M., and Belzunces, L. P. (2013). Neonicotinoids, bee disorders and the sustainability of pollinator services. *Curr. Opin. Environ. Sustain*. 5, 293–305. doi: 10.1016/j.cosust.2013.05.007

Westrich, P., and Dathe, H. H. (1997). Die bienenarten deutschlands (Hymenoptera, Apidae): ein aktualisiertes verzeichnis mit kritischen anmerkungen. *Mitt. Entomol. Verein Stuttg.* 32, 3–34.

Williamson, S. M., Willis, S. J., and Wright, G. A. (2014). Exposure to neonicotinoids influences the motor function of adult worker honeybees. *Ecotoxicology* 23, 1409–1418. doi: 10.1007/s10646-014-1283-x

[EPA] U.S. Environmental Protection Agency. (2003). *Clothianidin pesticide fact sheet*. Washington, D.C: United States Environmental Protection Agency.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 07 September 2021
doi: 10.3389/fevo.2021.669352

Specialized Metabolites in Floral Resources: Effects and Detection in Buff-Tailed Bumblebees

*Ombeline Sculfort[1], Maxence Gérard[2,3], Antoine Gekière[2], Denis Nonclercq[4], Pascal Gerbaux[5], Pierre Duez[6] and Maryse Vanderplanck[2]**

[1] Laboratoire Écologie, Évolution, Interactions des Systèmes Amazoniens, Université de Guyane, Centre National de la Recherche Scientifique, Institut Français de Recherche pour l'Exploitation de la Mer, Cayenne, France, [2] Laboratory of Zoology, Research Institute for Biosciences, University of Mons, Mons, Belgium, [3] Division of Functional Morphology, Department of Zoology, Stockholm University, Stockholm, Sweden, [4] Laboratory of Histology, Faculty of Medicine and Pharmacy, University of Mons, Mons, Belgium, [5] Organic Synthesis and Mass Spectrometry Laboratory, Research Institute for Biosciences, University of Mons, Mons, Belgium, [6] Unit of Therapeutic Chemistry and Pharmacognosy, Faculty of Medicine and Pharmacy, University of Mons, Mons, Belgium

The selection of appropriate food resources by bees is a critical aspect for the maintenance of their populations, especially in the current context of global change and pollinator decline. Wild bees have a sophisticated ability to forage selectively on specific resources, and can assess the quality of pollen using contact chemosensory perception (taste). While numerous studies have investigated the detection of pollen macronutrients in bees and their impact on bee health and reproductive success, only a few studies have described the gustatory responses of bees toward specialized metabolites. In addition, these studies mostly focused on the response to nectar and neglected pollen, which is the main food resource for both bee imagines and larvae. Whether bees have the ability to detect specialized toxic metabolites in pollen and then rapidly adapt their foraging behavior to avoid them is very little studied. In this study, we tested whether pollen specialized metabolites affect bumblebees at both the micro-colony and individual levels (i.e., bioassays using supplemented pollen), and whether foragers detect these specialized metabolites and potentially display an avoidance behavior (i.e., preference tests using supplemented syrup). Bumblebees were fed with either amygdalin-, scopolamine- or sinigrin-supplemented pollen diets in ratios that mimic 50%, 100%, and 200% of naturally occurring concentrations. We found no effect of these specialized metabolites on resource collection, reproductive success and stress response at the micro-colony level. At the individual level, bumblebees fed on 50%-amygdalin or 50%-scopolamine diets displayed the highest scores for damage to their digestive systems. Interestingly, during the preference tests, the solution with 50%-scopolamine displayed a phagostimulatory activity, whereas solution with 50%-amygdalin had a deterrent effect and could trigger an active avoidance behavior in bumblebees, with a faster proboscis retraction. Our results suggest that regulation of toxin intake is not as well-established and effective as the regulation of nutrient intake in bees. Bees are therefore not equally adapted to all specialized pollen metabolites that they can come into contact with.

Keywords: plant–pollinator interactions, amygdalin, sinigrin, scopolamine, detection, behavior, toxicity

OPEN ACCESS

Edited by:
Sara Diana Leonhardt, Technical University of Munich, Germany

Reviewed by:
Fabian Ruedenauer, Technical University of Munich, Germany
Sharoni Shafir, Hebrew University of Jerusalem, Israel

****Correspondence:***
Maryse Vanderplanck
maryse.vanderplanck@umons.ac.be

Specialty section:
This article was submitted to Chemical Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *18 February 2021*
Accepted: *05 August 2021*
Published: *07 September 2021*

Citation:
Sculfort O, Gérard M, Gekière A, Nonclercq D, Gerbaux P, Duez P and Vanderplanck M (2021) Specialized Metabolites in Floral Resources: Effects and Detection in Buff-Tailed Bumblebees. Front. Ecol. Evol. 9:669352. doi: 10.3389/fevo.2021.669352

INTRODUCTION

In the current context of global change and pollinator decline (Potts et al., 2016; Dicks et al., 2020), consumption of adequate food resources can provide bees with resilience to some environmental stressors, as recently demonstrated in bumblebees facing heat stress (Vanderplanck et al., 2019a), and in honeybees facing viral infections (Dolezal et al., 2019). The other side of the coin is that consumption of inadequate resources, even by an otherwise healthy organism, can lead to reduced survival and decreased immunity, as well as an increased susceptibility to pathogens and parasites (e.g., Alaux et al., 2010; DeGrandi-Hoffman et al., 2010; Di Pasquale et al., 2013; Roger et al., 2017; Vanderplanck et al., 2018). Selecting appropriate food resources is therefore a critical aspect for bees in order to maintain their populations (see Vaudo et al., 2015).

It is common knowledge that bees predominantly collect pollen to satisfy their nutritional and physiological requirements, it being essential for reproduction and for the health of imagines (Human et al., 2007; Di Pasquale et al., 2013; Cane, 2016; Cane et al., 2016), as well as for the development of their offspring (Génissel et al., 2002; Tasei and Aupinel, 2008a; Brodschneider and Crailsheim, 2010; Quezada-Euan et al., 2011). Pollen is a complex chemical mixture that contains both central (or primary) metabolites, which are vital for plant survival (e.g., proteins, amino acids and lipids; see Roulston et al., 2000; Weiner et al., 2010), and specialized (or secondary) metabolites, which play a key role in the interaction of the plant with the environment such as underpinning insect attraction or deterrence (e.g., alkaloids; see Kempf et al., 2010; Cook et al., 2013; Gosselin et al., 2013; Stegemann et al., 2018). Pollen composition is highly variable among plant species so bees face a high degree of variation in pollen quality (e.g., Roulston and Cane, 2000; Roulston et al., 2000; Weiner et al., 2010; Vaudo et al., 2015, 2020; Palmer-Young et al., 2019), with some pollen types being unsuitable for some bee species because of lack in essential nutrients, occurrence of toxic compounds or low digestibility leading to difficulties in extracting nutrients (e.g., Levin and Haydak, 1957; Praz et al., 2008b; Sedivy et al., 2011; Haider et al., 2013; Vanderplanck et al., 2014, 2018, 2020). This implies that even generalist bees cannot forage randomly on all available resources, but have to display selective foraging to increase their individual health and reproductive success.

Indeed, both social and solitary bees have been shown to forage selectively on different pollen types according to their nutritional quality. For instance, bumblebees preferentially collect pollen rich in proteins and amino acids (Rasheed and Harder, 1997; Robertson et al., 1999; Hanley et al., 2008; Kitaoka and Nieh, 2009; Leonhardt and Blüthgen, 2012). Such selective foraging on specific resources may arise from imprinting, with bees developing preference toward pollen types they fed on during their larval phase or as early imagines (e.g., Dobson and Peng, 1997; Cane and Sipes, 2006; Ruedenauer et al., 2020b). Additionally, social bee species could also rely on feedback from their nest, such as the rate of food consumption by larvae, in determining preferences (e.g., Ruedenauer et al., 2016). However, evidence is that pre-imaginal learning does not always prevail (e.g., Praz et al., 2008a), and that individual imagines can differentiate between different pollen types without relying on feedback from larvae (Ruedenauer et al., 2015). Wild bees have therefore a sophisticated ability to forage selectively on resources that allow them to achieve their nutritional optimum (Dobson and Bergström, 2000; Hanley et al., 2008; Ruedenauer et al., 2015, 2016). Such assessment of pollen quality could be done through olfactory (i.e., pollen odor; Dobson and Bergström, 2000), visual (i.e., pollen color; Lunau, 2000) or chemotactile cues (i.e., pollen taste; e.g., Pernal and Currie, 2002; Leonhardt and Blüthgen, 2012; Lunau et al., 2015; Ruedenauer et al., 2015, 2016; Muth et al., 2016), without knowing all components. Among these cues, it is more likely that bees would use smell (i.e., olfactory cues) and/or pollen taste (i.e., chemotactile cues) rather than pollen color to discriminate among food resources. Ruedenauer et al. (2015) have actually shown that bumblebees are able to differentiate between different nutrient concentrations using contact chemosensory perception, which is enabled via gustatory receptors on their antennae, mouthparts and tarsi (de Brito Sanchez, 2011). They are therefore able to regulate their nutrient intake by varying their foraging rate on different food resources. This selective foraging appears to be guided by the fat content and protein:lipid ratio of pollen rather than by the protein content alone (Vaudo et al., 2016a,b; Ruedenauer et al., 2020a). In particular, fatty acid cues appear to play a key role in fat regulation and foraging decisions in *Bombus terrestris* (Ruedenauer et al., 2020a). Hence, chemical composition of pollen might be involved in the selection and use of resources, at least in some bee species. However, the complete picture is lacking as studies on selective foraging have mainly focused on macronutrients (i.e., central metabolites), whereas only a few studies have described the gustatory responses of bees toward specialized metabolites in food (e.g., Ayestaran et al., 2010; Tiedeken et al., 2014). Moreover, these experiments have mainly used restrained bees (i.e., stressed individuals with non-natural feeding responses; Mommaerts et al., 2013), and have mostly considered specialized metabolites that occur only in nectar.

Despite this, there have been several reports on the presence of specialized metabolites in pollen of widespread plant species that represent important food resources for pollinators (Rivest and Forrest, 2020 and references cited). While the role of central metabolites as nutrients for pollinators is largely assumed (e.g., Hügel, 1962; Day et al., 1990; Herbert, 1992; Roulston et al., 2000), the role of specialized metabolites is still controversial (Manson et al., 2010; de Roode et al., 2013; Wright et al., 2013; Arnold et al., 2014; Hurst et al., 2014; Richardson et al., 2015; Stevenson et al., 2017; Stevenson, 2020). Originally, specialized metabolites evolved in plants as chemical defenses in response to selection imposed by herbivores and pathogens (Moore et al., 2014; Richards et al., 2015; Rivest and Forrest, 2020). Their occurrence in pollen could then play an important ecological role in plant–pollinator interactions, such as by favoring pollen specialization or pollen mixing behavior in bees; Eckhardt et al., 2014; Rivest and Forrest, 2020). These biologically active metabolites could improve the health status of pollinators (e.g., Palmer-Young et al., 2017) through anti-oxidant (e.g., Aličić et al., 2014) and anti-microbial properties (e.g., Compean and Ynalvez, 2014), but they

could also impede larval development (de Carvalho and Message, 2004; Arnold et al., 2014), induce malaise behavior (Hurst et al., 2014) and weaken the insect immune system through insecticidal properties (Baracchi et al., 2015). In some instances, specialized metabolites could even kill pollinators (Detzel and Wink, 1993; Adler, 2000; de Carvalho and Message, 2004). Consequently, it is critical that bees have the ability to detect pollen specialized metabolites, especially if they have negative impact on their health and fitness, and rapidly adapt their foraging behavior to avoid such toxic resources.

In this study, we performed a range of bioassays and behavioral experiments with freely moving workers of the bumblebee *Bombus terrestris* to determine (1) whether pollen specialized metabolites affect bumblebees at the micro-colony level (resource collection, reproduction and stress response) as well as at individual level (histological damage), (2) and whether bumblebees detect these specialized metabolites and potentially display an avoidance behavior. We focused on amygdalin, scopolamine and sinigrin; three nitrogen-containing metabolites synthesized by different plant families that are actively foraged upon by bumblebees (Erickson and Feeny, 1974; King, 1993; London-Shafir et al., 2003; Ares et al., 2015; Chowański et al., 2016; Sáez et al., 2020). We assume that bumblebees must be able to detect specialized metabolites (either through pre- or post-ingestive effects, or both) that are toxic for them at either the micro-colony or individual level. We further expect that bumblebees will consequently display an avoidance behavior, for instance by reducing their resource collection.

MATERIALS AND METHODS

Model System

Bombus terrestris is one of the most abundant and widespread bumblebee species of the West Palearctic. This social species is a highly polylectic bumblebee foraging on hundreds of different plant species belonging to numerous plant families (Kleijn and Raemakers, 2008; Rasmont et al., 2008; Leonhardt and Blüthgen, 2012). As a consequence, it has a very important role as a pollinator in wild and cultivated plant communities (Free, 1993; Velthuis and van Doorn, 2006). However, colonies do not show equivalent development on all pollen species (Vanderplanck et al., 2018), partly because of the occurrence of specialized metabolites. In this study, we focus on three nitrogen-containing metabolites, namely amygdalin, scopolamine and sinigrin.

Amygdalin belongs to the family of cyanogenic glycosides, which are chemical defenses characteristic of Rosaceae (Robinson, 1930; Conn, 1978). It occurs naturally at level of 1,889 ppm in pollen of *Prunus dulcis* Mill (London-Shafir et al., 2003). This *Prunus* species is one of the most economically valuable bee-pollinated crop species because of its high pollinator-dependence and high-market value (Sáez et al., 2020), bumblebees counting among the pollinators of almond crop fields (Dag et al., 2006; Marqués et al., 2019). Repeat consumption of such chemically defended pollen can be toxic to bees (Kevan and Ebert, 2005), especially if they are not able to detect the toxic substance. Upon enzymatic hydrolysis, amygdalin liberates cyanide; its toxicity may be explained by its metabolization to sulfocyanide, an inhibitor of the iodide pump.

Scopolamine belongs to the family of Solanaceae alkaloids, which display insecticidal and fungicidal properties characteristic of this plant family (Boulogne et al., 2012). It occurs naturally at level of 20,014 ppm in pollen of *Brugmansia aurea* Lagerh (Detzel and Wink, 1993). Plant species in the cosmopolitan family Solanaceae are among the most ecologically and economically important, particularly in terms of food production (e.g., potatoes and tomatoes), and are generally pollinated by bumblebees (i.e., buzz pollination) (King, 1993). The occurrence of such a biologically active compound could then render the pollen of Solanaceae species toxic to its pollinators, including bumblebees, which could be highly detrimental if the substance has no repellent effect. Scopolamine is a competitive antagonist of acetylcholine at muscarinic receptors (Fraenkel, 1959; Brown and Keith, 1987; Chowański et al., 2016). It was shown to bind to brain receptors and increase attacks on nestmates in honeybees (Gauthier et al., 1994; Ismail et al., 2008).

Sinigrin is one of the most widespread glucosinolates (mustard oil glycosides) occurring in many species of Brassicaceae and in a few other plant families (Erickson and Feeny, 1974; Mazumder et al., 2016). It has been detected at level of 1,892 ppm in bee pollen of *Brassica* sp. (Ares et al., 2015). Brassicaceae is a widespread plant family that includes numerous species of agricultural and medicinal interest (Kissen et al., 2009). This plant family is known to be largely bee-pollinated and bumblebees are likely exposed to the potential insecticidal properties of its glucosinolates, depending on their avoidance behavior. Upon enzymatic hydrolysis, sinigrin yields allyl isothiocyanate, a volatile and highly pungent compound that acts as plant defense by deterring herbivores (Erickson and Feeny, 1974; Shields and Mitchell, 1995; Frisch et al., 2015).

Bioassays

Pollen Diets

How specialized metabolites can impact pollinator behavior, performance and health was investigated by the use of a control diet as well as amygdalin-, scopolamine-, and sinigrin-supplemented diets (i.e., test diets). The test diets contained chemicals mixed with the control diet in ratios that mimic 50%, 100%, and 200% of the naturally occurring concentration (i.e., a total of nine test diets; see **Supplementary Table 1** for naturally occurring concentrations). The control diet consisted of ground pollen loads with a dominance of *Salix* sp. mixed with inverted sugar syrup (BIOGLUC®, Biobest) to obtain consistent ball-shaped candies. *Salix* pollen is described as an excellent resource for *B. terrestris* and is unlikely to display specialized metabolites at sublethal or lethal concentrations for bumblebees since it support their larval and colony development well (Tasei and Aupinel, 2008a; Moerman et al., 2017; Vanderplanck et al., 2018). Chromatographic analyses confirmed that amygdalin, scopolamine and sinigrin were absent from the control diet (for analytical details see London-Shafir et al., 2003; Ares et al., 2015). The test diets were prepared using commercial powders that were dissolved in aqueous ethanol solution (1:1) before addition

to the control diet. Aqueous ethanol was selected because, even if all tested metabolites are soluble in water, it improved their solubility at the highest concentrations and allowed for not over-moisturizing the pollen candies as ethanol quickly evaporates. All treatment diets (both control and test diets) contained aqueous ethanol (1:1; 0.4 mL/g of diet) to control for potential negative effects of the solvent when assessing the added chemical treatments. Pollen loads of *Salix* were purchased from the company "Ruchers de Lorraine," which were sold as organic nutrition complement (i.e., free of pesticides).

Experimental Design

The experiments were conducted at the University of Mons from February 2015 to May 2016. A first run of bioassays was performed in 2015 for amygdalin (i.e., four treatments; control, 50%-amygdalin, 100%-amygdalin, and 200%-amygdalin), and a second run in 2016 for scopolamine and sinigrin (i.e., seven treatments; control, 50%-scopolamine, 100%-scopolamine, 200%-scopolamine, 50%-sinigrin, 100%-sinigrin, and 200%-sinigrin). Ten queenless *B. terrestris* micro-colonies were established for each treatment using workers from five different colonies (Biobest *bvba,* Westerlo, Belgium) that were equally distributed among the treatments to ensure homogeneity of origin. A total of 110 micro-colonies were then monitored for all experiments. Each micro-colony was composed of five 2-day-old workers placed in different plastic boxes (10 cm × 16 cm × 16 cm) in a dark room at 27°C and 76% relative humidity. The micro-colonies were fed *ad libitum* with sugar syrup (BIOGLUC®, Biobest) and pollen candies that were freshly prepared and renewed every 2 days (0.5 g, 1.0 g, or 1.5 g depending on the age of the micro-colony) to avoid nutrient alteration and drying out during the experiment. Pollen and syrup collections were measured by weighing pollen candies and syrup container before their introduction into the micro-colony and after their removal. Ejected larvae were removed from the micro-colony; workers that died during the experiment were removed and replaced. Syrup and pollen supplies as well as micro-colonies monitoring were done in the darkroom under red light during the 35-day period following the first episode of egg laying of a worker. At the end of the experiment, workers were weighed. The total mass of workers was expressed as the sum of the weights of the five workers in each micro-colony, taking into account the time they spent in the micro-colony in case of death and replacement. The nest was then carefully dissected, and the number and mass of individuals were recorded for each brood stage.

Micro-Colony Performance

Feeding response and micro-colony development were evaluated based on: (i) composition (i.e., number of eggs, non-isolated larvae, isolated larvae, pupae, non-emerged and emerged drones) and fresh weight of offspring, (ii) larval ejection (i.e., number of larvae, alive and dead, removed from the nest by workers), (iii) pollen collection (i.e., amount of pollen consumed and stored) (fresh matter), (iv) pollen efficiency (i.e., the weight of hatched offspring divided by the total pollen collected per micro-colony), (v) syrup collection (i.e., amount of syrup consumed and stored) and (vi) pollen dilution (i.e., the total syrup collected divided by the total pollen collected per micro-colony) (parameters adapted from Tasei and Aupinel, 2008b). All weight parameters (i.e., brood weight, pollen collection, and syrup collection) were standardized by the total mass of workers in the micro-colonies to avoid potential bias from worker activities (i.e., consumption and brood care).

Digestive Damage

The general histology of the bumblebee digestive tract is described in details in Vanderplanck et al. (2020). It is composed of a cuticle-lined foregut (stomodaeum), a midgut (mesenteron) and a cuticle-lined hindgut (proctodaeum). Histological examination focused on the mesenteron, which is the principal site of digestion and absorption of both nutrients and ingested plant allelochemicals. It represents therefore the first line of defense against the absorption of specialized metabolites, with for instance the protective role of peritrophic membrane. Its epithelium also represents an important interface between the insect and its environment. It consists of discrete crypts and lies on connective tissue. Its major cell type is the columnar cells with numerous microvilli forming, at the apical pole, a brush-like border. These cells display a slightly granular cytoplasm and, at their center, a large ovoid and euchromatic nucleus (Calatayud and Rabhé, 2013; Sarwade and Bhawane, 2013).

Tissues for histological evaluation were prepared following the method described by Vanderplanck et al. (2020). For each treatment, four bumblebee individuals were randomly collected from the different micro-colonies and cold-anesthetized (n = 4 per treatment). Their abdomens were cut and incised to facilitate the fixation (Duboscq–Brazil fluid), dehydration and paraffin-embedding processes. Transverse serial sections of 5 µm thicknesses were performed with a microtome (Reichert-Jung® 2040 microtome) with the use of a softening agent (Mollifex™), and placed on silane-coated glass slides. After rehydration, the sections were stained with Masson's Trichrome staining method.

A single-blind microscopic evaluation was carried out using a research optical microscope (Leitz® Orthoplan). This allowed for eliminating biases due to knowledge of treatment. The parameters evaluated for damage score were the common histopathological alterations in the digestive tract (Vanderplanck et al., 2020), namely: (i) disorganization or loos of the brush-like border, (ii) vacuolization of the epithelial cells (hydropic degeneration), (iii) interstitial edema, (iv) apoptosis, and (v) necrosis. All parameters were scored from 0 (no damage) to 5 (extensive changes), except necrosis parameter that was scored from 0 to 6 (see **Supplementary Table 2** for criteria and score details). When necrosis parameter was set to at least 4 (i.e., sublethal damage), all other parameters were automatically set to the maximal value (5). Analysis was made of the damage score for each of the parameters on one hand, and of the total sum of damage scores (TDS) of the five parameters on the other hand. Thus, the TDS had a minimum possible total damage score of 0 and a maximum possible total damage score of 26.

Detection of Specialized Metabolites

We tested the hypothesis that bumblebees can detect the specialized metabolites using preference tests following the protocol from Ma et al. (2016). For each treatment, 15 bumblebee

individuals were randomly collected from five different colonies (i.e., three bumblebees per colony) and starved for 2–4 h in plastic vials (70 mm long, 25 mm inner diameter) in the rearing dark room at 27°C and 76% relative humidity. After this starvation period, bumblebees were transferred into a holding tube where they were able to move freely. The holding tube consisted in a modified 15 mL centrifuge tube fixed on a polystyrene holder as described in Ma et al. (2016). After a habituation phase of 3 min, the trial started and was recorded with a digital Dino-lite USB microscope camera fixed 5 cm above the tip of the holding tube. The trial was recorded using the software Dinocapture 2.0, with a 26.7 frames.sec^{-1} and a 25× magnification rate. A drop of sugar syrup (BIOGLUC®, Biobest) was presented to the bumblebee using a 1-mL syringe. Individuals that did not consume the syrup within 5 min were discarded. For responsive individuals, test solutions were presented using a 100 μL micro-capillary tube connected to a pumping system to ensure the presence of a permanent droplet of test solution at the top of the micro-capillary tube (Ma et al., 2016). Test solutions were prepared by diluting the commercial powders directly in sugar syrup (50%, 100%, and 200% of the naturally occurring concentrations, **Supplementary Table 1**). The control solutions consisted of pure sugar syrup (negative control) and a 1 mM quinine solution (positive control) that was proven to have a deterrent effect (Ma et al., 2016). A total of 165 workers (i.e., 15 workers per treatment and 11 treatments namely, negative control, positive control, 50%-amygdalin, 100%-amygdalin, 200%-amygdalin, 50%-scopolamine, 100%-scopolamine, 200%-scopolamine, 50%-sinigrin, 100%-sinigrin, and 200%-sinigrin) have been tested.

The 2-min test phase started as soon as the bumblebee's proboscis contacted the test or control solution inside the micro-capillary tube. The lengths of liquid inside the micro-capillary tube were measured before and after the test phase to calculate the volume of solution consumed. The volume of solution consumed as well as the number of feeding bouts, the cumulative duration of the feeding bouts, the total duration of effective feeding (i.e., contact with test or control solution) and the duration of the first contact (i.e., before the first proboscis retraction) were used to evaluate the phagostimulatory or the deterrent activity of the compounds tested. A feeding bout was defined as a contact between the extended proboscis and the test solution for at least 5 s (French et al., 2015).

Data Analysis

All analyses were performed in R version 3.4.0 (R Core Team, 2017).

Micro-Colony Performance

To test for differences in resource collection, reproduction (offspring mass; drone mass; number of individuals within each developmental stage), and stress response of bumblebees among diet treatments, we fitted general linear mixed effects models with concentrations as a fixed effect, and colony as a random factor. As the bioassays were conducted at different times with a significant difference among controls, separate models were fitted for each specialized metabolite. Pollen collection, syrup collection, pollen dilution, total offspring mass, and pollen efficiency per micro-colony were analyzed using models with a Gaussian error structure (i.e., normally distributed residuals, "lme" function, R-package "nlme"; Kuznetsova et al., 2017). Larval ejection was analyzed using a binomial model with the number of ejected larvae and the total number of living offspring produced per micro-colony as a bivariate response ("glmer" function, R-package "lmerTest"; Kuznetsova et al., 2017), with an observation-level random effect added to the model to account for overdispersion (i.e., each data point received a unique level of random effect that modeled the extra-parametric variation present in the data; Harrison, 2014). Numbers of individuals within each developmental stage per micro-colony were assessed using models with Poisson distribution for count data after checking for overdispersion ("glmer" function, R-package "lmerTest"; Kuznetsova et al., 2017). An observation-level random effect was added to the Poisson models when data overdispersion occurred (Harrison, 2014). When a significant effect was found ($p < 0.05$), multiple pairwise comparison tests were performed using Tukey contrasts and FDR (false discovery rate) adjustment to determine how diet treatments significantly differed from each other ("glht" function, R-package "multcomp"; Hothorn et al., 2008). Besides, Pearson (data being normally distributed) and Spearman (data not being normally distributed) correlation tests were used to evaluate the statistical significance (p-values) and the strength (correlation coefficients) of the correlation between pollen collection and total offspring mass for each specialized metabolite.

Digestive Damage

The ordinal method used for histological evaluation (i.e., scoring system) involved a non-normal distribution of data. Non-parametric analyses (i.e., Kruskal–Wallis test) were then considered to compare the damage score for each of the parameters (**Supplementary Table 2**) and the total sum of damage scores (TDS) among diet treatments (Gibson-Corley et al., 2013). When p-value was significant ($p < 0.05$), multiple pairwise comparisons (*post hoc* test) were performed ("kruskal" function, R-package "agricolae"; Mendiburu, 2020). Given the reduced sample size for this part of the study, a power analysis has been used to ensure sufficient power and reliability (power = 0.99; "kwpower" function, R-package "MultNonParam"; Kolassa and Jankowski, 2021).

Detection of Specialized Metabolites

To test for differences in the phagostimulatory or the deterrent activity of the treatments, we fitted general linear mixed effects models (GLMM) with treatment as a fixed effect, and colony as a random factor. The volume of solution consumed, cumulative duration of the feeding bouts, total duration of effective feeding (i.e., contact with test or control solution) and duration of the first contact (i.e., before the first proboscis retraction) were analyzed using models with a Gaussian error structure after log transformation (i.e., log-normally distributed residuals, "lme" function, R-package "nlme"; Kuznetsova et al., 2017). The number of feeding bouts was assessed using models with Poisson distribution for count data after checking

for overdispersion ("glmer" function, R-package "lmerTest"; Kuznetsova et al., 2017). When a significant effect was found ($p < 0.05$), multiple pairwise comparison tests were performed using Tukey contrasts to determine how treatments significantly differed from each other ("glht" function, R-package "multcomp"; Hothorn et al., 2008).

RESULTS

Micro-Colony Performance

Resource Collection

We found no significant effect of diet treatment on the collection of pollen (amygdalin, $\chi^2 = 6.78$, df = 3, $p = 0.079$, **Figure 1A**; scopolamine, $\chi^2 = 5.69$, df = 3, $p = 0.128$, **Figure 1B**; sinigrin, $\chi^2 = 1.79$, df = 3, $p = 0.618$, **Figure 1C**) or syrup (amygdalin, $\chi^2 = 4.82$, df = 3, $p = 0.185$; scopolamine, $\chi^2 = 2.12$, df = 3, $p = 0.549$; sinigrin, $\chi^2 = 2.19$, df = 3, $p = 0.534$) (**Supplementary Table 3**). Whatever the added specialized metabolite and its concentration, micro-colonies fed the test diets did not differ from micro-colonies fed the control diet. Pearson and Spearman correlation coefficients highlighted that total pollen collection (**Figures 1A–C**) correlated with the total mass of hatched offspring (**Figures 1D–F**), regardless of the treatment (amygdalin, $r = 0.775$, $p < 0.001$; scopolamine, $\rho = 0.584$, $p < 0.001$; sinigrin, $r = 0.771$, $p < 0.001$).

Reproduction

We found no significant effect of diet treatment on the total mass of hatched offspring (i.e., all developmental stages except eggs) produced by *B. terrestris* micro-colonies (amygdalin, $\chi^2 = 5.92$, df = 3, $p = 0.116$, **Figure 1D**; scopolamine, $\chi^2 = 5.64$, df = 3, $p = 0.131$, **Figure 1E**; sinigrin, $\chi^2 = 1.50$, df = 3, $p = 0.683$, **Figure 1F** and **Supplementary Table 3**). All micro-colonies produced eggs, non-isolated larvae, isolated larvae (pre- and post-defecating stages), pupae and non-emerged drones. We found no significant effects of treatment on numbers of individuals within each developmental stage per micro-colony ($p > 0.05$) except for the number of post-defecating larvae in the scopolamine bioassays ($\chi^2 = 11.31$, df = 3, $p = 0.010$), with *post hoc* Tukey analyses showing that micro-colonies fed the scopolamine diets at 100% and 200% of the naturally occurring concentration produced less post-defecating larvae than micro-colonies fed control diet (**Supplementary Table 3**). We then assessed if the diet treatment affected the ability of a micro-colony rearing their offspring to adulthood but found no significant difference in the number of emerged drones (amygdalin, $\chi^2 = 4.63$, df = 3, $p = 0.201$; scopolamine, $\chi^2 = 6.07$, df = 3, $p = 0.108$; sinigrin, $\chi^2 = 6.72$, df = 3, $p = 0.081$) (**Supplementary Table 3**).

Stress Response

In response to a diet stress, adult bumblebees may display peculiar behavior such as pollen dilution (Vanderplanck et al., 2018) or larval ejection from the brood (Tasei and Aupinel, 2008a). We found no significant effect of diet treatment either on the proportion of ejected larvae in micro-colonies (amygdalin, $\chi^2 = 3.19$, df = 3, $p = 0.363$, **Figure 1G**; scopolamine, $\chi^2 = 0.91$, df = 3, $p = 0.822$, **Figure 1H**; sinigrin, $\chi^2 = 6.30$, df = 3, $p = 0.098$, **Figure 1I**) or on pollen dilution (amygdalin, $\chi^2 = 7.55$, df = 3, $p = 0.056$; scopolamine, $\chi^2 = 3.77$, df = 3, $p = 0.287$; sinigrin, $\chi^2 = 2.30$, df = 3, $p = 0.513$) (**Supplementary Table 3**). Another evaluated stress response was pollen diet efficiency that highlights when a micro-colony needs to consume more pollen to produce offspring, which could then be indicative of digestibility constraint or nutrient deficiency. We found no significant effect of treatment on pollen diet efficiency (amygdalin, $\chi^2 = 0.10$, df = 3, $p = 0.991$; scopolamine, $\chi^2 = 0.74$, df = 3, $p = 0.864$; sinigrin, $\chi^2 = 0.83$, df = 3, $p = 0.844$), with micro-colonies fed the test diets having similar pollen efficiency than micro-colonies in control treatments (**Supplementary Table 3**). Such absence of significant difference in pollen diet efficiency was not surprising since total pollen collection (**Figures 1A–C**) correlated with the total mass of hatched offspring (**Figures 1D–F**) in our experiment, regardless of the treatment.

Digestive Damage

As expected, the control treatment did not cause damage to the digestive tract (**Supplementary Table 4**). The mesenteric epithelium displayed a normal organization, and the morphology of digestive cells appeared to be normal without cytoplasmic vacuolization or pyknotic nucleus. The nuclei had a smooth and regular appearance, and microvilli at the apex of the digestive cells were well-developed, without any partial degradation. No necrotic cells were observed both in the base and at the apex of the intestinal crypts that remained well shaped. Median scores of any histological criteria did not exceed 2 and the median TDS was 7.5 (**Figure 2** and **Supplementary Table 4**).

In comparison, the 50%-amygdalin and 50%-scopolamine treatments had a significantly higher damage score ($\chi^2 = 24.02$, df = 9, $p = 0.004$), median TDS being 25 for 50%-amygdalin treatment and 24.5 for 50%-scopolamine treatment (**Figure 2** and **Supplementary Table 4**). Both treatments induced marked higher histopathological alterations in the digestive tract compared to the control, with a significant augmentation of features of apoptosis (criterion 4) ($\chi^2 = 26.20$, df = 9, $p = 0.002$; **Supplementary Table 4**) as well as of necrosis (criterion 5) for 50%-amygdalin ($\chi^2 = 24.04$, df = 9, $p = 0.004$; **Supplementary Table 4**). Pyknotic nuclei were more numerous and several necrotic cells detached from the epithelium, forming large clusters in the mesenteron lumen. Cytoplasmic vacuolization (criterion 2) was also significantly more marked for both treatments ($\chi^2 = 23.79$, df = 9, $p = 0.005$; **Supplementary Table 4**), with hydropic degeneration in more than 50% of villus intestinal epithelial cells. Disorganization or loss of the brush-like border (criterion 1) were also more frequently observed ($\chi^2 = 23.42$, df = 9, $p = 0.005$; **Supplementary Table 4**) as well as interstitial edema (criterion 3) in the connective tissue that forms the central axes of intestinal crypts ($\chi^2 = 17.90$, df = 9, $p = 0.036$; **Supplementary Table 4**).

Damage to the digestive tract of bees exposed to the 100%-amygdalin treatment were less severe but significantly higher than in the control treatment ($\chi^2 = 24.02$, df = 9, $p = 0.004$), TDS

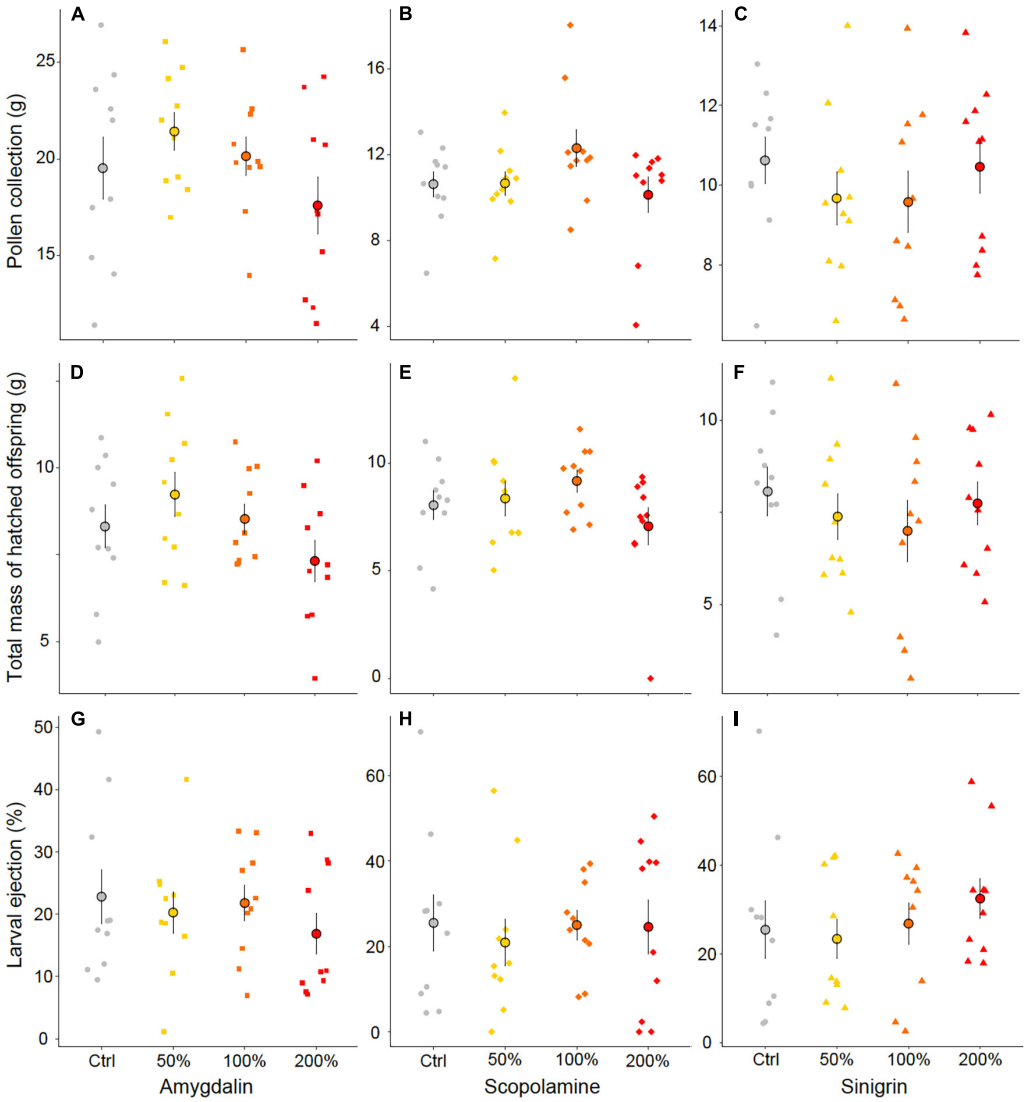

FIGURE 1 | Effects of diet treatments on resource collection, reproduction and stress response of *B. terrestris* in micro-colonies. **(A–C)** Pollen collection in each micro-colony across treatments. **(D–F)** Total mass of hatched offspring in each micro-colony across treatments. **(G–I)** Percentage of ejected larvae in each micro-colony across treatments. Each small data point represents a micro-colony and large points are mean values of each treatment. Error bars indicate the standard error of means. No significant effect was found on any parameter.

being 19.5 (**Figure 2** and **Supplementary Table 4**). The intestinal crypts were still well-organized without interstitial edema. However, occurrence of pyknotic nuclei was significantly higher than for control treatment although we did not observe hydropic degeneration despite cytoplasmic vacuolization (χ^2 = 23.79, df = 9, p = 0.005; **Supplementary Table 4**). Disorganization or loss of the brush-like border was observed in 25–50% of villus intestinal epithelial cells, which is significantly higher than in control treatment (χ^2 = 23.42, df = 9, p = 0.005; **Supplementary Table 4**).

No degeneration of epithelial cells was observed in the digestive tract of bees exposed to the other treatments, which displayed a TDS similar to the control (**Figure 2** and **Supplementary Table 4**). The mesenteron displayed a normal morphology as in the control treatment: the intestinal crypts were well-formed with a homogeneous brush-like border. Only some cells detached at the apex of the intestinal crypts in 100%-sinigrin and 200%-amygdalin treatments, which is probably due to normal cell renewal.

Detection of Specialized Metabolites

The treatments had a significant effect on the total volume of solution consumed (χ^2 = 30.42, df = 10, $p < 0.001$), whereby the consumption of quinine solution (positive control) was reduced in comparison to pure syrup solution (negative control) (**Figure 3A** and **Supplementary Table 5**). As expected, quinine displayed a deterrent activity in our assays based on food consumption. Bumblebees collected significantly more solution

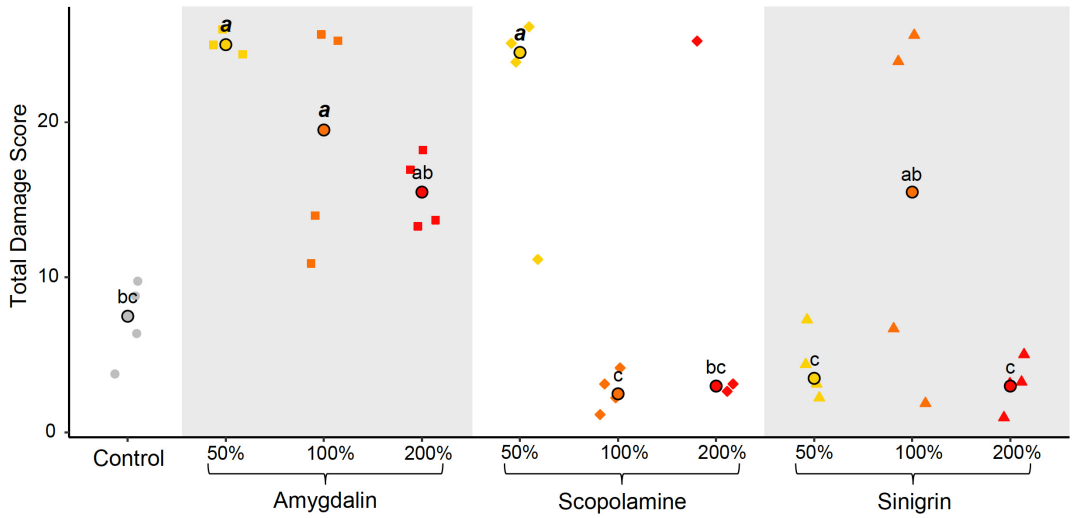

FIGURE 2 | Individual and median total damage scores. The dot-plot shows the total damage scores obtained in 38 individual tissue samples from the ten different diet treatments. Different letters indicate significant differences ($p < 0.05$) between diet treatments. Scores significantly higher than control are depicted in bold italic.

with scopolamine (50, 100, and 200%) and 200%-amygdalin than quinine solution, while they collected significantly less solution with 50%-sinigrin, 50%-amygdalin, and 200%-sinigrin than pure syrup solution (**Figure 3A** and **Supplementary Table 5**). These results suggest that solutions with scopolamine (50, 100, and 200%) and 200%-amygdalin displayed a phagostimulatory activity for bumblebees, whereas solutions with 50%-sinigrin, 50%-amygdalin, and 200%-sinigrin had a deterrent effect. The consumption of solution with 100%-sinigrin and 100%-amygdalin did not differ from any controls, suggesting neither phagostimulatory nor deterrent effects.

Similarly, the treatments had a significant effect on the duration of the first contact of proboscis with the solution ($\chi^2 = 57.64$, df = 10, $p < 0.001$). The duration of the first contact with quinine solution (positive control) was significantly reduced in comparison to pure syrup solution (negative control) (**Figure 3B** and **Supplementary Table 5**), suggesting that quinine triggered an active avoidance behavior in bumblebees with a faster proboscis retraction. While the duration of the first contact with solutions containing 200%-amygdalin, 100%-sinigrin, and scopolamine (50, 100, and 200%) was significantly longer in comparison to quinine solution (positive control) (i.e., phagostimulatory activity of test solutions), it was significantly reduced with the solutions containing 100%-amygdalin, 50%-amygdalin, and 50%-sinigrin in comparison to pure syrup solution (negative control) (i.e., deterrent activity of test solutions) (**Figure 3B** and **Supplementary Table 5**). Only the duration of the first contact with the 200%-sinigrin solution did not differ from any controls.

Likewise, the treatments had a significant effect on the cumulative duration of feeding bouts ($\chi^2 = 74.38$, df = 10, $p < 0.001$). As expected, the cumulative duration of feeding bouts with quinine solution (positive control) was significantly reduced in comparison to pure syrup solution (negative control) (**Supplementary Table 5**). *Post hoc* Tukey test showed that the cumulative duration of feeding bouts with all test solutions (i.e., amygdalin, scopolamine and sinigrin) was significantly longer than with the quinine solution and similar to the pure sugar syrup solution (i.e., no deterrent effect based on this parameter, **Supplementary Table 5**). The treatments had also a significant effect on the total duration of effective feeding ($\chi^2 = 86.77$, df = 10, $p < 0.001$), whereby the total duration of effective feeding with quinine solution (positive control) was significantly reduced in comparison to pure syrup solution (negative control). For all test solutions (i.e., amygdalin, scopolamine and sinigrin), the total duration of effective feeding was significantly longer than with the quinine solution and similar to the pure sugar syrup solution (i.e., no deterrent effect based on this parameter, **Supplementary Table 5**). Only bumblebees in contact with the solution containing 50%-sinigrin spent significantly less time for effective feeding than the negative control but significantly more than the positive one (**Supplementary Table 5**).

The treatments had also a significant effect on the frequency of feeding bouts ($\chi^2 = 91.63$, df = 10, $p < 0.001$), whereby the number of bouts with test solutions containing scopolamine was significantly higher in comparison to other treatments. However, the number of bouts to feed the negative control did not significantly differ from the positive one, suggesting that this parameter was not the most suitable to assess the deterrent effects of substances in such assay (**Supplementary Table 5**).

DISCUSSION

Because floral visitors of a given plant species vary in their contribution to plant pollination (Thomson and Thomson, 1992), plants have evolved several mechanisms through which they filter their pollinators (Westerkamp and Claben-Bockhoff, 2007). Such "filtering" mechanisms allow for interactions with the most efficient

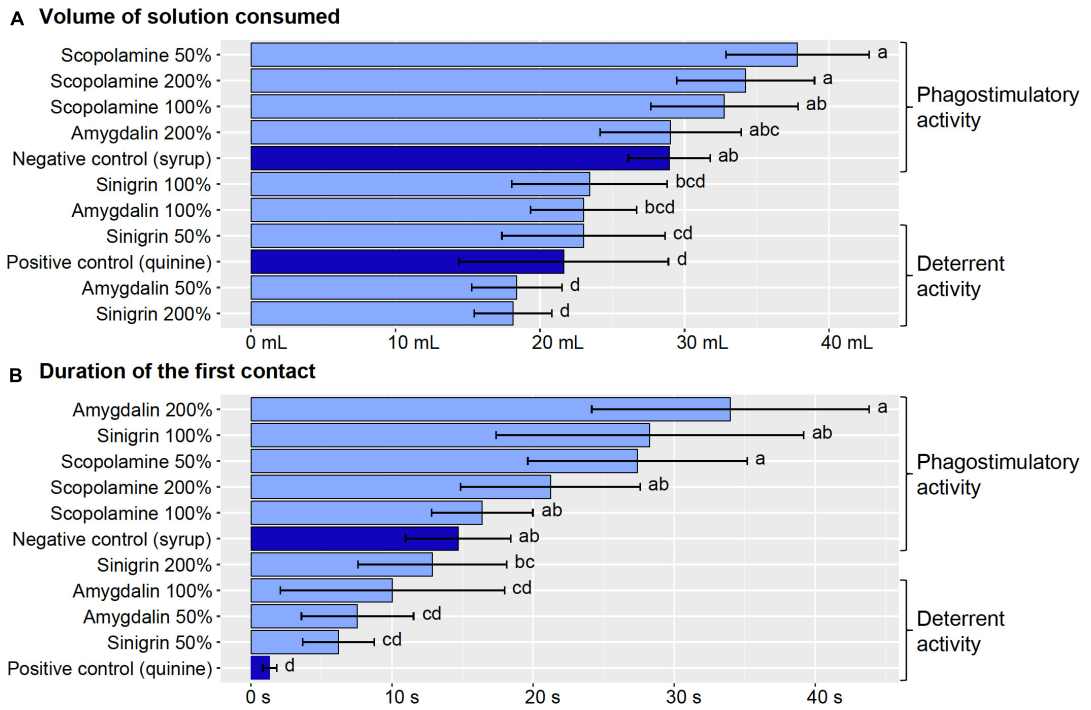

FIGURE 3 | Phagostimulatory or deterrent activity of the specialized metabolites. **(A)** Volume of solution consumed, and **(B)** duration of the first contact with the solution during preference experiments. Different letters indicate significant differences ($p < 0.05$) between treatments. Controls are depicted in dark blue, all test solutions are depicted in light blue.

pollinators to be maximized, while minimizing pollen loss due to excessive harvesting by pollen-feeding visitors (Müller, 1996). In the absence of specialized flower morphology, such "filtering" may occur through chemical properties of pollen, for instance occurrence of specialized metabolites that render the resource unsuitable to some bee species (i.e., fitness loss caused by pollen consumption) (Praz et al., 2008b; Trunz et al., 2020; Vanderplanck et al., 2020). In order to be an effective defense mechanism, these specialized metabolites have compulsorily to generate an avoidance behavior in bees (i.e., floral visits without pollen consumption), which then ensures the availability of pollen for plant reproduction. Such avoidance behavior by bees may have different mechanistic origins that are either pre-ingestive, post-ingestive, or both. Although both mechanisms involve the sensory system of pollinators, the former results from signal detection (i.e., olfactory and chemotactile cues) without consuming the resource, while the latter results from an association between such signal and the malaise induced by resource consumption, and hence ingestion of specialized metabolites (Wright et al., 2010). Pre-ingestive detection is therefore less costly for both bees and plants, as it does not impair their reproductive success or their physiological state. Such deterrent and toxic effects of specialized metabolites from floral resources have largely been assessed in honeybees, with a bias toward nectar specialized metabolites (Detzel and Wink, 1993; Wink, 1993). Besides, studies on pollen specialized metabolites and on other bee species, including the common buff-tailed bumblebee species *Bombus terrestris,* remain scarce, which does not allow for a complete picture of the ecological role of specialized metabolites. One main aspect that needs to be solved is the ability of generalist pollinators to detect pollen specialized metabolites and avoid them in case of deleterious effects. Here we have assessed the ability of workers of *B. terrestris* to avoid specialized metabolites naturally occurring in pollen by distinguishing (i) post-ingestive effects at both micro-colony (i.e., resource collection, reproduction success and stress response) and individual (i.e., digestive damage) levels, as well as (ii) pre-ingestive effects at both micro-colony (i.e., resource collection) and individual levels (i.e., detection of specialized metabolites through preference tests). As this study is one of the first to address these aspects in bumblebees, we critically discuss its limitations and suggest some parameters that would be of great interest when assessing the effects of pollen specialized metabolites (i.e., comprehensive evaluation). Caution has also to be paid regarding the significance of our results as some of the tested concentrations (i.e., 50%- and 200% treatments) may fall outside the natural concentration ranges encountered in plant pollen.

Considering the post-ingestive effects assessed at the micro-colony level, neither resource collection, reproduction nor stress response of the micro-colonies were affected by any of the tested specialized metabolites, whatever their concentration. However, caution has to be paid regarding the interpretation of post-ingestive effect based on the total resource collection, as any potential adjustment of resource collection (e.g., regulation of pollen intake following brood feedback or worker malaise)

during the experiment could have been obscured by behavioral changes. For example, micro-colonies that may have had high pollen consumption at the beginning of the experiment but which may have decreased consumption following post-ingestive regulation would display the same total pollen collection as micro-colonies that may have had low pollen consumption at the beginning of the experiment but could have increased it during colony development. A more specific analysis of pollen collection over time may have highlighted changes in resource collection during the experiment, which would have been able to better capture post-ingestive effects than the total pollen collection metric (Brochu et al., 2020; Vanderplanck et al., 2020). Moreover, total pollen collection alone does not allow for clearly distinguishing post-ingestive regulation of pollen intake from pre-ingestive regulation. Again, an analysis of pollen collection over time would allow for untangling these effects. Regarding syrup collection, no difference was detected between the supplementation and control treatments, indicating that workers did not increase their syrup intake when fed pollen supplemented with specialized metabolites. Hence occurrence of the tested specialized metabolites did not appear to affect worker resource collection (i.e., both pollen and syrup). In the same way, both reproduction and stress response of micro-colonies were not impacted during the supplementation assays. Specifically, brood development and individual mass within each developmental stage were similar between micro-colonies fed with the control and the pollen supplemented with specialized metabolites. Likewise, no mitigation of unsuitable chemical properties was achieved through pollen mixing behavior during our experiment (i.e., "toxin" dilution by excessive syrup addition; Vanderplanck et al., 2018), and larval ejection was similar among micro-colonies, regardless of the treatment.

While no post-ingestive effects were detected at the micro-colony level, some post-ingestive deleterious effects were clearly observed at the individual level through histological evaluation. Whereas digestive damage (i.e., quantified by the TDS) was significantly higher for workers exposed to the 50%- amygdalin, 100%- amygdalin and 50%-scopolamine treatments compared to those from the control treatment, no sinigrin treatments induced digestive damage. Astonishingly, the smallest concentrations triggered the most severe damage since no damage was observed in the 200% treatments, regardless of the specialized metabolite tested. Such absence of digestive damage at very high concentrations might be due to an activation of the complex machinery of endogenous immune defenses, which could occur only above a certain threshold. Indeed, as such endogenous defenses are energetically costly (Moret and Schmid-Hempel, 2000), they are likely to be activated only when the fitness loss caused by specialized metabolites is higher than the cost associated with the use of such endogenous defenses (i.e., a trade-off between the cost of endogenous defenses and the fitness loss caused by stressors; Janashia and Alaux, 2016). Besides digestive damage, evaluation of post-ingestive deleterious effects at the individual level might also be assessed through other parameters. Indeed, specialized metabolites could also affect the health status of bees, either directly by impacting their immune system (insecticidal properties) or indirectly by affecting their gut microbiota (anti-microbial properties). As in numerous organisms, bacterial symbiont communities have a substantial impact on bee physiology and ecology (Feldhaar, 2011; Engel and Moran, 2013; Bonilla-Rosso and Engel, 2018). For instance, they are involved in detoxification, protection against pathogens, digestion of food components, and activation of host immunity. Although the microbiota could provide bee host with resistance to cope with toxic metabolites (e.g., degradation; Kešnerová et al., 2017), its functional roles could be disrupted (i.e., dysbiosis) by the ingestion of naturally occurring metabolites displaying anti-microbial activities, as already shown for pesticides (Paris et al., 2020). Indeed, in the same way that pollen nutrients can modulate the bacterial composition in the bumblebee gut (Billiet et al., 2015), specialized metabolites in pollen could induce significant changes in the gut microbiota that indirectly affect bee health. Besides, direct effects of specialized metabolites on bee individual immunity might also be assessed by measuring parameters related to immunocompetence (defined as the capacity to mount an immune response), such as hemocyte concentration, fat body content, and gut melanization (Alaux et al., 2010; Roger et al., 2017; Brochu et al., 2020). Specifically, hemocytes are involved in the encapsulation of parasite followed by the melanization process (cellular immunocompetence), while the fat body is the main tissue involved in the synthesis of immunoproteins (humoral immunocompetence) (Alaux et al., 2010). Such assessment of the baseline immunocompetence could also allow for testing the hypothesis of the activation threshold of the endogenous immune defenses. The impacts of pollen specialized metabolites on the bee gut microbiota and immunity should therefore also be taken into consideration when investigating their post-ingestive effects at individual level.

Considering the pre-ingestive effects assessed at the micro-colony level, total resource collection did not differ between the treatments and the control, indicating that workers did not avoid to collect pollen supplemented with specialized metabolites based on olfactory and chemotactile cues. However, preference tests showed that workers seemed able to detect these specialized metabolites through pre-ingestive mechanisms. Caution has nevertheless to be paid when comparing these two experiments since specialized metabolites were mixed within willow pollen for assessing pre-ingestive effects at the micro-colony level (i.e., bioassays), whereas they were mixed within sugar syrup for assessing pre-ingestive effects at the individual level (i.e., preference tests). This might account for the contrasting results obtained, as bees may perceive compounds in nectar but not in pollen, likely due to the higher chemical complexity of pollen (Ruedenauer et al., 2015, 2020a). Further bioassays with addition of specialized metabolites within nectar would allow for highlighting any significant effect on nectar intake at the micro-colony level, annihilating the matrix effect of pollen. Regarding the preference tests, the effects depended on the tested specialized metabolite and its concentration (i.e., pre-ingestive effects at individual level). While scopolamine at all tested concentrations, amygdalin at 200%, and sinigrin at 100% appeared to elicit phagostimulatory responses; amygdalin at 50 and 100%, as well as sinigrin at 50 and 200% rather appeared to induce deterrent effects based on the volume of solution consumed or the duration

of the first contact with the solution. The fact that scopolamine at 50% can be phagostimulatory despite its induced digestive damage is not so aberrant. For instance, it has been shown that glucosinolates may be feeding cues for the fly *Scaptomyza nigrita* despite their deterrent and defensive properties (Humphrey et al., 2016). The phagostimulatory activity of scopolamine remains, however, quite unexpected regarding the results of a previous study that found a deterrent effect of this specialized metabolite toward honeybees (Detzel and Wink, 1993). Such discrepancy could be explained by the different concentrations used in both studies on one hand (minimum 10,007 ppm herein; maximum 300 ppm in Detzel and Wink, 1993), and by the different bee models used on the other hand (*Bombus terrestris* herein; *Apis mellifera* in Detzel and Wink, 1993). The possibility for different responses to the same metabolite between both bee models is supported by the finding that a same sterol, beta-sitosterol, is known to have antifeedant effects on *A. mellifera* whereas it is freely consumed by *B. terrestris* (Rasmont et al., 2005). Besides, the hypothesis of concentration effect is directly supported by our results since amygdalin had a phagostimulatory effect at 200%, and a deterrent effect at both 50 and 100%. In the same way, a concentration effect was detected for sinigrin since workers displayed a deterrent response for the 50% and 200% solutions, and a phagostimulatory response for the 100% solution. This suggests that while a given specialized metabolite may have a deterrent effect at some concentrations, it can turn out to have a phagostimulatory activity once a certain concentration threshold or range is reached. Likewise, Burden et al. (2019) found compound- and concentration-dependent responses, at both pre- and post-ingestive levels, in honeybees exposed to distinct heavy metals. Actually, if we confronted results from several studies, it clearly appears that concentration effects may occur, which renders impossible any extrapolation of previous studies led in different experimental conditions. For instance, Tiedeken et al. (2014) demonstrated that *B. terrestris* suffered from amygdalin deterrent effect when its concentration was at least 450 ppm. Similarly, London-Shafir et al. (2003) showed that *A. mellifera* reduced its consumption when amygdalin concentration was at 500 ppm whereas the honeybees were not sensitive to amygdalin concentration of 50 ppm (Singaravelan et al., 2005). Such findings that not only the nature of the specialized metabolite but also its concentration may influence bee foraging decisions (and hence fitness), and that these effects are likely to depend on the bee species, are particularly relevant in the current context of bee decline. Indeed, among the main drivers of bee decline, pollinators must cope with land-use changes that lead to crop homogenization and monotonous diet (Ricketts et al., 2008; Winfree et al., 2009; Goulson et al., 2015). Bees are then probably exposed to either very small quantities of specialized metabolites (if a particular resource is scarce) or substantial quantities (if a resource is abundant). In both cases, the reduction in the diversity of available floral resources is likely to prevent bees from displaying pollen mixing behavior to balance their diet and regulate their intake of specialized metabolites, regardless of their ability to detect them (see Eckhardt et al., 2014).

It is important to underline that although phagostimulatory and deterrent effects have been highlighted during the preference tests, no difference in total resource collection has been observed during the bioassays. Such contradictory results between the two experiments may arise from the co-occurrence of pre- and post-ingestive mechanisms during the bioassays, whereas only pre-ingestive mechanisms can occur during the preference tests. Indeed, post-ingestive mechanisms are enabled (1) by the possibility for brood feedback in the bioassays while preference tests only included one individual without any brood (Ruedenauer et al., 2016), and (2) by the possibility for conditional taste aversion in the bioassays (35 days) while preference tests comprise short feedings trials (2 min) that are too short for such learning to occur (Reilly and Schachtman, 2009; Wright et al., 2010). Therefore, an initial phagostimulatory response to a given specialized metabolite might be hidden during the long-time bioassays after negative brood feedback or associative learning in workers (Ruedenauer et al., 2016, 2020a), which was not necessarily reflected by the micro-colony performance nor any of the measured parameters. In the same way, although an initial avoidance response to a given specialized metabolite may have occurred, workers might have increased their resource intake over a long-time period after brood feedback (nutritional requirements) or associative learning in workers (no physiological damage associated with the deterrence). Moreover, specialized metabolites were presented differently to the bumblebees according to the experiments, as above-mentioned: they were mixed within willow pollen during the bioassays whereas they were mixed within sugar syrup during the preference tests. While the co-occurrence with pollen nutrients may have allowed for their intake for nutritional purpose, when they were dissolved in sugar syrup this may have facilitated their perception by the gustatory sensilla on the mouthparts of bumblebees (de Brito Sanchez, 2011). Besides, some specialized metabolites are considered to enhance feeding only in the presence of other phagostimulants such as sucrose, which may account for the differences observed between our experiments (Nayar and Thorsteinson, 1963; Shields and Mitchell, 1995; Mazumder et al., 2016). The presence of a given specialized metabolite at a given concentration in nectar or in pollen may then influence the detection ability of bees, as well as their foraging decision. This hypothesis is strengthened by the ecological "*raison d'être*" of specialized metabolites from the plant perspective: while a phagostimulatory activity of nectar through the occurrence of specialized metabolites might attract more specialized pollinators and subsequently enhance the plant fitness, a phagostimulatory activity of pollen would have a reverse effect by compromising the plant's reproductive success (e.g., Gosselin et al., 2013; Trunz et al., 2020). This highlights the importance for investigating not only the nectar specialized metabolites but also the pollen ones using appropriate and biologically relevant experimental designs. For instance, some of the metabolites, such as sinigrin and amygdalin tested herein, need specialized enzymatic activation by plant cytoplasmic enzymes, which come into contact with their substrate upon cell disruption. It should then be verified (i) whether the enzymes are effectively present in the pollen of origin, and (ii) whether the bees have the enzymatic capacity to liberate the active cyanide and allyl isothiocyanate, and if so, at which level of activity.

While we largely discussed the advantage of detecting specialized metabolites to avoid them, another evolutionary framework might also prevail from the pollinators' perspective: an active intake of specialized metabolites for medicinal purposes. Indeed, besides neutral or negative impact on bees, pollen specialized metabolites could also improve their health status through antioxidant and anti-microbial properties (e.g., Aličić et al., 2014; Palmer-Young et al., 2017). Such active intake of dietary chemicals suitable to improve health status corresponds to the concept of "self-medication" (Beaulieu and Schaefer, 2013), which occurs in many taxa (Povey et al., 2008; Forbey et al., 2009; Singer et al., 2009; Hart, 2011; Parker et al., 2011). While nutritional resilience to some environmental stressors has been already demonstrated in bees (e.g., heat waves, Vanderplanck et al., 2019a; parasites, Richardson et al., 2015; Vanderplanck et al., 2019b), there is only limited evidence for their ability to recognize and use specialized metabolites as medicinal resources when exposed to environmental stressors. There is then an urgent need to repeat similar experiments assessing the detection and effects of specialized metabolites in bees exposed to environmental stressors such as pesticides, diseases and parasites. Such experimental ecology could allow for developing operational research and proposing nature-based solutions in the current context of global bee declines.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Data Dryad, doi: 10.5061/dryad.2jm63xspz.

AUTHOR CONTRIBUTIONS

MV conceptualized the project and led the experiments. OS, MG, and MV conducted the biological assays. OS, DN, PG, and PD collected, analyzed, and interpreted histological and chromatographic data. MV, AG, and MG conducted the statistical analyses data and wrote the manuscript. All authors reviewed and edited the manuscript.

FUNDING

Contributions from MV were supported by the ARC project "METAFLORE." MG was supported by the Swedish Research Council grant number 2018-06238. Open access publication costs were supported by the UMONS Research Institute for Biosciences.

ACKNOWLEDGMENTS

We would like to thank M. Bourguignon, E. Couture, M. Dehon, D. Evrard, H. Gilles, J. Kieffer, M. Poivre, N. Roger, and P.-L. Zerck for their assistance, as well as T. Wood (native speaker) for English proofreading. We gratefully acknowledge financial support from the UMONS Research Institute for Biosciences for open access publication charges.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.669352/full#supplementary-material

REFERENCES

Adler, L. S. (2000). The ecological significance of toxic nectar. *Oikos* 91, 409–420. doi: 10.1034/j.1600-0706.2000.910301.x

Alaux, C., Ducloz, F., Crauser, D., and Le Conte, Y. (2010). Diet effects on honeybee immunocompetence. *Biol. Let.* 6, 562–565. doi: 10.1098/rsbl.2009.0986

Aličić, D., Šubarić, D., Jašić, M., Pašalić, H., and Ačkar, Đ (2014). Antioxidant properties of pollen. *Hrana u Zdravlju i Bolesti Znanstveno-struèni Èasopis za Nutricionizam i Dijetetiku* 3, 6–12.

Ares, A. M., Nozal, M. J., and Bernal, J. (2015). Development and validation of a liquid chromatography-tandem mass spectrometry method to determine intact glucosinolates in bee pollen. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 1000, 49–56. doi: 10.1016/j.jchromb.2015.07.017

Arnold, S. E. J., Idrovo, M. E. P., Arias, L. J. L., Belmain, S. R., and Stevenson, P. C. (2014). Herbivore defence compounds occur in pollen and reduce bumblebee colony fitness. *J. Chem. Ecol.* 40, 878–881. doi: 10.1007/s10886-014-0467-4

Ayestaran, A., Giurfa, M., and de Brito Sanchez, M. G. (2010). Toxic but drank: gustatory aversive compounds induce post-ingestional malaise in harnessed honeybees. *PLoS One* 5:e15000. doi: 10.1371/journal.pone.0015000

Baracchi, D., Brown, M. F. J., and Chittka, L. (2015). Behavioural evidence for self-medication in bumblebees? *F1000 Res.* 4:73. doi: 10.12688/f1000research.6262.2

Beaulieu, M., and Schaefer, H. M. (2013). Rethinking the role of dietary antioxidants through the lens of self-medication. *Anim. Behav.* 86, 17–24. doi: 10.1016/j.anbehav.2013.05.022

Billiet, A., Meeus, I., Van Nieuwerburgh, F., Deforce, D., Wäckers, F., and Smagghe, G. (2015). Impact of sugar syrup and pollen diet on the bacterial diversity in the gut of indoor-reared bumblebees (*Bombus terrestris*). *Apidologie* 47, 548–560. doi: 10.1007/s13592-015-0399-1

Bonilla-Rosso, G., and Engel, P. (2018). Functional roles and metabolic niches in the honey bee gut microbiota. *Curr. Opin. Microbiol.* 43, 69–76. doi: 10.1016/j.mib.2017.12.009

Boulogne, I., Petit, F., Ozier-Lafontaine, H., Desfontaines, L., and Loranger-Merciris, G. (2012). Insecticidal and antifungal chemicals produced by plants: a review. *Environ. Chem. Lett.* 10, 325–347. doi: 10.1007/s10311-012-0359-1

Brochu, K. K., van Dyke, M. T., Milano, N. J., Petersen, J. D., McArt, S. H., Nault, B. A., et al. (2020). Pollen defenses negatively impact foraging and fitness in a generalist bee (*Bombus impatiens:* Apidae). *Sci. Rep.* 10:3112.

Brodschneider, R., and Crailsheim, K. (2010). Nutrition and health in honey bees. *Apidologie* 41, 278–294. doi: 10.1051/apido/2010012

Brown, C., and Keith, S. (1987). Chemistry at the Solanaceae/Ithomiinae Interface. *Ann. Mo. Bot. Gard.* 74, 359–397. doi: 10.2307/2399406

Burden, C. M., Morgan, M. O., Hladun, K. R., Amdam, G. V., Trumble, J. J., and Smith, B. H. (2019). Acute sublethal exposure to toxic heavy metals alters honey bee (*Apis mellifera*) feeding behavior. *Sci. Rep.* 9:4253.

Calatayud, P. A., and Rabhé, Y. (2013). "Chapitre 6. Le tube digestif des insects," in *Interactions Insectes-Plantes*, eds N. Sauvion, C. Paul-André, D. Thiéry, and F. Marion-Pol (Marseille: IRD).

Cane, J. H. (2016). Adult pollen diet essential for egg maturation by a solitary Osmia bee. *J. Insect Physiol.* 95, 105–109. doi: 10.1016/j.jinsphys.2016.09.011

Cane, J. H., Dobson, H. E. M., and Boyer, B. (2016). Timing and size of daily pollen meals eaten by adult females of a solitary bee (*Nomia melanderi*) (Apiformes: Halictidae). *Apidologie* 48, 17–30. doi: 10.1007/s13592-016-0444-8

Cane, J. H., and Sipes, S. (2006). "Characterizing floral specialization by bees: analytical methods and a revised lexicon for oligolecty," in *Plant-Pollinator Interactions: from Specialization to Generalization*, eds N. M. Waser and J. Ollerton (Chicago, IL: The University of Chicago Press), 99–112.

Chowański, S., Adamski, Z., Marciniak, P., Rosiński, G., Büyükgüzel, E., Büyükgüzel, K., et al. (2016). A Review of bioinsecticidal activity of Solanaceae alkaloids. *Toxins* 8:60. doi: 10.3390/toxins8030060

Compean, K. L., and Ynalvez, R. A. (2014). Antimicrobial activity of plant secondary metabolites: a review. *Res. J. Med. Plant* 8, 204–213. doi: 10.3923/rjmp.2014.204.213

Conn, E. E. (1978). "Cyanogenesis, the production of hydrogen cyanide, by plants," in *Effects of Poisonous Plants on Livestock*, eds R. F. Keeler, K. R. Van kampen, and L. F. James (New York, NY: Academic Press), 301–310. doi: 10.1016/b978-0-12-403250-7.50035-2

Cook, D., Manson, J. S., Gardner, D. R., Welch, K. D., and Irwin, R. E. (2013). Norditerpene alkaloid concentrations in tissues and floral rewards of larkspurs and impacts on pollinators. *Biochem. Syst. Ecol.* 48, 123–131. doi: 10.1016/j.bse.2012.11.015

Dag, A., Zipori, I., and Pleser, Y. (2006). Using bumblebees to improve almond pollination by the honeybee. *J. Apic. Res.* 45, 215–216. doi: 10.3896/ibra.1.45.4.08

Day, S., Beyer, R., Mercer, A., and Ogden, S. (1990). The nutrient composition of honeybee collected pollen in Otago. *N. Z. J. Apic. Res.* 29, 138–146. doi: 10.1080/00218839.1990.11101210

de Brito Sanchez, M. G. (2011). Taste perception in honey bees. *Chem. Senses* 36, 675–692. doi: 10.1093/chemse/bjr040

de Carvalho, A. C. P., and Message, D. (2004). A scientific note on the toxic pollen of *Stryphnodendron polyphyllum* (Fabaceae, Mimosoideae) which causes sacbrood-like symptoms. *Apidologie* 35, 89–90. doi: 10.1051/apido:2003059

de Roode, J. C., Lefèvre, T., and Hunter, M. D. (2013). Ecology. Self-medication in animals. *Science* 340, 150–151. doi: 10.1126/science.1235824

DeGrandi-Hoffman, G., Chen, Y., Huang, E., and Huang, M. H. (2010). The effect of diet on protein concentration, hypopharyngeal gland development and virus load in worker honey bees (*Apis mellifera* L.). *J. Insect Physiol.* 56, 1184–1191. doi: 10.1016/j.jinsphys.2010.03.017

Detzel, A., and Wink, M. (1993). Attraction, deterrence or intoxication of bees (*Apis mellifera*) by plant allelochemicals. *Chemoecology* 4, 8–18. doi: 10.1007/bf01245891

Di Pasquale, G., Salignon, M., Le Conte, Y., Belzunces, L. P., Decourtye, A., Kretzschmar, A., et al. (2013). Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? *PLoS One* 8:e72016. doi: 10.1371/journal.pone.0072016

Dicks, L., Breeze, T., Ngo, H., Senapathi, D., An, J., Aizen, M., et al. (2020). A global assessment of drivers and risks associated with pollinator decline. *Res. Sq.* doi: 10.21203/rs.3.rs-90439/v1

Dobson, H. E. M., and Bergström, G. (2000). The ecology and evolution of pollen odors. *Plant Syst. Evol.* 222, 63–87. doi: 10.1007/978-3-7091-6306-1_4

Dobson, H. E. M., and Peng, Y. S. (1997). Digestion of pollen components by larvae of the flower- specialist bee *Chelostoma florisomne* (Hymenoptera: Megachilidae). *J. Insect Physiol.* 43, 89–100. doi: 10.1016/s0022-1910(96)00024-8

Dolezal, A. G., Carrillo-Tripp, J., Judd, T. M., Miller, W. A., Bonning, B. C., and Toth, A. L. (2019). Interacting stressors matter: diet quality and virus infection in honeybee health. *R. Soc. Open Sci.* 6:181803. doi: 10.1098/rsos.181803

Eckhardt, M., Haider, M., Dorn, S., and Müller, A. (2014). Pollen mixing in pollen generalist solitary bees: a possible strategy to complement or mitigate unfavourable pollen properties? *J. Anim. Ecol.* 83, 588–597. doi: 10.1111/1365-2656.12168

Engel, P., and Moran, N. A. (2013). The gut microbiota of insects–diversity in structure and function. *FEMS Microbiol. Rev.* 37, 699–735. doi: 10.1111/1574-6976.12025

Erickson, M., and Feeny, P. (1974). Sinigrin: a chemical barrier to the black swallowtail butterfly, *Papilio polyxenes*. *Ecology* 55, 103–111. doi: 10.2307/1934622

Feldhaar, H. (2011). Bacterial symbionts as mediators of ecologically important traits of insects hosts. *Ecol. Entomol.* 36, 533–543. doi: 10.1111/j.1365-2311.2011.01318.x

Forbey, J. S., Harvey, A. L., Huffman, M. A., Provenza, F. D., Sullivan, R., and Tasdemir, D. (2009). Exploitation of secondary metabolites by animals: a response to homeostatic challenge. *Integr. Comp. Biol.* 49, 314–328. doi: 10.1093/icb/icp046

Fraenkel, G. S. (1959). The raison d'être of secondary plant substances. *Science* 129, 1466–1470. doi: 10.1126/science.129.3361.1466

Free, J. B. (1993). *Insect Pollination of Crops*, 2nd Edn. London: Academic Press, 768.

French, A. S., Sellier, M. J., Ali Agha, M., Guigue, A., Chabaud, M. A., Reeb, P. D., et al. (2015). Dual mechanism for bitter avoidance in *Drosophila*. *J. Neurosci.* 35, 3990–4004. doi: 10.1523/jneurosci.1312-14.2015

Frisch, T., Motawia, M. S., Olsen, C. E., Agerbirk, N., Møller, B. L., and Bjarnholt, N. (2015). Diversified glucosinolate metabolism: biosynthesis of hydrogen cyanide and of the hydroxynitrile glucoside alliarinoside in relation to sinigrin metabolism in *Alliaria petiolata*. *Front. Plant Sci.* 6:926. doi: 10.3389/fpls.2015.00926

Gauthier, M., Cano-Lozano, V., Zaoujal, A., and Richard, D. (1994). Effects of intracranial injections of scopolamine on olfactory conditioning retrieval in the honeybee. *Behav. Brain Res.* 63, 145–149. doi: 10.1016/0166-4328(94)90085-x

Génissel, A., Aupinel, P., Bressac, C., Tasei, J. N., and Chevrier, C. (2002). Influence of pollen origin on performance of *Bombus terrestris* microcolonies. *Entomol. Exp. Appl.* 104, 329–336. doi: 10.1046/j.1570-7458.2002.01019.x

Gibson-Corley, K. N., Olivier, A. K., and Meyerholz, D. K. (2013). Principles for valid histopathologic scoring in research. *Vet. Pathol.* 50, 1007–1015. doi: 10.1177/0300985813485099

Gosselin, M., Michez, D., Vanderplanck, M., Roelants, D., Glauser, G., and Rasmont, P. (2013). Does *Aconitum septentrionale* chemically protect floral rewards to the advantage of specialist bumblebees? *Ecol. Entomol.* 38, 400–407. doi: 10.1111/een.12032

Goulson, D., Nicholls, E., Botias, C., and Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* 347:1255957. doi: 10.1126/science.1255957

Haider, M., Dorn, S., and Müler, A. (2013). Intra- and interpopulational variation in the ability of a solitary bee species to develop on non-host-pollen: implications for host range expansion. *Funct. Ecol.* 27, 255–263. doi: 10.1111/1365-2435.12021

Hanley, M. E., Franco, M., Pichon, S., Darvill, B., and Goulson, D. (2008). Breeding system, pollinator choice and variation in pollen quality in British herbaceous plants. *Funct. Ecol.* 22, 592–598. doi: 10.1111/j.1365-2435.2008.01415.x

Harrison, X. A. (2014). Using observation-level random effects to model overdispersion in count data in ecology and evolution. *PeerJ* 2:e616. doi: 10.7717/peerj.616

Hart, B. L. (2011). Behavioural defences in animals against pathogens and parasites: parallels with the pillars of medicine in humans. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 366, 3406–3417. doi: 10.1098/rstb.2011.0092

Herbert, E. W. (1992). "Honey bee nutrition," in *The Hive and the Honeybee*, ed. J. M. Graham (Hamilton, IL: Dadant), 197–233.

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biom. J.* 50, 346–363. doi: 10.1002/bimj.200810425

Human, H., Nicolson, S. W., Strauss, K., Pirk, C. W. W., and Dietemann, V. (2007). Influence of pollen quality on ovarian development in honeybee workers (*Apis mellifera scutellata*). *J. Insect Physiol.* 53, 649–655. doi: 10.1016/j.jinsphys.2007.04.002

Humphrey, P. T., Gloss, A. D., Alexandre, N. M., Villalobos, M. M., Fremgen, M. R., Groen, S. C., et al. (2016). Aversion and attraction to harmful plant secondary compounds jointly shape the foraging ecology of a specialist herbivore. *Ecol. Evol.* 6, 3256–3268. doi: 10.1002/ece3.2082

Hurst, V., Stevenson, P. C., and Wright, G. A. (2014). Toxins induce 'malaise' behaviour in the honeybee (*Apis mellifera*). *J. Comp. Physiol. A.* 200, 881–890. doi: 10.1007/s00359-014-0932-0

Hügel, M. F. (1962). Etude de quelques constituants du pollen. *Ann. l'Abeille* 5, 97–133. doi: 10.1051/apido:19620201

Ismail, N., Christine, S., Robinson, G. E., and Fahrbach, S. E. (2008). Pilocarpine improves recognition of nestmates in young honey bees. *Neurosci. Lett.* 439, 178–181. doi: 10.1016/j.neulet.2008.05.014

Janashia, I., and Alaux, C. (2016). Specific immune stimulation by endogenous bacteria in honey bees (Hymenoptera: Apidae). *J. Econ. Entomol.* 109, 1474–1477. doi: 10.1093/jee/tow065

Kempf, M., Heil, S., Haßlauer, I., Schmidt, L., von der Ohe, K., Theuring, C., et al. (2010). Pyrrolizidine alkaloids in pollen and pollen products. *Mol. Nutr. Food Res.* 54, 292–300. doi: 10.1002/mnfr.200900289

Kešnerová, L., Mars, R. A. T., Ellegaard, K. M., Troilo, M., Sauer, U., and Engel, P. (2017). Disentangling metabolic functions of bacteria in the honey bee gut. *PLoS Biol.* 15:e2003467. doi: 10.1371/journal.pbio.2003467

Kevan, P. G., and Ebert, T. (2005). Can almond nectar and pollen poison honey bees? *Am. Bee J.* 145, 507–509.

King, M. J. (1993). Buzz foraging mechanism in bumble bees. *J. Apic. Res.* 32, 41–49. doi: 10.1080/00218839.1993.11101286

Kitaoka, T. K., and Nieh, J. C. (2009). Bumble bee pollen foraging regulation: role of pollen quality, storage levels, and odor. *Behav. Ecol. Sociobiol.* 63, 501–510. doi: 10.1007/s00265-008-0684-3

Kissen, R., Rossiter, J. T., and Bones, A. M. (2009). The "Mustard Oil Bomb": not so easy to assemble?! localization, expression and distribution of the components of the myrosinase enzyme system. *Phytochem. Rev.* 8, 69–86. doi: 10.1007/s11101-008-9109-1

Kleijn, D., and Raemakers, I. (2008). A retrospective analysis of pollen host plant use by stable and declining bumble bee species. *Ecology* 89, 1811–1823. doi: 10.1890/07-1275.1

Kolassa, J. E., and Jankowski, S. (2021). *MultNonParam: Mutlivariate Nonparametric Methods. R package Version 1.3.5*. Available online at: https://cran.r-project.org/package=MultNonParam. (Accessed June 3, 2021).

Kuznetsova, A., Brockhoff, P. B., and Christensen, R. H. B. (2017). lmerTest package: tests in linear mixed effects models. *J. Stat. Softw.* 82, 1–26.

Leonhardt, S. D., and Blüthgen, N. (2012). The same, but different: pollen foraging in honeybee and bumblebee colonies. *Apidologie* 43, 449–464. doi: 10.1007/s13592-011-0112-y

Levin, M. D., and Haydak, M. H. (1957). Comparative value of different pollens in the nutrition of *Osmia lignaria* Say (Hymenoptera: Apoidea). *Bee World* 38, 221–226. doi: 10.1080/0005772x.1957.11095007

London-Shafir, I., Shafir, S., and Eisikowitch, D. (2003). Amygdalin in almond nectar and pollen–facts and possible roles. *Plant Syst. Evol.* 238, 87–95. doi: 10.1007/s00606-003-0272-y

Lunau, K. (2000). The ecology and evolution of visual pollen signals. *Plant Syst. Evol.* 222, 89–111. doi: 10.1007/978-3-7091-6306-1_5

Lunau, K., Piorek, V., Krohn, O., and Pacini, E. (2015). Just spines–mechanical defense of malvaceous pollen against collection by corbiculate bees. *Apidologie* 46, 144–149. doi: 10.1007/s13592-014-0310-5

Ma, C., Kessler, S., Simpson, A., and Wright, G. A. (2016). Novel behavioral assay to investigate gustatory responses of individual, freely-moving bumble bees (*Bombus terrestris*). *J. Vis. Exp.* 113:54233.

Manson, J. S., Otterstatter, M. C., and Thomson, J. D. (2010). Consumption of a nectar alkaloid reduces pathogen load in bumble bees. *Oecologia* 162, 81–89. doi: 10.1007/s00442-009-1431-9

Marqués, A., Juan, A., Ruíz, M., Traveset, A., and Leza, M. (2019). Improvement of almond production using *Bombus terrestris* (Hymenoptera: Apidae) in Mediterranean conditions. *J. Appl. Entomol.* 143, 1132–1142. doi: 10.1111/jen.12690

Mazumder, A., Dwivedi, A., and du Plessis, J. (2016). Sinigrin and its therapeutic benefits. *Molecules* 21:416. doi: 10.3390/molecules21040416

Mendiburu, F. (2020). *Agricolae: Statistical Procedures for Agricultural Research. R Package Version 1.3-3*. Available online at: http://CRAN.R-project.org/package=agricolae. (Accessed June 6, 2021)

Moerman, R., Vanderplanck, M., Fournier, D., Jacquemart, A.-L., and Michez, D. (2017). Pollen nutrients better explain bumblebee colony development than pollen diversity. *Insect Conserv. Divers.* 10, 171–179. doi: 10.1111/icad.12213

Mommaerts, V., Wackers, F., and Smagghe, G. (2013). Assessment of gustatory responses to different sugars in harnessed and free-moving bumblebee workers (*Bombus terrestris*). *Chem. Senses* 38, 399–407. doi: 10.1093/chemse/bjt014

Moore, B. D., Andrew, R. L., Külheim, C., and Foley, W. J. (2014). Explaining intraspecific diversity in plant secondary metabolites in an ecological context. *New Phytol.* 201, 733–750. doi: 10.1111/nph.12526

Moret, Y., and Schmid-Hempel, P. (2000). Survival for immunity: the price of immune system activation for bumblebee workers. *Science* 290, 1166–1168. doi: 10.1126/science.290.5494.1166

Müller, A. (1996). Convergent evolution of morphogical specializations in Central European bee and honey wasp species as an adaptation to the uptake of pollen from nototribic flowers (Hymenoptera, Apoidea and Masaridae). *Biol. J. Linn. Soc.* 57, 235–252. doi: 10.1006/bijl.1996.0013

Muth, F., Francis, J. S., and Leonard, A. S. (2016). Bees use the taste of pollen to determine which flowers to visit. *Biol. Lett.* 12:20160356. doi: 10.1098/rsbl.2016.0356

Nayar, J. K., and Thorsteinson, A. J. (1963). Further investigations into the chemical basis of insect-host plant relationships in an oligophagous insect, *Plutella maculipennis* (Curtis) (Lepidoptera: Plutellidae). *Can. J. Zool.* 41, 923–929. doi: 10.1139/z63-066

Palmer-Young, E. C., Farrell, I. W., Adler, L. S., Milano, N. J., Egan, P., Juncker, R., et al. (2019). Chemistry of floral rewards: intra- and interspecific variability of nectar and pollen secondary metabolites across taxa. *Ecol. Monogr.* 89:e01335.

Palmer-Young, E. C., Tozkar, C. O., Schwarz, R. S., Chen, Y., Irwin, R. E., Adler, L. S., et al. (2017). Nectar and pollen phytochemicals stimulate honey bee (Hymenoptera: Apidae) immunity to viral infection. *J. Econ. Entomol.* 110, 1959–1972. doi: 10.1093/jee/tox193

Paris, L., Peghaire, E., Moné, A., Diogon, M., Debroas, D., Delbac, F., et al. (2020). Honeybee gut microbiota dysbiosis in pesticide/parasite co-exposures is mainly induced by *Nosema ceranae*. *J. Invertebr. Pathol.* 172:107348. doi: 10.1016/j.jip.2020.107348

Parker, B. J., Barribeau, S. M., Laughton, A. M., de Roode, J. C., and Gerardo, N. M. (2011). Non-immunological defense in an evolutionary framework. *Trends Ecol. Evol.* 26, 242–248. doi: 10.1016/j.tree.2011.02.005

Pernal, S., and Currie, R. (2002). Discrimination and preferences for pollen-based cues by foraging honeybees, *Apis mellifera* L. *Anim. Behav.* 63, 369–390. doi: 10.1006/anbe.2001.1904

Potts, S. G., Imperatriz-Fonseca, V., Ngo, H., Aizen, M., Biesmeijer, J., Breeze, T., et al. (2016). Safeguarding pollinators and their values to human well-being. *Nature* 540, 220–229. doi: 10.1038/nature20588

Povey, S., Cotter, S. C., Simpson, S. J., Lee, K. P., and Wilson, K. (2008). Can the protein costs of bacterial resistance be offset by altered feeding behaviour? *J. Anim. Ecol.* 78, 437–446. doi: 10.1111/j.1365-2656.2008.01499.x

Praz, C. J., Müller, A., and Dorn, S. (2008a). Host recognition in a pollen-specialist bee: evidence for a genetic basis. *Apidologie* 39, 547–557. doi: 10.1051/apido:2008034

Praz, C. J., Müller, A., and Dorn, S. (2008b). Specialized bees fail to develop on non-host pollen: do plants chemically protect their pollen? *Ecology* 89, 795–804. doi: 10.1890/07-0751.1

Quezada-Euan, J. J. G., Lopez-Velasco, A., Perez-Balam, J., Moo-Valle, H., Velazquez-Madrazo, A., and Paxton, R. J. (2011). Body size differs in workers produced across time and is associated with variation in the quantity and composition of larval food in *Nannotrigona perilampoides* (Hymenoptera. *Meliponini). Insectes Soc.* 58, 31–38. doi: 10.1007/s00040-010-0113-2

R Core Team (2017). *R: a Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rasheed, S. A., and Harder, L. D. (1997). Economic motivation for plant species preferences of pollen-collecting bumble bees. *Econ. Entomol.* 2, 209–219. doi: 10.1046/j.1365-2311.1997.t01-1-00059.x

Rasmont, P., Coppée, A., Michez, D., and De Meulemeester, T. (2008). An overview of the *Bombus terrestris* (L. 1758) subspecies (Hymenoptera: Apidae). *Ann. Soc. Entomol. Fr.* 44, 243–250. doi: 10.1080/00379271.2008.10697559

Rasmont, P., Regali, A., Ings, T. C., Lognay, G., Baudart, E., Marlier, M., et al. (2005). Analysis of pollen and nectar of *Arbutus unedo* as a food source for *Bombus terrestris* (Hymenoptera: Apidae). *J. Econ. Entomol.* 98, 656–663.

Reilly, S., and Schachtman, R. (2009). *Conditioned Taste Aversion: Behavioral and Neural Processes*. Oxford: Oxford University Press.

Richards, L. A., Dyer, L. A., Forister, M. L., Smilanich, A. M., Dodson, C. D., Leonard, M. D., et al. (2015). Phytochemical diversity drives plant–insect community diversity. *Proc. Natl. Acad. Sci. U.S.A.* 112, 10973–10978. doi: 10.1073/pnas.1504977112

Richardson, L. L., Adler, L. S., Leonard, A. S., Andicoechea, J., Regan, K. H., Anthony, W. E., et al. (2015). Secondary metabolites in floral nectar reduce parasite infections in bumblebees. *Proc. R. Soc. B* 282:20142471. doi: 10.1098/rspb.2014.2471

Ricketts, T. H., Regetz, J., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., Bogdanski, A., et al. (2008). Landscape effects on crop pollination services: are there general patterns? *Ecol. Lett.* 11, 499–515. doi: 10.1111/j.1461-0248.2008.01157.x

Rivest, S., and Forrest, J. R. K. (2020). Defence compounds in pollen: why do they occur and how do they affect the ecology and evolution of bees? *New Phytol.* 225, 1053–1064. doi: 10.1111/nph.16230

Robertson, A. W., Mountjoy, C., Faulkner, B. E., Roberts, M. V., and Macnair, M. R. (1999). Bumblebee selection of *Mimulus guttatus* flowers: the effects of pollen quality and reward depletion. *Ecology* 80, 2594–2606. doi: 10.2307/177243

Robinson, M. E. (1930). Cyanogenesis in plants. *Biol. Rev.* 5, 126–141. doi: 10.1111/j.1469-185x.1930.tb00896.x

Roger, N., Michez, D., Wattiez, R., Sheridan, C., and Vanderplanck, M. (2017). Diet effects on bumblebee health. *J. Insect Physiol.* 96, 128–133. doi: 10.1016/j.jinsphys.2016.11.002

Roulston, T. H., and Cane, J. H. (2000). Pollen nutritional content and digestibility for animals. *Plant Syst. Evol.* 222, 187–209. doi: 10.1007/978-3-7091-6306-1_10

Roulston, T. H., Cane, J. H., and Buschmann, S. L. (2000). What governs protein content of pollen: pollinator preferences, pollen-pistil interactions, or phylogeny? *Ecol. Monogr.* 70, 617–643. doi: 10.2307/2657188

Ruedenauer, F. A., Raubenheimer, D., Kessner-Beierlen, D., Grund-Muller, N., Noack, L., Spaethe, J., et al. (2020a). Best be(e) on low fat: linking nutrient perception, regulation and fitness. *Ecol. Lett.* 23, 545–554. doi: 10.1111/ele.13454

Ruedenauer, F. A., Spaethe, J., and Leonhardt, S. D. (2015). How to know which food is good for you: bumblebees use taste to discriminate between different concentrations of food differing in nutrient content. *J. Exp. Biol.* 218, 2233–2240. doi: 10.1242/jeb.118554

Ruedenauer, F. A., Spaethe, J., and Leonhardt, S. D. (2016). Hungry for quality—individual bumblebees forage flexibly to collect high-quality pollen. *Behav. Ecol. Sociobiol.* 70, 1209–1217. doi: 10.1007/s00265-016-2129-8

Ruedenauer, F. A., Sydow, D., Spaethe, J., and Leonhardt, S. D. (2020b). Young bumblebees may rely on both direct pollen cues and early experience when foraging. *Proc. R. Soc. B* 287:20201615. doi: 10.1098/rspb.2020.1615

Sáez, A., Aizen, M. A., Medici, S., Viel, M., Villalobos, E., and Negri, P. (2020). Bees increase crop yield in an alleged pollinator-independent almond variety. *Sci. Rep.* 10:3177.

Sarwade, A. B., and Bhawane, G. P. (2013). Anatomical and histological structure of digestive tract of adult *Platynotus belli* (Coleoptera: Tenebrionidae). *Biol. Forum Int. J.* 5, 47–55.

Sedivy, C., Müller, A., and Dorn, S. (2011). Closely related pollen generalist bees differ in their ability to develop on the same pollen diet: evidence for physiological adaptations to digest pollen. *Funct. Ecol.* 25, 718–725. doi: 10.1111/j.1365-2435.2010.01828.x

Shields, V. D. C., and Mitchell, B. K. (1995). Sinigrin as a feeding deterrent in two crucifer-feeding, polyphagous lepidopterous species and the effects of feeding stimulant mixtures on deterrency. *Philos. Trans. R. Soc. Lond. B* 347, 439–446. doi: 10.1098/rstb.1995.0035

Singaravelan, N., Ne'eman, G., Inbar, M., and Izhaki, I. (2005). Feeding responses of free-flying honeybees to secondary compounds mimicking floral nectars. *J. Chem. Ecol.* 31, 2791–2804. doi: 10.1007/s10886-005-8394-z

Singer, M. S., Mace, K. C., and Bernays, E. A. (2009). Self-medication as adaptative plasticity: increased ingestion of plant toxins by parasitized caterpillars. *PLoS One* 4:e4796. doi: 10.1371/journal.pone.0004796

Stegemann, T., Kruse, L. H., Brütt, M., and Ober, D. (2018). Specific distribution of pyrrolizidine alkaloids in floral parts of comfrey (*Symphytum officinale*) and its implications for flower ecology. *J. Chem. Ecol.* 45, 128–135. doi: 10.1007/s10886-018-0990-9

Stevenson, P. C. (2020). For antagonists and mutualists: the paradox of insect toxic secondary metabolites in nectar and pollen. *Phytochem. Rev.* 19, 603–614. doi: 10.1007/s11101-019-09642-y

Stevenson, P. C., Nicolson, S. W., and Wright, G. A. (2017). Plant secondary metabolites in nectar: impacts on pollinators and ecological functions. *Funct. Ecol.* 31, 65–75. doi: 10.1111/1365-2435.12761

Tasei, J. N., and Aupinel, P. (2008a). Nutritive value of 15 single pollens and pollen mixes tested on larvae produced by bumble bee workers (*Bombus terrestris*, Hymenoptera: Apidae). *Apidologie* 39, 397–409. doi: 10.1051/apido:2008017

Tasei, J. N., and Aupinel, P. (2008b). Validation of a method using queenless *Bombus terrestris* micro-colonies for testing the nutritive value of commercial pollen mixes by comparison with queenright colonies. *J. Econ. Entomol.* 101, 1737–1742. doi: 10.1603/0022-0493-101.6.1737

Thomson, J. D., and Thomson, B. A. (1992). "Pollen presentation and viability schedules in animal-pollinated plants: consequences for reproductive success," in *Ecology and Evolution of Plant Reproduction: New Approaches*, ed. R. Wyatt (New York, NY: Chapman & Hall), 1–24.

Tiedeken, E. J., Stout, J. C., Stevenson, P. C., and Wright, G. A. (2014). Bumblebees are not deterred by ecologically relevant concentrations of nectar toxins. *J. Exp. Biol.* 217, 1620–1625.

Trunz, V., Lucchetti, M. A., Bénon, D., Dorchin, A., Desurmont, G. A., Kast, C., et al. (2020). To bee or not to bee: the 'raison d'être' of toxic secondary compounds in the pollen of Boraginaceae. *Funct. Ecol.* 34, 1345–1357. doi: 10.1111/1365-2435.13581

Vanderplanck, M., Declèves, S., Roger, N., Decroo, C., Caulier, G., Glauser, G., et al. (2018). Is non-host pollen suitable for generalist bumblebees? *Insect Sci.* 25, 259–272. doi: 10.1111/1744-7917.12410

Vanderplanck, M., Gilles, H., Nonclercq, D., Duez, P., and Gerbaux, P. (2020). Asteraceae paradox: chemical and mechanical protection of *Taraxacum* pollen. *Insects* 11:304. doi: 10.3390/insects11050304

Vanderplanck, M., Martinet, B., Carvalheiro, L. G., Rasmont, P., Barraud, A., Renaudeau, C., et al. (2019a). Ensuring access to high-quality resources reduces the impacts of heat stress on bees. *Sci. Rep.* 9:12596.

Vanderplanck, M., Moerman, R., Rasmont, P., Lognay, G., Wathelet, B., Wattiez, R., et al. (2014). How does pollen chemistry impact development and feeding behaviour of polylectic bees? *PLoS One* 9:e86209. doi: 10.1371/journal.pone.0086209

Vanderplanck, M., Roger, N., Moerman, R., Ghisbain, G., Gérard, M., Popowski, D., et al. (2019b). Bumble bee parasite prevalence but not genetic diversity impacted by the invasive plant *Impatiens glandulifera*. *Ecosphere* 10:e02804.

Vaudo, A. D., Patch, H. M., Mortensen, D. A., Tooker, J. F., and Grozinger, C. M. (2016a). Macronutrient ratios in pollen shape bumble bee (*Bombus impatiens*) foraging strategies and floral preferences. *Proc. Natl. Acad. Sci. U.S.A.* 113, E4035–E4042.

Vaudo, A. D., Stabler, D., Patch, H. M., Tooker, J. F., Grozinger, C. M., and Wright, G. A. (2016b). Bumble bees regulate their intake of essential protein and lipid pollen macronutrients. *J. Exp. Biol.* 219, 3962–3970.

Vaudo, A. D., Tooker, J. F., Grozinger, C. M., and Patch, H. M. (2015). Bee nutrition and floral resource restoration. *Curr. Opin. Insect Sci.* 10, 133–141. doi: 10.1016/j.cois.2015.05.008

Vaudo, A. D., Tooker, J. F., Patch, H. M., Biddinger, D. J., Coccia, M., Crone, M. K., et al. (2020). Pollen protein:lipid macronutrient ratios may guide broad patterns of bee species floral preferences. *Insects* 11:132. doi: 10.3390/insects11020132

Velthuis, H. H. W., and van Doorn, A. (2006). A century of advances in bumblebee domestication and the economic and environmental aspect of its commercialization for pollination. *Apidologie* 37, 421–451. doi: 10.1051/apido:2006019

Weiner, C. N., Hilpert, A., Werner, M., Linsenmair, K. E., and Bluthgen, N. (2010). Pollen amino acids and flower specialisation in solitary bees. *Apidologie* 41, 476–487. doi: 10.1051/apido/2009083

Westerkamp, C., and Claben-Bockhoff, R. (2007). Bilabiate flowers: the ultimate response to bees? *Ann. Bot.* 100, 361–374. doi: 10.1093/aob/mcm123

Winfree, R., Aguilar, R., Vasquez, D. P., LeBuhn, G., and Aizen, M. A. (2009). A meta-analyis of bees'responses to anthropogenic disturbance. *Ecology* 90, 2068–2076. doi: 10.1890/08-1245.1

Wink, M. (1993). "Allelochemical properties or the raison d'être of Alkaloids," in *The Alkaloids*, Vol. 43, ed. J. Cordell (New York, NY: Academic Press), 1–118. doi: 10.1016/s0099-9598(08)60134-0

Wright, G. A., Baker, D. D., Palmer, M. J., Stabler, D., Mustard, J. A., Power, E. F., et al. (2013). Caffeine in floral nectar enhances a pollinator's memory of reward. *Science* 339, 1202–1204. doi: 10.1126/science.1228806

Wright, G. A., Mustard, J. A., Simcock, N. K., Ross-Taylor, A. A., McNicholas, L. D., Popescu, A., et al. (2010). Parallel reinforcement pathways for conditioned food aversions in the honeybee. *Curr. Biol.* 20, 2234–2240. doi: 10.1016/j.cub.2010.11.040

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Ecology and Evolution

BRIEF RESEARCH REPORT
published: 23 September 2021
doi: 10.3389/fevo.2021.731626

How Much Pigment Should Flowers Have? Flowers With Moderate Pigmentation Have Highest Color Contrast

*Casper J. van der Kooi**

Groningen Institute for Evolutionary Life Science, University of Groningen, Groningen, Netherlands

Floral pigments are a core component of flower colors, but how much pigment a flower should have to yield a strong visual signal to pollinators is unknown. Using an optical model and taking white, blue, yellow and red flowers as case studies, I investigate how the amount of pigment determines a flower's color contrast. Modeled reflectance spectra are interpreted using established insect color vision models. Contrast as a function of the amount of pigment shows a pattern of diminishing return. Low pigment amounts yield pale colors, intermediate amounts yield high contrast, and extreme amounts of pigment do not further increase, and sometimes even decrease, a flower's color contrast. An intermediate amount of floral pigment thus yields the highest visibility, a finding that is corroborated by previous behavioral experiments on bees. The implications for studies on plant-pollinator signaling, intraspecific flower color variation and the costs of flower color are discussed.

Keywords: pigmentation, color vision, pollination, reflection, absorbance, contrast, diminishing return

OPEN ACCESS

Edited by:
Isabel Marques,
University of Lisbon, Portugal

Reviewed by:
Yuval Sapir,
Tel Aviv University, Israel
Steven Johnson,
University of KwaZulu-Natal,
South Africa

***Correspondence:**
Casper J. van der Kooi
C.J.van.der.Kooi@rug.nl
orcid.org/0000-0003-0613-7633

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *27 June 2021*
Accepted: *24 August 2021*
Published: *23 September 2021*

Citation:
van der Kooi CJ (2021) How Much Pigment Should Flowers Have? Flowers With Moderate Pigmentation Have Highest Color Contrast. Front. Ecol. Evol. 9:731626. doi: 10.3389/fevo.2021.731626

INTRODUCTION

The coloration of flowers is a major component of plant-pollinator signaling. Flower coloration is due to two optical principles: reflection and scattering of light by the flower's surface and interior structures, and wavelength-selective absorption of scattered light by floral pigments (Kay et al., 1981; Kevan and Backhaus, 1998; van der Kooi et al., 2016, 2019). The degree of filtering of the reflected light by floral pigments determines a flower's color contrast to a (green leaf) background. For example, a low amount of pigment yields a pale color, whereas a higher amount of pigment generally results in a more marked color. Whether and how scattering structures and floral pigments are tuned so to yield visually contrasting floral displays to pollinators remains an open question.

To understand if and how different aspects of floral optical properties are tuned, it is important to consider how much the pigmentation and scattering properties vary—both within and between species. Pigmentation is probably more evolutionarily and developmentally labile than are the structural properties of flowers. This difference in variation of pigmentation vs scattering structures can be understood from both a mechanistic and functional point of view. First, the flower pigmentation of numerous species exhibits considerable intraspecific variation [reviewed by Sapir et al. (2021)]. Intraspecific flower color variation can be discrete (Schemske and Bierzychudek, 2007; Streinzer et al., 2019; von Witt et al., 2020; Buide et al., 2021) or continuous, e.g., covering

the spectrum of white, pink and purple in *Trifolium pratense* (**Figure 1**), orchids (Sletvold et al., 2016; Dormont et al., 2019) and the *Iris atropurpurea* complex (Roguz et al., 2020). The amount of pigment that is synthesized is a quantitative trait, and therefore small changes in the pigment synthesis pathway can yield appreciable changes in color (Shrestha et al., 2014; van der Kooi et al., 2019). Molecular studies also suggest that the synthesis of floral pigment is an evolutionary labile trait (Koes et al., 1994; Rausher, 2008; Wessinger et al., 2014; Sapir et al., 2021). In contrast, the structural aspects that determine how light is reflected, i.e., the number and type of cells, and flower thickness, are most likely phylogenetically and developmentally constrained (Martin and Gerats, 1993; van der Kooi et al., 2016). Indeed, in related species with differently pigmented flowers, the cellular structures are similar (Martin and Gerats, 1993; Stavenga et al., 2021). Further, whereas pigmentation virtually solely serves for visibility, the thickness and interior structure of flowers are also important for the flower's mechanical strength. Given the amount of pigment can vary within a species, for a flower with a certain backscattering, how much pigment yields the highest color contrast?

Based on evidence from studies on the optical properties of flowers and insect vision, I hypothesize that intermediate amounts of floral pigment yield the most conspicuous colors. Floral pigment absorption spectra generally feature a broadband unimodal distribution (see Figure 3 in van der Kooi et al., 2016). A low amount of pigment will yield a pale color, but because of the broadband absorption, extremely high amounts of floral pigment often yield dark, dull colors. For example, different amounts of a highly similar floral pigment create different shades of red in *Papaver* flowers and yellow/orange *Meconopsis cambrica* flowers (see Figure 6 in van der Kooi and Stavenga, 2019). Papiorek et al. (2013), investigating how quickly honeybees and bumblebees detect artificial stimuli with different concentrations of blue or orange pigment, found that bees most easily detect stimuli with intermediate concentrations of floral pigment.

Here, I provide a framework that enables exploration of how the amount of pigment determines a flower's visibility to pollinators, with the aim to test whether there are optima in the amount of pigment. Using our previously developed optical model (Stavenga and van der Kooi, 2016), I investigate how systematic changes in the amount of pigment change the reflectance spectrum of white, blue, yellow and red flowers. The resulting reflectance spectra are interpreted with a "pollinator-subjective view" using established models for animal color vision (Chittka, 1992; Vorobyev and Osorio, 1998). It is thus found that the highest color contrast is often obtained by moderate amounts of pigment. Finally, I discuss how this approach could help to further our understanding of the optical properties and costs of floral displays.

MATERIALS AND METHODS

Model Species

Four species with different flower colors were chosen: the white campion *Silene latifolia* (ssp. *alba*) Poir. (Caryophyllaceae), the Chilean bellflower *Nolana paradoxa* Lindl. (Solanaceae), the Missouri evening primrose *Oenothera macrocarpa* Nutt. (also known as *O. missouriensis*, Onagraceae) and the common poppy *Papaver rhoeas* L. (Papaveraceae) (**Figure 2**). Together these species span the spectrum of flower colors that are common in nature. *S. latifolia* and *O. macrocarpa* are pollinated by nocturnal moths (e.g., Young, 2002; Krakos and Austin, 2020), and *P. rhoeas* in Europe is pollinated by bees (van der Kooi and Stavenga, 2019). For *N. paradoxa* I could not find reliable data on the pollinating

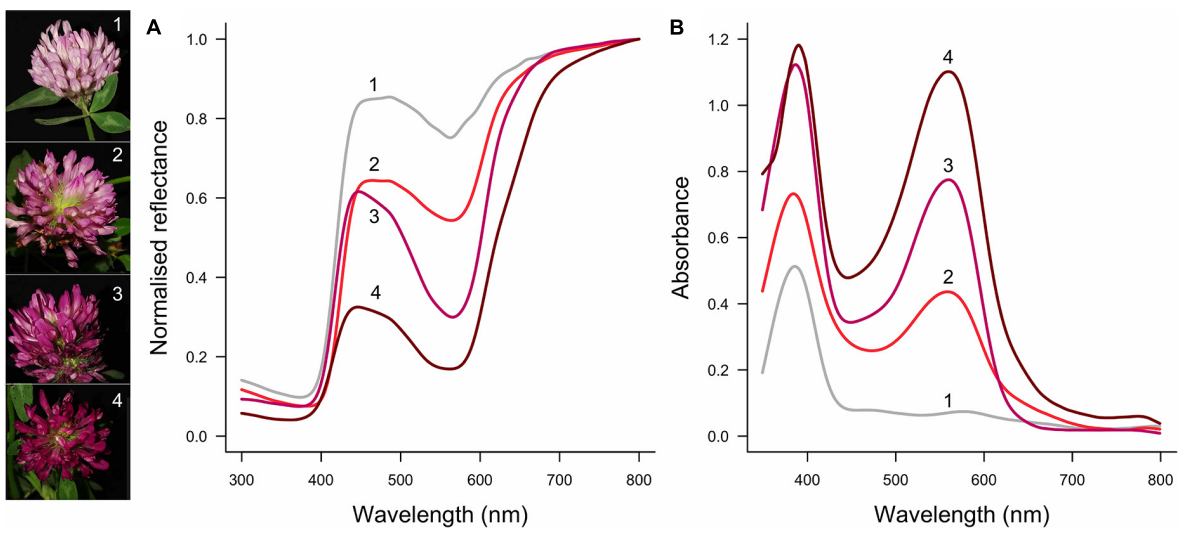

FIGURE 1 | Example of intraspecific flower color variation in *Trifolium pratense*. Flower color ranges from light pink (1) *via* pink (2, 3) to dark red (4). From light to dark, modulation of the reflectance spectra increases, particularly between 500 and 600 nm **(A)**, which is due to an increase in the amount of pigment with peak absorption at ~550 nm **(B)**. Flowers of this species also have a UV-absorbing pigment, the amount of which varies less than the pigment that absorbs in the green wavelength range. The numbered spectra correspond to the left pictures (photo credit: Alina Höwener).

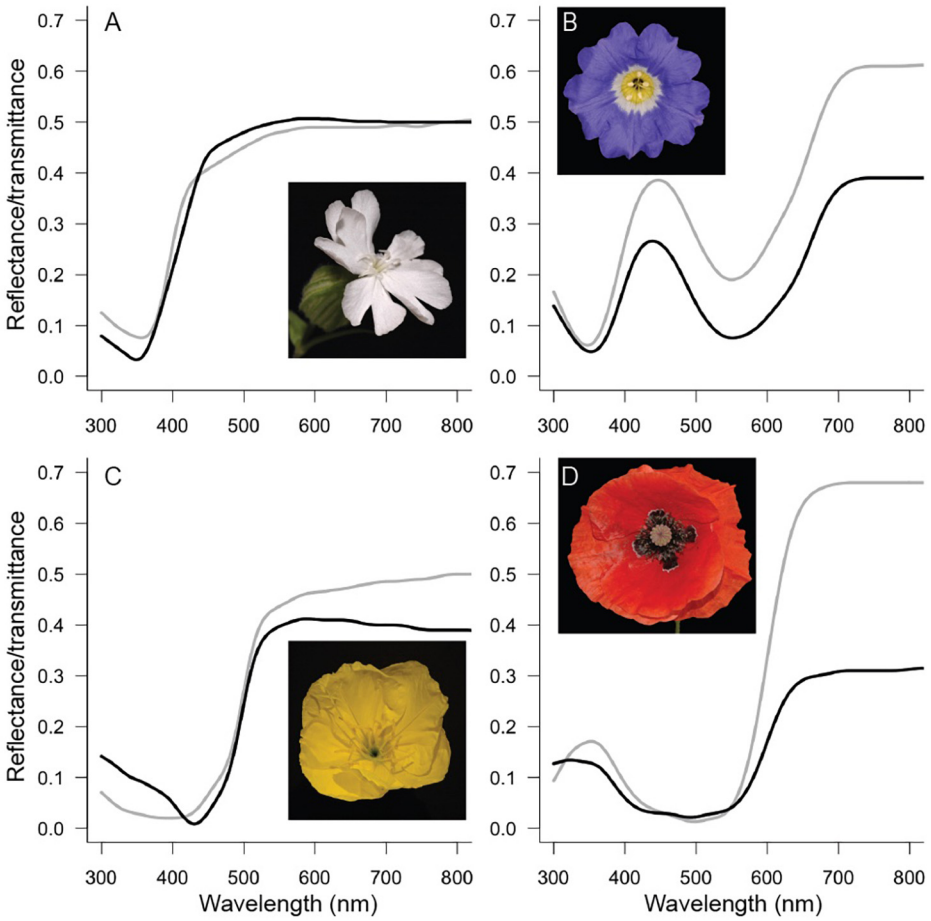

FIGURE 2 | Flowers investigated in this study. Measured reflectance (black curves) and transmittance (gray curves) spectra for *Silene latifolia* **(A),** *Nolana paradoxa* **(B)**, *Oenothera macrocarpa* **(C)**, and *Papaver rhoeas* **(D)**.

species. For *N. paradoxa* and *P. rhoeas*, we previously applied the optical model to study the optical properties (particularly the backscattering) of these flowers (Stavenga and van der Kooi, 2016; van der Kooi and Stavenga, 2019).

Spectroscopy

Reflectance spectra of the four species were measured with an integrating sphere (AvaSphere-50-Refl) and a deuterium-halogen lamp [AvaLight-D(H)-S] and the spectrometer an AvaSpec-2048 (Avantes, Netherlands). A piece of flower was directionally and about perpendicularly illuminated from within the sphere at an area with diameter ∼5 mm. A white diffuse tile (Avantes WS-2) was used as a reference. For transmittance measurements, the sample was illuminated from outside the sphere with a fiber, with an illumination spot size of ∼1 mm. To model the spectra, it is necessary to have the (absolute) amounts of transmittance and reflectance, which can be obtained with an integrating sphere and not with a reflection probe. For *T. pratense* (**Figure 1A**) the reflectance spectra were obtained with a bifurcated reflection probe, because the flowers were too small to be measured with the sphere. Petal absorbance spectra (**Figure 1B**) were obtained using a microspectrophotometer equipped with a xenon arc light source (for details, see Stavenga and van der Kooi, 2016).

Modeling

To obtain reflectance spectra of similar shape but with different degrees of modulation, I applied our previously developed optical model (Stavenga and van der Kooi, 2016). That model combines the Kubelka-Munk theory for scattering and absorbing media (Kubelka and Munk, 1931; Allen et al., 1969) with a layer-stack model (Stavenga et al., 2006). Two key aspects of the model are the scattering and absorption parameters, S^* and K^*, which can be derived from the measured reflectance (R) and transmittance (T) (Yamada and Fujimura, 1991; Stavenga and van der Kooi, 2016):

$$S^* = [ln\{(1-(a-b)R)/T\}]/b \quad (1a)$$

$$K^* = (a-1)S^* \quad (1b)$$

with

$$a = (1+R^2-T^2)/(2R),\ b = \sqrt{a^2-1}. \quad (1c)$$

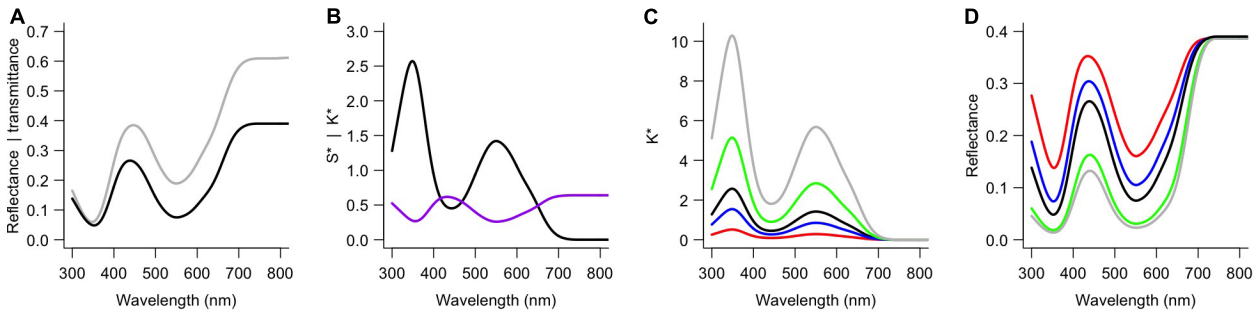

FIGURE 3 | Example spectra and calculations for the blue flowers of *Nolana paradoxa*. **(A)** Measured reflectance (black) and transmittance (gray) spectra (as in **Figure 2B**). **(B)** Absorption (K^*, black) and scattering parameter (S^*, purple) calculated using the measured spectra from panel **(A)**. **(C)** Different absorption parameters used for the modeling; $0.2K^*$ (red), $0.6K^*$ (blue), K^* [black, as in panel **(B)**], $2K^*$ (green) and $4K^*$ (gray). **(D)** Reflectance spectra obtained using the absorption parameters as in panel **(C)** (with corresponding colors).

S^* and K^* are proportional to the amount of scattering and absorption, respectively. After the scattering and absorption parameters of a flower have been calculated using measured spectra (**Figures 3A,B**), modeled reflectance spectra can be obtained using a series of matrix calculations (see the appendix of Stavenga and van der Kooi, 2016; also see the R script). Spectra of similar shape but with different modulation were obtained by systematically varying K^* but leaving S^* unchanged, because K^* is the main factor that determines the modulation. The contribution of K^* in the model was set to be 0.2, 0.4, 0.6 . . . 4 (**Figure 3C**). The chosen parameter range yields spectra that are similar to those that can be found in real flowers and enables modeling cases of extreme amounts of pigment. What follows is a series of reflectance spectra, which are similar to those of flowers with different amounts of the same pigment (**Figure 3D**). S^* was kept identical to the value obtained for real flowers, so the modulation of the reflectance spectrum varied independently from the total amount of reflectance (see the convergence in the long wavelength range, **Figure 3D**).

Different optical processes determine the modulation of the reflected light and the applied optical model can be used to quantitatively investigate that, but the approach used here is not to study these factors. The aim of this study is to investigate the consequences of variation in absorption by pigments, but not *via* which anatomical ways this is achieved, because that requires detailed species-specific anatomical information. For example, increases in modulation of the reflected light can be obtained by increasing the total amount of pigment as well as by concentrating a smaller amount of pigment in the outer layer at the side of viewing (see Figure 5 in van der Kooi et al., 2016). To standardize the degree of modulation among the four different species, I used fractions and multiples of the empirical K^* (**Figure 3**). The different cases are classified by a "pigment index," with pigment index = 5 being identical to the measured spectra (**Figure 3**) and lower and higher indices being the more weakly and strongly modulated cases, respectively.

It is important to point out that scattering and absorption, and so S^* and K^*, are not strictly separated, because presence of pigments (slightly) influences the refractive index of a medium, and thereby the medium's scattering. Nevertheless, the obtained spectra are highly similar to those found in nature, so the chosen approach is sufficient to study the effect of modulation for visibility to pollinators.

To interpret the different reflectance spectra with a pollinator subjective view, I deployed two commonly used insect vision models, i.e., the hexagon and the receptor noise-limited model (Chittka, 1992; Vorobyev and Osorio, 1998), using honeybee (*Apis mellifera*) and hawk moth (*Deilephila elpenor*) spectral sensitivities (van der Kooi et al., 2021a) and an average green leaf background under D65 ambient illumination. Both models yield a color contrast value, which is broadly considered a good proxy for visibility to a wide range of animals (Giurfa et al., 1996; Spaethe et al., 2001; Kelber et al., 2003; Dyer and Chittka, 2004).

RESULTS

Color Contrast Follows a Pattern of Diminishing Return

When color contrast is modeled as a function of the amount of pigment, for all four cases there is a non-linear relationship. At low levels (i.e., for pale flowers), an increase in the amount of pigment enhances the flower's contrast to the background. However, at a certain (species-specific) point, the color contrast curve plateaus, and further increases in the amount of pigment will not increase or even decrease color contrast (**Figure 4**)—indicating a pattern of diminishing return. The relationship of the color contrast and the amount of pigment depends on the type of pigment, but at least for the modeled white, blue and yellow colors the overall effect is similar. For the (ultraviolet reflecting) red *P. rhoeas* flowers the amount of pigment does not drastically change the flower's contrast, meaning that within the current set of parameters changing the amount of pigment has a comparatively small effect on the visibility. Nevertheless, also for this species, the overall observation of diminishing returns in contrast with the amount of pigment is supported.

The results are largely vision model-independent, that is, within one color category, color contrast as a function of the amount of pigment is similar regardless of whether the hexagon or the receptor noise-limited model was applied

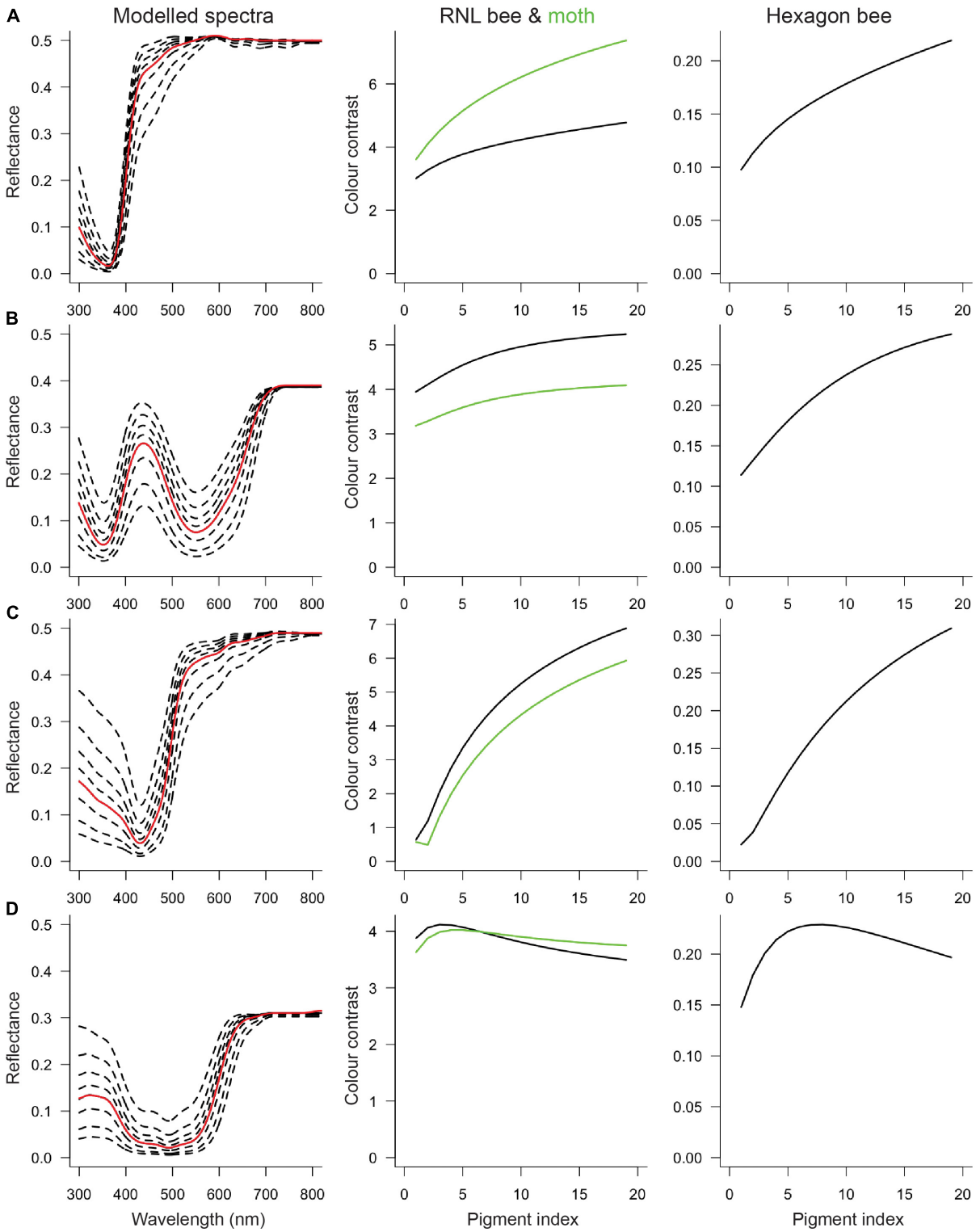

FIGURE 4 | Color contrast for reflectance spectra with different degrees of modulation. The first column shows the measured reflectance spectra (red curve) and a selection of the modeled cases (dashed curves), including the most weakly and strongly modulated spectra. The measured reflectance is identical to the fifth modeled spectrum, so the empirical color contrast values are at pigment index = 5. The second column shows bee (black) and moth (green) color contrast calculated with the receptor noise-limited (RNL) model. The third column shows bee color contrast calculated with the hexagon model. **(A)** *Silene latifolia*, **(B)** *Nolana paradoxa*, **(C)** *Oenothera macrocarpa*, and **(D)** *Papaver rhoeas*.

(compare columns 2 and 3 in **Figure 4**). The effect of the amount of pigment on color contrast is also similar for bees and hawk moths (compare black and green in column 2, **Figure 4**), though there is one interesting exception. For moth-pollinated *S. latifolia*, color contrast increases more for moths than for bees (**Figure 4A**), suggesting that increasing the amount of pigment would disproportionally improve visibility to pollinators. However, in the other moth-pollinated species, *O. macrocarpa*, the effect may be opposite with (slightly) larger increases for bees than for moths.

Intriguingly, for almost all species, empirical contrast values (pigment index = 5) are not the highest that are theoretically possible. For the (ultraviolet reflecting) red poppy flower, the empirical color contrast is very close to the theoretical optimum, but for white, blue and yellow flowers, more wavelength-specific absorption by pigment would increase visibility. On the other hand, the empirical color contrast values are (well) above the detection threshold for bees, which are ∼0.10 for the hexagon model (Dyer and Chittka, 2004) and ∼2 for the RNL model (Vorobyev et al., 2001).

DISCUSSION

Floral pigments are a crucial component of flower coloration. Whereas numerous studies showed that the type of pigment determines flower color and visibility to pollinators (e.g., Rausher, 2008; Lunau et al., 2011; Shrestha et al., 2013), it is virtually unknown how much pigment is needed to produce a conspicuous flower. The amount of pigment can, however, vary within and between species (Introduction), meaning that the amount of absorption of light by floral pigments can be tuned to the scattering structures.

Using a previously developed optical model, I systematically varied the amount of pigment for four differently colored flowers. Interpretation with established bee and moth vision models revealed that more pigment does not necessarily improve, and may even reduce, a flower's visibility. This means that pale flowers have a relatively low contrast to the background and flowers with an intermediate amount of pigment a high contrast. Above a certain amount of pigment, the reflected light is sufficiently modulated to be clearly visible to pollinators and more pigment does not increase the flower's visibility to pollinators. Owing to the broadband absorption range that is typical for floral pigments (van der Kooi et al., 2016), a very high amount of pigment may render the flower dull and little contrasting with the green background. The modeling results dovetail those of Papiorek et al. (2013) who found that honeybees and bumblebees most easily detect artificial stimuli with intermediate amounts of blue and orange pigments.

The observation that for three of the four cases, the flowers do not exhibit the amount of pigment that would yield the highest contrast that is theoretically possible suggests that there is a trade-off between visibility and investment in floral pigments. Although more research is needed to infer whether this is a general phenomenon, it is tempting to speculate why flowers would not exhibit maximum contrast. Increases in pigmentation may be not worth the energy investment once the threshold at which pollinators readily detect the flower has been reached. Color contrast does not scale linearly with detectability by animals, but generally shows a sigmoidal relationship (Olsson et al., 2015; Garcia et al., 2017, 2020; Santiago et al., 2020). Above the detection threshold, which is rapidly reached in the cases considered here, further increases in color contrast may not increase the likelihood of detection by pollinators. Alternatively, for plant species that share pollinators and have the same floral pigment, differences in the amount of pigment may help to obtain more dissimilar flower colors and so enhance character displacement.

The Amount of Pigment and Perspectives for Future Studies on Flower Color

The observation that, in addition to the type of pigment, the amount of pigment is important for flower visibility is relatively overlooked in studies on flower color. These results open perspectives for future research in various directions.

Intraspecific trait variation (provided that it is heritable) is the cornerstones of evolution, and studies on the mechanistic underpinnings and consequences of such variation are paramount in understanding trait evolution (van der Kooi et al., 2021b). Generally, yellow colors are generated by carotenoids, white flowers by flavonols, and blue, purple, and red colors by anthocyanin pigments. Of these types of pigments, anthocyanins are most commonly associated with flower color polymorphisms (Sapir et al., 2021). Indeed, many species for which flower color variation was reported (Frey, 2004; Rakosy et al., 2012; Renoult et al., 2013; Sletvold et al., 2016; Dormont et al., 2019; Paine et al., 2019; Streinzer et al., 2019; Gómez et al., 2020; Whitney et al., 2020) have blue, pink or purple colors that are commonly due to anthocyanin pigments. It would be interesting to know, for these species, how standing variation in pigmentation relates to the flower's conspicuousness to their pollinators.

The four cases considered here revealed (subtle) differences between types of pigment, as well as between bee and moth visual systems in how close the empirical amount of pigment is to the theoretical optimum and the variation around that optimum. Comparative studies covering species that are serviced by pollinators with different visual systems can elucidate the role of the amount of pigment in tuning flower color to pollinator vision. Something to consider is that the perceived flower color variation probably differs across pollinators. Whitney et al. (2020), for example, recently showed that for the same set of flowers, birds perceive more intraspecific variation than bees. It is known that different pollinators select for different flower colors (hues) owing to variation in spectral sensitivity and/or color preferences (Lunau et al., 2011; Shrestha et al., 2013; van der Kooi et al., 2019), but pollinators may also impose different selective pressures on the degree of variation around a certain hue. In other words, the degree of stabilizing selection on flower color probably varies with the type of pollinator, which would translate to different optima ranges in the amount of pigment.

To conclude, the amount of pigment is an important though often overlooked aspect of flower coloration. The amount of pigment shows a pattern of diminishing return and, corroborated by behavioral experiments with bees (Papiorek et al., 2013), I conclude that intermediate amounts often yield sufficient, if not maximal, visibility to pollinators. Future studies on how much floral pigment is needed to create conspicuous flowers will further our understanding on (the maintenance of) flower color variation and the costs of floral displays (Obeso, 2002; Roddy et al., 2020).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

The author was funded by the Dutch Science Foundation (016.Veni.181.025) and by AFOSR/EOARD (FA9550-19-1-7005).

ACKNOWLEDGMENTS

The author would like to thank Doekele Stavenga and Johannes Spaethe for interesting discussions and comments on an early version of this manuscript. Two reviewers provided suggestions that greatly improved the manuscript. The author would also like to thank Alina Höwener for providing the pictures of *T. pratense* used in **Figure 1**.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.731626/full#supplementary-material

REFERENCES

Allen, W. A., Gausman, H. W., Richardson, A. J., and Thomas, J. R. (1969). Interaction of isotropic light with a compact plant leaf. *J. Opt. Soc. Am.* 59, 1376–1379. doi: 10.1364/josa.59.001376

Buide, M. L., Del Valle, J. C., Prado-Comesaña, A., and Narbona, E. (2021). The effects of pollination, herbivory and autonomous selfing on the maintenance of flower colour variation in *Silene littorea*. *Plant Biol.* 23, 275–284. doi: 10.1111/plb.13209

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543.

Dormont, L., Joffard, N., and Schatz, B. (2019). Intraspecific variation in floral color and odor in orchids. *Int. J. Plant Sci.* 180, 1036–1058. doi: 10.1086/705589

Dyer, A. G., and Chittka, L. (2004). Biological significance of distinguishing between similar colours in spectrally variable illumination: bumblebees (*Bombus terrestris*) as a case study. *J. Comp. Physiol. A* 190, 105–114. doi: 10.1007/s00359-003-0475-2

Frey, F. M. (2004). Opposing natural selection from herbivores and pathogens may maintain floral color variation in *Claytonia virginica* (Portulacaceae). *Evolution* 58, 2426–2437. doi: 10.1111/j.0014-3820.2004.tb00872.x

Garcia, J. E., Phillips, R. D., Peter, C. I., and Dyer, A. G. (2020). Changing how biologists view flowers-colour as a perception not a trait. *Front. Plant Sci.* 11:1775.

Garcia, J. E., Spaethe, J., and Dyer, A. G. (2017). The path to colour discrimination is S-shaped: behaviour determines the interpretation of colour models. *J. Comp. Physiol. A* 203, 983–997. doi: 10.1007/s00359-017-1208-2

Giurfa, M., Vorobyev, M., Kevan, P., and Menzel, R. (1996). Detection of coloured stimuli by honeybees: minimum visual angles and receptor specific contrasts. *J. Comp. Physiol. A* 178, 699–709.

Gómez, J. M., Perfectti, F., Armas, C., Narbona, E., González-Megías, A., Navarro, L., et al. (2020). Within-individual phenotypic plasticity in flowers fosters pollination niche shift. *Nat. Commun.* 11:4019. doi: 10.1038/s41467-020-17875-1

Kay, Q. O. N., Daoud, H. S., and Stirton, C. H. (1981). Pigment distribution, light reflection and cell structure in petals. *Bot. J. Linn. Soc.* 83, 57–83. doi: 10.1111/j.1095-8339.1981.tb00129.x

Kelber, A., Vorobyev, M., and Osorio, D. (2003). Animal colour vision–behavioural tests and physiological concepts. *Biol. Rev.* 78, 81–118. doi: 10.1017/s1464793102005985

Kevan, P. G., and Backhaus, W. G. K. (1998). "Color vision: ecology and evolution in making the best of the photic environment," in *Color Vision: Perspectives from Different Disciplines*, eds W. G. K. Backhaus, R. Kliegl, and J. S. Werner (Berlin: De Gruyter).

Koes, R. E., Quattrocchio, F., and Mol, J. N. M. (1994). The flavonoid biosynthetic pathway in plants: function and evolution. *BioEssays* 16, 123–132. doi: 10.1002/bies.950160209

Krakos, K. N., and Austin, M. W. (2020). Testing pollination syndromes in oenothera (Onagraceae). *J. Pollinat. Ecol.* 26, 52–66.

Kubelka, P., and Munk, F. (1931). Ein beitrag zur optik der farbanstriche. *Zeitschrift für Tech. Phys.* 12, 593–601.

Lunau, K., Papiorek, S., Eltz, T., and Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *J. Exp. Biol.* 214, 1607–1612. doi: 10.1242/jeb.052688

Martin, C., and Gerats, T. (1993). Control of pigment biosynthesis genes during petal development. *Plant Cell* 5:1253. doi: 10.1105/tpc.5.10.1253

Obeso, J. R. (2002). The costs of reproduction in plants. *New Phytol.* 155, 321–348. doi: 10.1046/j.1469-8137.2002.00477.x

Olsson, P., Lind, O., and Kelber, A. (2015). Bird colour vision: behavioural thresholds reveal receptor noise. *J. Exp. Biol.* 218, 184–193. doi: 10.1242/jeb.111187

Paine, K. C., White, T. E., and Whitney, K. D. (2019). Intraspecific floral color variation as perceived by pollinators and non-pollinators: evidence for pollinator-imposed constraints? *Evol. Ecol.* 33, 461–479. doi: 10.1007/s10682-019-09991-2

Papiorek, S., Rohde, K., and Lunau, K. (2013). Bees' subtle colour preferences: how bees respond to small changes in pigment concentration. *Naturwissenschaften* 100, 633–643. doi: 10.1007/s00114-013-1060-3

Rakosy, D., Streinzer, M., Paulus, H. F., and Spaethe, J. (2012). Floral visual signal increases reproductive success in a sexually deceptive orchid. *Arthropod. Plant. Interact.* 6, 671–681. doi: 10.1007/s11829-012-9217-0

Rausher, M. D. (2008). Evolutionary transitions in floral color. *Int. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Renoult, J. P., Thomann, M., Schaefer, H. M., and Cheptou, P. O. (2013). Selection on quantitative colour variation in *Centaurea cyanus*: the role of

the pollinator's visual system. *J. Evol. Biol.* 26, 2415–2427. doi: 10.1111/jeb.12234

Roddy, A. B., Martínez-Perez, C., Teixido, A. L., Cornelissen, T. G., Olson, M. E., Oliveira, R. S., et al. (2020). Towards the flower economics spectrum. *New Phytol.* 229, 665–672. doi: 10.1111/nph.16823

Roguz, K., Gallagher, M. K., Senden, E., Bar-Lev, Y., Lebel, M., Heliczer, R., et al. (2020). All the colors of the rainbow: diversification of flower color and intraspecific color variation in the genus *Iris*. *Front. Plant Sci.* 11:1519.

Santiago, C., Green, N. F., Hamilton, N., Endler, J. A., Osorio, D. C., Marshall, N. J., et al. (2020). Does conspicuousness scale linearly with colour distance? A test using reef fish. *Proc. R. Soc. B* 287:20201456. doi: 10.1098/rspb.2020.1456

Sapir, Y., Gallagher, M. K., and Senden, E. (2021). What maintains flower colour variation within populations? *Trends Ecol. Evol.* 36, 507–519. doi: 10.1016/j.tree.2021.01.011

Schemske, D. W., and Bierzychudek, P. (2007). Spatial differentiation for flower color in the desert annual *Linanthus parryae*: was Wright right? *Evolution* 61, 2528–2543. doi: 10.1111/j.1558-5646.2007.00219.x

Shrestha, M., Dyer, A. G., Bhattarai, P., and Burd, M. (2014). Flower colour and phylogeny along an altitudinal gradient in the Himalayas of Nepal. *J. Ecol.* 102, 126–135. doi: 10.1111/1365-2745.12185

Shrestha, M., Dyer, A. G., Boyd-Gerny, S., Wong, B. B. M., and Burd, M. (2013). Shades of red: bird-pollinated flowers target the specific colour discrimination abilities of avian vision. *New Phytol.* 198, 301–310. doi: 10.1111/nph.12135

Sletvold, N., Trunschke, J., Smit, M., Verbeek, J., and Ågren, J. (2016). Strong pollinator-mediated selection for increased flower brightness and contrast in a deceptive orchid. *Evolution* 70, 716–724. doi: 10.1111/evo.12881

Spaethe, J., Tautz, J., and Chittka, L. (2001). Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3898–3903. doi: 10.1073/pnas.071053098

Stavenga, D. G., Giraldo, M. A., and Hoenders, B. J. (2006). Reflectance and transmittance of light scattering scales stacked on the wings of pierid butterflies. *Opt. Express* 14, 4880–4890. doi: 10.1364/oe.14.004880

Stavenga, D. G., Leertouwer, H. L., Dudek, B., and van der Kooi, C. J. (2021). Colouration of flowers by flavonoids and consequences of pH dependent absorption. *Front. Plant Sci.* 11:2148. doi: 10.3389/fpls.2020.600124

Stavenga, D. G., and van der Kooi, C. J. (2016). Coloration of the Chilean bellflower, *Nolana Paradoxa*, interpreted with a scattering and absorbing layer stack model. *Planta* 243, 171–181. doi: 10.1007/s00425-015-2395-0

Streinzer, M., Roth, N., Paulus, H. F., and Spaethe, J. (2019). Color preference and spatial distribution of glaphyrid beetles suggest a key role in the maintenance of the color polymorphism in the peacock anemone (*Anemone pavonina*, Ranunculaceae) in Northern Greece. *J. Comp. Physiol. A* 205, 735–743. doi: 10.1007/s00359-019-01360-2

van der Kooi, C. J., Dyer, A. G., Kevan, P. G., and Lunau, K. (2019). Functional significance of the optical properties of flowers for visual signalling. *Ann. Bot.* 123, 263–276. doi: 10.1093/aob/mcy119

van der Kooi, C. J., Elzenga, J. T. M., Staal, M., and Stavenga, D. G. (2016). How to colour a flower: on the optical principles of flower coloration. *Proc. R. Soc. B* 283:20160429. doi: 10.1098/rspb.2016.0429

van der Kooi, C. J., and Stavenga, D. G. (2019). Vividly coloured poppy flowers due to dense pigmentation and strong scattering in thin petals. *J. Comp. Physiol. A* 205, 363–372. doi: 10.1007/s00359-018-01313-1

van der Kooi, C. J., Stavenga, D. G., Belusic, G., Arikawa, K., and Kelber, A. (2021a). Evolution of insect color vision: from spectral sensitivity to visual ecology. *Annu. Rev. Entomol.* 66, 435–462. doi: 10.1146/annurev-ento-061720-071644

van der Kooi, C. J., Vallejo-Marín, M., and Leonhardt, S. D. (2021b). Mutualisms and (a)symmetry in plant-pollinator interactions. *Curr. Biol.* 31, R91–R99. doi: 10.1016/j.cub.2020.11.020

von Witt, C. G., Anderson, B., Durbach, I. N., and Johnson, S. D. (2020). Breeding systems of floral colour forms in the *Drosera cistiflora* species complex. *Plant Biol.* 22, 992–1001. doi: 10.1111/plb.13159

Vorobyev, M., Brandt, R., Peitsch, D., Laughlin, S. B., and Menzel, R. (2001). Colour thresholds and receptor noise: behaviour and physiology compared. *Vision Res.* 41, 639–653. doi: 10.1016/s0042-6989(00)00288-1

Vorobyev, M., and Osorio, D. (1998). Receptor noise as a determinant of colour thresholds. *Proc. R. Soc. London B* 265, 351–358. doi: 10.1098/rspb.1998.0302

Wessinger, C. A., Hileman, L. C., and Rausher, M. D. (2014). Identification of major quantitative trait loci underlying floral pollination syndrome divergence in *Penstemon*. *Philos. Trans. R. Soc. B Biol. Sci.* 369:20130349. doi: 10.1098/rstb.2013.0349

Whitney, K. D., Smith, A. K., Williams, C., and White, T. E. (2020). Birds perceive more intraspecific color variation in bird-pollinated than bee-pollinated flowers. *Front. Plant Sci.* 11:1806.

Yamada, N., and Fujimura, S. (1991). Nondestructive measurement of chlorophyll pigment content in plant leaves from three-color reflectance and transmittance. *Appl. Opt.* 30, 3964–3973. doi: 10.1364/ao.30.003964

Young, H. J. (2002). Diurnal and nocturnal pollination of *Silene alba* (Caryophyllaceae). *Am. J. Bot.* 89, 433–440. doi: 10.3732/ajb.89.3.433

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 04 October 2021
doi: 10.3389/fevo.2021.734128

Differences in Floral Scent and Petal Reflectance Between Diploid and Tetraploid *Chamerion angustifolium*

*Björn Palmqvist[1], Hannah A. Brazeau[2,3] and Amy L. Parachnowitsch[1,3]**

[1] Plant Ecology and Evolution, Uppsala University, Uppsala, Sweden, [2] Department of Integrative Biology, University of Guelph, Guelph, ON, Canada, [3] Department of Biology, University of New Brunswick, Fredericton, NB, Canada

Genome duplication in plants is thought to be a route to speciation due to cytotype incompatibility. However, to reduce cross-pollination between cytotypes in animal-pollinated species, distinctive floral phenotypes, which would allow pollinator-mediated assortative mating between flowers, are also expected. *Chamerion angustifolium* is a Holarctic species that forms a hybrid zone between diploid and tetraploid populations in the North American Rocky Mountains. Extensive research has shown that these cytotypes differ in many ways, including some floral traits, and that pollinators can discriminate between cytotypes, leading to assortative mating. However, two signals commonly used by insect pollinators have not been measured for this species, namely petal colour and floral scent. Using greenhouse-grown diploids and tetraploids of *C. angustifolium* from the ploidy hybrid-zone in the North American Rocky Mountains, we show that both floral scent signals and petal reflectance differ between cytotypes. These differences, along with differences in flower size shown previously, could help explain pollinator-mediated assortative mating observed in previous studies. However, these differences in floral phenotypes may vary in importance to pollinators. While the differences in scent included common floral volatiles readily detected by bumblebees, the differences in petal reflectance may not be perceived by bees based on their visual sensitivity across the spectra. Thus, our results suggest that differences in floral volatile emissions are more likely to contribute to pollinator discrimination between cytotypes and highlight the importance of understanding the sensory systems of pollinators when examining floral signals.

Keywords: fireweed, assortative mating, floral scent, volatiles, reflectance, *Apis* visual model

OPEN ACCESS

Edited by:
Casper J. Van Der Kooi,
University of Groningen, Netherlands

Reviewed by:
Karin Gross,
University of Salzburg, Austria
João Marcelo Robazzi Bignelli
Valente Aguiar,
University of São Paulo, Brazil
Zong-Xin Ren,
Kunming Institute of Botany, Chinese Academy of Sciences (CAS), China

***Correspondence:**
Amy L. Parachnowitsch
aparachn@unb.ca

Specialty section:
This article was submitted to Chemical Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *30 June 2021*
Accepted: *13 September 2021*
Published: *04 October 2021*

Citation:
Palmqvist B, Brazeau HA and Parachnowitsch AL (2021) Differences in Floral Scent and Petal Reflectance Between Diploid and Tetraploid Chamerion angustifolium. Front. Ecol. Evol. 9:734128. doi: 10.3389/fevo.2021.734128

INTRODUCTION

Genome duplication is an important mechanism of speciation in plants (Rieseberg and Willis, 2007; Soltis et al., 2009; Barker et al., 2016) and is notable as one of the few roads to speciation that can occur in sympatry (e.g., Vallejo-Marín et al., 2016). Polyploidy can arise from the hybridisation of two progenitor species (allopolyploidy) or be the result of whole genome duplication from a single progenitor (autopolyploidy). In general allopolyploids have received relatively more attention than autopolyploids (Soltis et al., 2007; Parisod et al., 2010; Spoelhof et al., 2017), however, both types of polyploids must be reproductively isolated from their progenitors to coexist. Autopolyploids

may face even larger challenges because they are often regarded as being morphologically similar to their diploid progenitors and as such have historically been lumped as the same species (Soltis et al., 2007; Spoelhof et al., 2017). Mixed populations of autopolyploids and diploid progenitors provide an opportunity to understand the evolution of polyploidy, however, many questions remain (Kolář et al., 2017). Moreover the ecological consequences of polyploidy are relatively unexplored despite the role of ecology in evolutionary processes (Ramsey and Ramsey, 2014; Segraves, 2017).

Newly formed autopolyploids are at a reproductive disadvantage relative to their progenitors due to their rarity, i.e., minority cytotype exclusion (Levin, 1975; Husband, 2000). If polyploids do not differ significantly in floral traits from progenitors, pollinator fidelity to a particular cytotype is unlikely. The resulting random visitation from pollinators across cytotypes means that polyploids are more likely to reproduce with diploids than other polyploids, resulting in reduced polyploid fitness and minority cytotype exclusion (Husband, 2000). Therefore, coexistence and spread of polyploids is likely driven by mechanisms of reproductive isolation. For example, increased selfing could facilitate separation between ploidies (Barringer, 2007; Husband et al., 2008; Segraves and Anneberg, 2016). For outcrossing species, mechanisms such as separation in flowering time (e.g., Husband and Schemske, 2000; Jersáková et al., 2010; Castro et al., 2011; Münzbergová et al., 2015; Münzbergová and Skuhrovec, 2017; Pegoraro et al., 2019) or geography/niche differentiation (e.g., Raabová et al., 2008; Maherali et al., 2009; Muñoz-Pajares et al., 2018; Decanter et al., 2020) could contribute to isolation. Natural selection could also act on floral traits for differentiated pollinator signals (e.g., Nuismer and Cunningham, 2005). For pollinators to act as a mechanism to facilitate coexistence of cytotypes (Kolář et al., 2017; Sutherland et al., 2020; Laport et al., 2021), the flowers of the cytotypes must differ in ways that pollinators can perceive (e.g., Segraves and Thompson, 1999) or have sufficient morphological differences to facilitate isolation (e.g., Borges et al., 2012). For *Gymnadenia conopsea* (Orchidaceae), cytotypes differ in floral signals, mainly floral scent (Jersáková et al., 2010; Gross and Schiestl, 2015), and assortative mating has also been observed in some populations (Gross and Schiestl, 2015), suggesting that these mechanisms may function as reproductive isolation mechanisms in mixed populations of autotetraploids. Alternatively, post-pollination mechanisms of isolation may be more important in keeping polyploids distinctive, as in *Aster amellus* (Asteraceae) (Castro et al., 2011) and for some species little evidence of reproductive isolation is observed (e.g., Barringer and Galloway, 2017). Although pollinator-mediated reproductive isolation between cytotypes has strong theorical support and empirical evidence for some species where it has been measured (e.g., Kennedy et al., 2006; Thompson and Merg, 2008; Roccaforte et al., 2015; Sutherland et al., 2020; Laport et al., 2021), few studies have examined differences in multifaceted floral signals between cytotypes making generalisations challenging.

Chamerion angustifolium has become a model system for studying autopolyploidy largely due to the work of Brian Husband's group. Considerable advances have been made in the study of autopolyploidy using this system to measure reproductive isolation of the cytotypes (Husband and Sabara, 2004), assortative mating (Husband, 2000; Kennedy et al., 2006), fitness differences (Burton and Husband, 2000), climatic niche differences of the cytotypes (Thompson et al., 2014), and their physiology (Maherali et al., 2009). Reproductive isolation does occur between the cytotypes in wild populations (Kennedy et al., 2006) and experimental plots (Husband, 2000) and pollinator fidelity is an important isolating mechanism in the system (Husband and Sabara, 2004), suggesting that differences in floral traits may explain pollinator behaviour on these inflorescences. In natural mixed populations, tetraploids often display more and larger flowers than diploids, although greenhouse grown plants have shown the opposite pattern for flower number (Husband, 2000). However, a detailed examination of the floral phenotypes has not been undertaken for the species. In this study we examine floral signals pollinators could use to discriminate between diploid and tetraploid *C. angustifolium*. We compare flower size, spectral reflectance of petals, and scent between the two ploidies. These signalling traits can be important in driving pollinator preferences and fidelity, either individually or in an integrated fashion (Katzenberger et al., 2013; Schiestl and Johnson, 2013; Junker and Parachnowitsch, 2015). Signaling differences are likely for *C. angustifolium* cytotypes from mixed populations because pollinators do contribute to reproductive isolation in the species, however, which traits aid those decisions are largely unknown.

MATERIALS AND METHODS

Study System

Chamerion angustifolium (formerly *Epilobium angustifolium*) is a protandrous herbaceous perennial native to temperate regions throughout the northern hemisphere (Mosquin, 1966). Tetraploid and diploid populations of *C. angustifolium* occur throughout North America, with diploids occupying regions at higher latitudes than tetraploids (Thompson et al., 2014) and adaptation to elevation of both cytotypes is observed (Martin and Husband, 2013). Although a contact zone between the two cytotypes exists in the Rocky Mountains, this contact zone features a patchy mosaic with small-scale variation in cytotype, rather than a smooth transition from tetraploid at low altitudes and diploids at higher altitudes (Husband and Schemske, 1998; Sabara et al., 2013) and mixed populations have been detected elsewhere in North America (Thompson et al., 2014). The history and phylogeography of populations is largely unknown but it can be assumed that diploids and tetraploids have had some opportunity to evolve in these populations (e.g., Maherali et al., 2009). Where diploids and tetraploids occur in sympatry, triploids can be found (Husband and Schemske, 1998; Sabara et al., 2013), suggesting that mating between cytotypes occurs. Reproduction happens both clonally and by seed (Baldwin and Husband, 2013). Hymenoptera serve as the primary pollinators throughout the range (Husband and Schemske, 2000; Kennedy et al., 2006; Ollerton et al., 2007) and *Bombus* spp. are the most common effective pollinators

in North America (Galen and Plowright, 1985; Kennedy et al., 2006). While bees seem to be the more effective pollinators for *C. angustifolium,* their flowers can be visited by a diversity of additional animals such as Lepidoptera, syrphid flies, and occasionally hummingbirds.

Greenhouse Conditions

We grew diploid and tetraploid *C. angustifolium* from seed in a greenhouse at Uppsala University in 2015. Seeds were collected from populations in the Rocky Mountains in North America, and were from a bulk collection of pure diploid, pure tetraploid and mixed populations as used in Thompson et al. (2015). Cytotype for the bulk collections was determined by estimating DNA content using flow cytometry in previous work and the classification was highly accurate [60/60 confirmed diploid, 59/60 confirmed tetraploid (Thompson et al., 2015)], however, we did not directly test the individuals used in our experiment. Seeds were planted in compost pellets (one seed per pellet). Germination success was lower in tetraploids, resulting in a sample size of 30 tetraploid and 50 diploid plants. Seedlings were transplanted into a 3:1 mixture of vegetable garden soil and expanded clay in 10 cm wide pots after 28 days and placed randomly on two self-watering greenhouse tables. The automated watering system delivered additional nutrients and water for 30 min every 48 h for the first month after transplant, then increased to 2 h of watering every 24 h to accommodate increased water demands as plants grew. Due to a technical problem with the automated watering system, one greenhouse table did not receive fertiliser and this was only discovered when plants showed signs of nutrient deficiency (e.g., chlorosis). To account for differences in pre-flowering fertiliser levels we include this factor in all analyses, however, both tables received the same water and fertiliser amounts during the flower sampling period and the deficient plants ($N = 12$ tetraploids, 26 diploids) quickly recovered.

Floral Morphology

We measured petal width, petal length, and style length on three haphazardly selected flowers on each plant following the protocols of Husband and Schemske (2000). We used the lower right petal of each flower for petal measurements, and all measurements were collected from female phase flowers to ensure petals were fully expanded. When possible, we selected flowers on the main stem. We measured style length as the distance from the base of the style to the point where stigmatic lobes separated. For each of the three morphological traits, we used plant mean values in analyses.

Floral Spectral Reflectance

During peak flowering, we haphazardly selected three female phase flowers from each plant for spectral reflectance measurements. As for petal size, we used the lower right petal of each flower using a UV-VIS spectrophotometer (Ocean Optics), however, these were not necessarily the same flowers used for morphology measurements. We measured reflectance spectra for 300–700 nm, taking at least three readings for each petal to account for slight variations in reflectance measures. We binned spectra readings to have one measure per nm and used the average reflectance of all flowers and readings for each plant (due to missing data of petal reflectance for four diploid plants, $N = 30$ tetraploids and 46 diploids).

Floral Volatile Sampling and Analyses

We collected floral volatiles using dynamic headspace sampling (Raguso and Pellmyr, 1998). To collect floral volatiles, the entire inflorescence was placed inside a 14.5 × 35 cm ICA® oven bag (ICA Sverige AB, Solna, Sweden) attached to a Teflon tube scent trap filled with 10 mg Tenax GR® filter (Sigma-Aldrich, St Louis, MO, United States). Scent traps were connected via a Cole-Parmer (Vernon Hills, IL, United States) 65-mm direct-reading flow meter to a custom-built air pump (GroTech, Gothenburg, Sweden) for 5 h at a flow rate of 200 ml/min (as in Friberg et al., 2013). For each sampling period, an air control was collected from an empty oven bag to assess background scent levels in our open sampling set-up and at least one diploid and one tetraploid were haphazardly chosen for a leaf sample to help determine floral-specific volatiles. Volatiles found in roughly equal amounts in air controls and plant samples were excluded from our analyses. For volatiles present in air controls but in greater amounts in plant samples, we subtracted the air control amounts from all samples taken that day (any negative numbers were converted to zero). At sampling, the number of open flowers sampled per plant was recorded and used to calculate the emission of floral volatiles in ng/h/flower. In order to assess temporal variation in volatile emissions, 10 plants ($N = 6$ tetraploid, 4 diploid with one sampled 3 times) were haphazardly selected for repeated sampling, however, we found no significant differences between repeated samples and so used the mean across multiple samples in our analysis. Compounds were extracted from scent traps using 200 μl of hexane and concentrated to 50 μl with nitrogen gas and stored at −20°C until analysis. We added 5 μl of a 0.03% toluene (1.3 μg) solution in hexane to samples for quantification. Samples were analyzed on a Finnigan Trace GC ultra 2000 gas chromatograph connected to a Finnigan Trace DSQ mass spectrometer (both Thermo Fisher Scientific, United States) with a 30 m × 0.250 mm × 0.25 μm DB-Wax column (Agilent Technologies, United States). Helium was used as carrier gas at a constant velocity of 1 ml/min. The temperature program was as follows: 3 min hold at 50°C, followed by a 10°C/min increase for 20 min until the oven reached a maximum temperature of 250°C. Chromatograms were integrated using Xcalibur™ (version 1.4, ®Thermo Electron Corporation). Compounds were identified using NIST library searches, as well as available standards. Floral volatile emission in ng/h/flower was calculated following the equation in Svensson et al. (2005).

Statistical Analyses

All analyses were conducted in R (R Core Team, 2021).

Flower Size Analyses

We used a two-way multivariate analysis of variance (MANOVA) to compare petal length, petal width, and style length between diploids and tetraploids, while accounting for fertiliser level

during growth. To determine differences for the individual traits, we used linear models (*lm* function) to conduct ANOVAs that included ploidy level and fertiliser and their interaction, followed by the Dunn–Šidák correction for multiple comparisons. All traits met the assumptions of the tests and so no transformations were used.

Reflectance Analyses

To identify differences in spectral reflectance between diploids and tetraploids, we first used the *adonis* function in the R package "vegan" (Oksanen et al., 2019) to conduct a permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis dissimilarities with 5,000 permutations, including fertiliser condition as an interaction term. This analysis considered the entire curve between 300 and 700 nm (spectral reflectance for each wavelength was treated as a single data point) to assess whether there was an overall difference. To test whether differences in reflectance are likely detected by pollinators, we applied the visual sensitivity model based on Chittka (1992) to the spectral reflectance data. The visual sensitivity model was implemented using the *vismodel* function in "Pavo2" R package using the recommended settings for the colour hexagon based on a green background, daylight illumination and for simplicity we used the built-in "*Apis*" model for visual sensitivity (Maia et al., 2019). The honeybee visual sensitivity is often used to represent Hymenoptera vision which is evolutionarily conserved (Briscoe and Chittka, 2001) and *Apis mellifera* can also be a common visitor to *C. angustifolium*. Similar results were found using the visual sensitivities of *Bombus* species *(not shown)*, which are common *C. angustifolium* pollinators and also have very similar visual sensitivity (Skorupski and Chittka, 2010). We used the *bootcoldist* function of Pavo2 to calculate unweighted Euclidean distances between ploidies based on the hexagon to show the mean difference in colour contrast.

Scent Analyses

We first tested whether total scent emission differed between the two ploidies using a linear model with fertiliser condition included as an interaction term. We also compared scent profiles from the leaf and flower samples to determine floral specific volatiles; we considered volatiles as floral specific if they were at least twice as prevalent in the floral samples as in the leaf samples, as in Parachnowitsch et al. (2012). To assess whether variation in emission rates of specific compounds differed between the ploidies, we used a PERMANOVA on Bray–Curtis dissimilarities with 5,000 permutations. To determine which compounds contributed substantially to differences between diploid and tetraploids we used a similarity percentage (SIMPER) analysis using the *simper* function in "vegan" (Oksanen et al., 2019). To visualise these differences, we used non-metric multidimensional scaling ordination (NMDs) based on Bray–Curtis distances in "vegan."

RESULTS

Floral traits of *C. angustifolium* diploids and tetraploids differed across morphology, colour, and scent. Interestingly, petal size did not differ between ploidies. While the MANOVA detected differences between ploidies in morphology ($P < 0.001$), ANOVAs for petal length ($F_{1,76} = 1.9$, $P = 0.17$), petal width ($F_{1,76} = 1.7$, $P = 0.20$), and style length ($F_{1,76} = 33.16$, $P < 0.001$), showed that style length was responsible for these morphological differences (**Figure 1**). Style length differences remain significant after correction for multiple comparisons. Fertiliser during growth and its interaction with ploidy had no effect on morphology. We found significant differences in petal reflectance between diploids and tetraploids (**Table 1**), particularly in the 400–500 nm and 600–700 nm range (**Figure 2**). However, as the range 600–700 nm is not an important component of the Hymenoptera visual system, which could help explain why the signficant effect of ploidy on petal reflectance was lost when a bee vision model was applied (**Figure 2**). The differences detected in the 400–500 range were obviously not enough for bees to detect and this was not surprising given that the mean colour contrast in hexagon units between diploids and tetraploids was 0.002 (CI = 0.0008–0.012).

We detected 21 compounds emitted from *C. angustifolium* from a range of compound classes such as terpenes, aromatics, fatty acid derivatives, and aliphatics (**Table 2**). Most compounds were emitted in much greater amounts in the floral samples than the leaf samples, suggesting that these were floral-specific compounds. The exceptions were the fatty acid derivatives *cis*-3-hexenyl butyrate, *cis*-3-hexenyl acetate, and 3-hexen-1-ol benzoate, the monoterpenes ocimene and linalool, as well as methyl salicylate and nonadecane. No compounds were exclusively detected only in diploids or tetraploids, suggesting that these scent bouquets generally overlap in composition (**Figure 3**, inset). Moreover, total scent emission did not differ with ploidy ($F_{1,76} = 1.6$, $P = 0.21$), and nutrient deficiency during growth or its interaction with ploidy did not affect total scent emission ($Ps > 0.5$). However, despite the overlap in scent bouquet, there was significant differences between diploids and tetraploids when emission amounts of all compounds were considered, as shown with the PERMANOVA (**Table 3**), suggesting scent ratios within the floral scent bouquet differed between ploidies. This remains true if we limit our analyses to only volatiles that are found in greater abundances in the floral samples (PERMANOVA: ploidy $F_{1,76} = 6.63$, $P = 0.0002$) suggesting floral-specific bouquet differs between ploidies. However, because floral visitors experience the whole plant scent, we focus on the analyses that included all volatiles. Generally, diploids emit more 2-nonanone and 2-hexen-1-ol while tetraploids emit more phenyl acetaldehyde and cis-3-hexenyl acetate (**Figure 3**). Together these four compounds explain ∼55% of the difference in scent between diploids and tetraploids (**Supplementary Table 1**) and were the most abundant volatiles in the floral bouquet.

DISCUSSION

We found differences in floral signals between diploid and tetraploid *C. angustifolium* grown in controlled greenhouse conditions that suggest pollinators might distinguish between

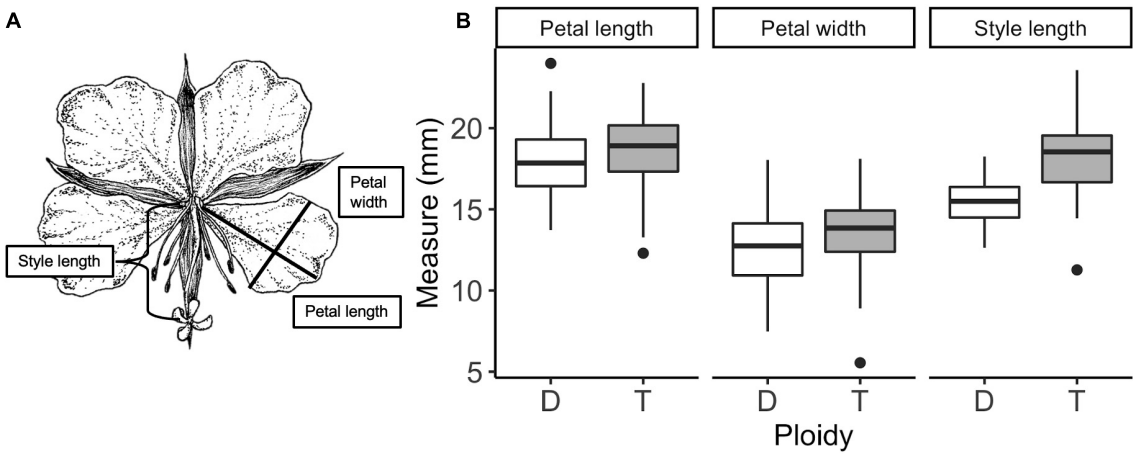

FIGURE 1 | Drawing of *Chamerion angustifolium* with the three floral measures depicted **(A)** and **(B)** the variation in flower size measures of diploid (D) and tetraploid (T) plants. Petal measurements were taken from the lower right petal of female-phase flowers. Only style length showed significant differences between diploids (white) and tetraploids (grey). Boxplots show median and the spread of the data. Illustration by Lucie Vézina.

TABLE 1 | PERMANOVA results for the spectral reflectance of *Chamerion angustifolium* flower petals before and after application of a *Apis* visual sensitivity model, testing for the effects of ploidy, fertiliser during growth, and their interaction.

	Factor	DF	Sum of squares	Mean squares	*F*	R^2	*P*
Spectral reflectance	Ploidy	1	0.022	0.022	7.15	0.087	0.003
	Fertiliser	1	0.006	0.006	2.02	0.025	0.14
	Ploidy:Fertiliser	1	0.003	0.003	1.13	0.014	0.29
	Residuals	72	0.218	0.003		0.88	
Apis model	Ploidy	1	0.00026	0.0002	0.25	0.0034	0.63
	Fertiliser	1	0.0001	0.00012	0.18	0.0024	0.68
	Ploidy:Fertiliser	1	0.0013	0.0013	2.03	0.028	0.16
	Residuals	72	0.0472	0.00075		0.97	

these cytotypes. However, not all signals showed the same pattern of variation. Flower petal size did not differ between the ploidies, but we did see a trend that flowers in tetraploids were generally bigger with longer styles (**Figure 1**). Significant flower size differences are detectable in field populations of *C. angustifolium* and are a common result of genome wide duplication (Porturas et al., 2019). While floral petal reflectance showed a significant statistical difference between ploidies, using a bee vision model suggests that these differences are unlikely to be perceived by Hymenopteran pollinators (**Figure 2**). Instead our results suggest that floral scent may be the most likely signal to differentiate between cytotypes in mixed populations (**Figure 3**).

Neither diploid nor tetraploid *C. angustifolium* emitted unique floral volatiles and the overall scent bouquets are not distinctive (**Figure 3**) suggesting that these two cytotypes strongly overlap in their scent. However, insect pollinators can respond to differences in volatile ratios to distinguish between flowers (Raguso, 2008) and we did detect differences in the specific compounds in the volatile bouquets (**Figure 3**). Both 2-nonanone and 2-hexen-1-ol were emitted in higher amounts from diploids and are common floral scents (Knudsen et al., 2006). Interestingly, 2-nonanone is also a component of the *Bombus terrestris* queen pheromone (Krieger et al., 2006) and can be produced by nectar fungi (Rering et al., 2018). Because our plants were grown in the greenhouse without access to pollinators to inoculate the nectar, it is unlikely that 2-nonanone was nectar fungi-produced. It would be interesting to see if these differences remain in wild plants where nectar microbes could more readily interact with the flowers and influence the floral scent.

Tetraploids tended to emit more phenylacetaldehyde and *cis*-3-hexenyl acetate than diploids (**Figure 3**). Phenylacetaldehyde is also a common floral volatile (Knudsen et al., 2006), detected by a range of insects such as bees and moths (Huber et al., 2005; Dötterl and Vereecken, 2010; Knauer and Schiestl, 2015) and has a significant positive effect on the number of bumble bee visits to *Brassica rapa* inflorescences (Knauer and Schiestl, 2017). Together these suggest a strong attractive role for the compound in floral scent bouquets. Conversely, *cis*-3-hexenyl acetate is more commonly associated with green leaf volatiles (GLVs) released after herbivore damage (Kessler and Baldwin, 2001; Frost et al., 2008), although that can also be true for most of the aliphatics detected here. For *C. angustifolium*, this compound was not floral specific and was found in similar amounts in leaf and floral samples. While GLVs can be associated with handling plants to sample for scent, there is no reason to think tetraploids experienced different damage levels

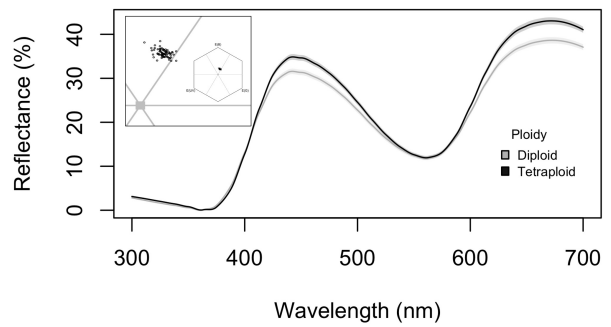

FIGURE 2 | Flower reflectance differences for diploid and tetraploid *Chamerion angustifolium* plotted from 300 to 700 nm; lines are the treatment mean and shading represents the SE. Inset: flower colour plotted using the colour hexagon for Hymenoptera colour vision to illustrate the overlap of diploid and tetraploid reflectance (empty circles = diploid, filled circles = tetraploid), corners represent blue = E(B), green = E(G), and ultraviolet = E(UV).

during our sampling. Variation in response to plant damage is detected in many plant species (Karban, 2020) and ploidy can influence herbivory (Halverson et al., 2008; Boalt et al., 2010; Segraves and Anneberg, 2016), however, differences in herbivore-induced volatiles between ploidies should be specifically tested in *C. angustifolium* to be conclusive. Furthermore, while our results suggest that the two cytotypes differ in scent emission of compounds that are common to floral bouquets and likely readily detected by the wide range of insect pollinators to *C. angustifolium*, behaviour tests are needed to determine if these signals are used in foraging decisions such as in Husband (2000) and Kennedy et al. (2006).

TABLE 2 | Floral scents detected in diploid and tetraploid *Chamerion angustifolium*.

Compound	RT	Floral/leaf	ID method
Aliphatics			
Methylhexanone	7.04	floral	RT, NIST
Cis-3-hexenyl acetate	9.15	leaf	RT, NIST
3-Hexen-1-ol	9.90	floral	RT, STD
2-Hexen-1-ol	10.10	floral	RT, NIST
2-Nonanone	10.20	floral	RT, NIST
Cis-3-hexenyl butyrate	11.25	leaf	RT, NIST
Cis-3-hexenyl isovalerate	11.38	floral	RT, NIST
2-Nonanol	11.90	floral	RT, NIST
Nonadecane	12.90	leaf	RT, NIST
3-Hexen-1-ol benzoate	18.75	leaf	RT, NIST
Aromatics			
Benzaldehyde	11.85	floral	RT, STD
Phenylacetaldehyde	13.30	floral	RT, NIST
Ethyl benzoate	13.68	floral	RT, NIST
Methyl salicylate	14.90	leaf	RT, STD
Benzyl alcohol	16.15	floral	RT, NIST
Phenylethyl alcohol	16.54	floral	RT, STD
Monoterpenes			
Ocimene	8.05	leaf	RT, STD
Linalool	12.30	leaf	RT, STD
Sesquiterpenes			
Caryophyllene	14.44	floral	RT, STD
Farnesene	14.72	floral	RT, STD
Cadinene	14.80	floral	RT, NIST

Compounds are grouped based on compound class. Floral volatiles were higher in floral samples compared to leaf (see text) and compounds were identified using retention time (RT), comparisons with the NIST library, and where possible, known synthetic standards (STD).

TABLE 3 | PERMANOVA results for the 21 floral volatiles emitted by *Chamerion angustifolium* individuals, testing for the effects of ploidy, fertiliser during growth, and their interaction.

Factor	DF	Sum of Squares	Mean Squares	*F*	R^2	*P*
Ploidy	1	1.50	1.50	6.65	0.079	0.0002
Fertiliser	1	0.18	0.18	0.80	0.0095	0.60
Ploidy:Fertiliser	1	0.28	0.28	1.25	0.015	0.23
Residuals	76	17.18	0.22		0.90	

Flower size differences between cytotypes have been found for *C. angustifolium* in the field (Kennedy et al., 2006), and although we saw a trend for tetraploids to have larger flowers, we did not detect the same statistical differences as previous work. As pollinators can learn to distinguish between flowers based on size, although possibly not as easily as colour and floral scent signals (e.g., Blarer et al., 2002), further behavioural studies examining foraging behaviour could prove illuminating. Interestingly, the reflectance differences we observed are unlikely to be perceived by bee pollinators to *C. angustifolium* (**Figure 2**). While Hymenoptera do show sensitivity between the 400 and 500 nm wavelengths where tetraploids have higher reflectance than diploids (**Figure 2**), these differences were presumably not pronounced enough to detect and the mean difference in hexagon units between ploidy is likely below the discrimination level for bees (Dyer and Chittka, 2004; Aguiar et al., 2020). Lepidoptera can also visit these flowers and their vision incorporates the 600–700 nm range (van der Kooi et al., 2021), so perhaps they could better distinguish between these cytotypes. However, Lepidoptera visits to *C. angustifolium* are unlikely to drive reproductive isolation between cytotypes due to their relatively infrequent visits (Lack, 1982) and behaviour on the flowers (personal observation, ALP). Similarly, Diptera can be common visitors to *C. angustifolium* but based on pollen removal and deposition rates compared to bees, Diptera are poor pollinators of this species (Ollerton et al., 2007). Interestingly, the difference in amplitude in reflectance suggests that the reason these reflectances differ is due to tissues or cells changing the backscattering structure rather than pigment amount or placement (e.g., Figure 3 in van der Kooi et al., 2019). Larger cell size and nuclear volume are common with polyploidy and might help explain the reflectance patterns. An unanswered question for the system is whether any *C. angustifolium* visitors perceive the reflectance variation from the petals or if it matters. Additionally, it is possible pollinators may respond to visual signals that were not represented by our point measure of

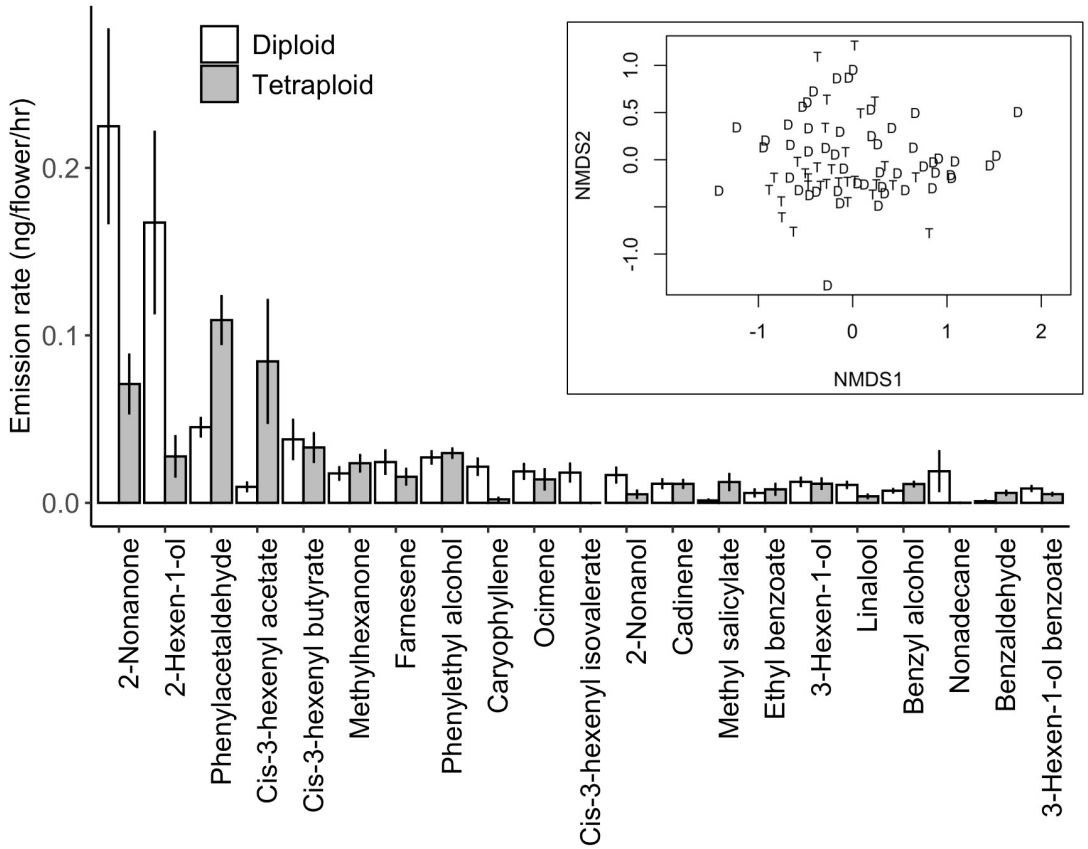

FIGURE 3 | Floral volatile compound mean emission rate (+/− SE) from diploid and tetraploid *Chamerion angustifolium*. Compounds are ordered by their contributions to differences between diploids (white bars) and tetraploids (grey bars) based on SIMPER (**Supplementary Table 1**). Inset: Overlap of floral scent bouquets for diploids (D) and tetraploids (T) based on all compounds emitted.

spectral reflectance, such as ultraviolet patterns (Koski and Ashman, 2014; Trunschke et al., 2021). While we only measured a single petal per flower, to our knowledge there are no UV patterns on *C. angustifolium* petals (Kaz Ohashi, personal communication) and the low reflectance in the 300–400 nm range also supports this interpretation. Regardless of whether the reflectance differences drive behavioral differences on the flowers, it could be interesting to examine the effects of genome duplication on floral pigments in autotetraploids and whether these are similar to somatic ploidy seen in the petals of some species (Schepper et al., 2001).

The two *C. angustifolium* cytotypes have different physiological responses (e.g., to water stress, Maherali et al., 2009), and so tend to occupy different microclimates (Martin and Husband, 2013; Thompson et al., 2014). These microclimatic differences could also drive phenotypic variation in wild plants, for example scent emission can vary with temperature (Farré-Armengol et al., 2014). Thus, wild plants may show greater, or at least different, variation than the greenhouse grown ones we measured here. However, the controlled greenhouse environment does mean the differences we detected are more likely to have a genetic basis than are due to phenotypic plasticity. Our bulked seeds mean that our study represents general differences with ploidy in the Rocky Mountains. Evolutionary history, especially in mixed populations where reproductive isolation likely plays a larger role, could mean wild populations show different patterns of variation. Furthermore, plants in this experiment were not directly tested for ploidy, so it is possible that we have misclassified some individuals or included triploids. Although given the accuracy for other individuals in the bulk seed collections (Thompson et al., 2015), it seems unlikely that this would play a large role in the results we found.

Our experiment unintentionally included differences in nutrient availability during development that could have impacted the floral traits and differences between cytotypes. However, generally this 'treatment' was not significant in our comparisons, perhaps because it was corrected before flowering began. Nutrients can of course have strong effects on plant growth (e.g., in *C. angustifolium* Bennett et al., 2004; Pinno et al., 2013), and these effects can impact diploid and tetraploid *C. angustifolium* differently (Bales and Hersch-Green, 2019). Nutrients can also impact floral traits. These effects are often seen as increases in display size (e.g., Friberg et al., 2017) directly related to an increase of overall biomass in high nutrient environments. Nutrients can also affect floral scent for some species (Majetic et al., 2017), but scent may be less affected

than other floral traits (Friberg et al., 2017; Luizzi et al., 2021). Timing of nutrient deficits could be important, especially for physiologically plastic traits such as scent emission, and may explain the lack of differences with fertiliser seen here. Although our experiment cannot be seen as a definitive test of fertiliser effects on floral traits in *C. angustifolium*, it does suggest that these weedy plants can recover quickly from nutrient deficits.

Coexistence of polyploids with their diploid progenitors requires reproductive isolation to maintain separation of the cytotypes. Pollinator fidelity is estimated to play a significant role in reproductive isolation of *C. angustifolium* (Husband and Sabara, 2004) and therefore traits that allow pollinators to distinguish between cytotypes are expected. Flower size and display size may play a role in these decisions in wild populations (Kennedy et al., 2006), however, our results suggest that floral scent may also be an important trait to distinguish between cytotypes. Floral trait differences may be a direct result of genome duplication but could also be the result of subsequent evolution in the cytotypes. For our study, we cannot distinguish these effects but other work with neo-tetraploid *C. angustifolium*, shows that differences in response to water stress between the cytotypes is likely due to evolution rather than the duplication event (Maherali et al., 2009). Presumably, the pollinators are also motivated by more than just subtle differences in floral signals and an important next step would be to compare rewards such as nectar and pollen in *C. angustifolium*. The connection with rewards and signals is generally understudied in pollination biology, especially in an evolutionary ecology context (Parachnowitsch et al., 2019), therefore evaluating reward differences between cytotypes could have broad value to understanding assortative mating. Our results are similar to those in the orchid *G. conopsea* where floral scent, but not petal reflectance, differs between cytotypes (Gross and Schiestl, 2015). Mixed autoploidy systems provide opportunities for furthering our understanding of ploidy (Kolář et al., 2017), however, many more of these systems will need to be measured for floral traits to know whether the pattern of floral scent differences between cytotypes in *G. conopsea* and *C. angustifolium* is common or rare. These two examples also incorporate evolutionary history after genome-wide duplication and testing synthesised neo-polyploids would help determine whether the difference observed in these species are likely the result of the duplication or natural selection to reinforce reproductive isolation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

BP and AP conceived and designed the study. BP collected all data and wrote the first draft of the manuscript for his masters thesis. BP, HB, and AP performed the statistical analysis. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

The Parachnowitsch lab is supported by a Natural Sciences and Engineering Research Council of Canada Discovery Grant (2018-04148) as well as funding from the Swedish Research Council (VR 2016-04508).

ACKNOWLEDGMENTS

We thank Brian Husband for the seed that allowed this study to happen, Paul Kron for information on the seed source, Kaz Ohashi for sharing observations on the UV patterns (or rather lack of) of the flowers, and Robert Junker for sharing R code and advice for floral reflectance analyses. CV and three reviewers provided helpful feedback that improved the manuscript. We acknowledge that the universities in which we work and the land from which these seeds were harvested are the traditional lands of First Nations People.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.734128/full#supplementary-material

REFERENCES

Aguiar, J. M. R. B. V., Giurfa, M., and Sazima, M. (2020). A cognitive analysis of deceptive pollination: associative mechanisms underlying pollinators' choices in non-rewarding colour polymorphic scenarios. *Sci. Rep.* 10:9476. doi: 10.1038/s41598-020-66356-4

Baldwin, S. J., and Husband, B. C. (2013). The association between polyploidy and clonal reproduction in diploid and tetraploid *Chamerion angustifolium*. *Mol. Ecol.* 22, 1806–1819. doi: 10.1111/mec.12217

Bales, A. L., and Hersch-Green, E. I. (2019). Effects of soil nitrogen on diploid advantage in fireweed, *Chamerion angustifolium* (Onagraceae). *Ecol. Evol* 9, 1095–1109. doi: 10.1002/ece3.4797

Barker, M. S., Husband, B. C., and Pires, J. C. (2016). Spreading Winge and flying high: the evolutionary importance of polyploidy after a century of study. *Am. J. Bot.* 103, 1139–1145. doi: 10.3732/ajb.1600272

Barringer, B. C. (2007). Polyploidy and self-fertilization in flowering plants. *Am. J. Bot.* 94, 1527–1533. doi: 10.3732/ajb.94.9.1527

Barringer, B. C., and Galloway, L. F. (2017). The reproductive ecology of diploid and tetraploid *Galax urceolata*. *Am. Midl. Nat.* 177, 299–308. doi: 10.1674/0003-0031-177.2.299

Bennett, J. N., Lapthorne, B. M., Blevins, L. L., and Prescott, C. E. (2004). Response of *Gaultheria shallon* and *Epilobium angustifolium* to large additions of nitrogen and phosphorus fertilizer. *Can. J. For. Res.* 34, 502–506. doi: 10.1139/x03-219

Blarer, A., Keasar, T., and Shmida, A. (2002). Possible mechanisms for the formation of flower size preferences by foraging bumblebees. *Ethology* 108, 341–351.

Boalt, E., Arvanitis, L., Lehtilä, K., and Ehrlén, J. (2010). The association among herbivory tolerance, ploidy level, and herbivory pressure in *Cardamine pratensis*. *Evol. Ecol.* 24, 1101–1113. doi: 10.1007/s10682-010-9364-7

Borges, L. A., Souza, L. G. R., Guerra, M., Machado, I. C., Lewis, G. P., and Lopes, A. V. (2012). Reproductive isolation between diploid and tetraploid cytotypes of Libidibiaferrea (= *Caesalpinia ferrea*) (Leguminosae): ecological and taxonomic implications. *Plant Syst. Evol.* 298, 1371–1381. doi: 10.1007/s00606-012-0643-3

Briscoe, A. D., and Chittka, L. (2001). The evolution of color vision in insects. *Annu. Rev. Entomol.* 46, 471–510. doi: 10.1146/annurev.ento.46.1.471

Burton, T. L., and Husband, B. C. (2000). Fitness differences among diploids, tetraploids, and their triploid progeny in *Chamerion angustifolium*: mechanisms of inviability and implications for polyploid evolution. *Evolution* 54, 1182–1191. doi: 10.1111/j.0014-3820.2000.tb00553.x

Castro, S., Münzbergová, Z., Raabová, J., and Loureiro, J. (2011). Breeding barriers at a diploid–hexaploid contact zone in *Aster amellus*. *Evol. Ecol.* 25, 795–814. doi: 10.1007/s10682-010-9439-5

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543. doi: 10.1007/BF00199331

Decanter, L., Colling, G., Elvinger, N., Heiðmarsson, S., and Matthies, D. (2020). Ecological niche differences between two polyploid cytotypes of *Saxifraga rosacea*. *Am. J. Bot.* 107, 423–435. doi: 10.1002/ajb2.1431

Dötterl, S., and Vereecken, N. J. (2010). The chemical ecology and evolution of bee–flower interactions: a review and perspectives. *Can. J. Zool.* 88, 668–697. doi: 10.1139/Z10-031

Dyer, A. G., and Chittka, L. (2004). Biological significance of distinguishing between similar colours in spectrally variable illumination: bumblebees (*Bombus terrestris*) as a case study. *J. Comp. Physiol. A* 190, 105–114. doi: 10.1007/s00359-003-0475-2

Farré-Armengol, G., Filella, I., Llusià, J., Niinemets, Ü, and Peñuelas, J. (2014). Changes in floral bouquets from compound-specific responses to increasing temperatures. *Glob. Change Biol.* 20, 3660–3669. doi: 10.1111/gcb.12628

Friberg, M., Schwind, C., Raguso, R. A., and Thompson, J. N. (2013). Extreme divergence in floral scent among woodland star species (*Lithophragma* spp.) pollinated by floral parasites. *Ann. Bot.* 111, 539–550. doi: 10.1093/aob/mct007

Friberg, M., Waters, M. T., and Thompson, J. N. (2017). Nutrient availability affects floral scent much less than other floral and vegetative traits in *Lithophragma bolanderi*. *Ann. Bot.* 120, 471–478. doi: 10.1093/aob/mcx069

Frost, C. J., Mescher, M. C., Dervinis, C., Davis, J. M., Carlson, J. E., and Moraes, C. M. D. (2008). Priming defense genes and metabolites in hybrid poplar by the green leaf volatile cis-3-hexenyl acetate. *New Phytol.* 180, 722–734. doi: 10.1111/j.1469-8137.2008.02599.x

Galen, C., and Plowright, R. C. (1985). The effects of nectar level and flower development on pollen carry-over in inflorescences of fireweed (*Epilobium angustifolium*) (Onagraceae). *Can. J. Bot.* 63, 488–491.

Gross, K., and Schiestl, F. P. (2015). Are tetraploids more successful? Floral signals, reproductive success and floral isolation in mixed-ploidy populations of a terrestrial orchid. *Ann. Bot.* 115, 263–273. doi: 10.1093/aob/mcu244

Halverson, K., Heard, S. B., Nason, J. D., and Stireman, J. O. (2008). Differential attack on diploid, tetraploid, and hexaploid *Solidago altissima* L. by five insect gallmakers. *Oecologia* 154, 755–761. doi: 10.1007/s00442-007-0863-3

Huber, F. K., Kaiser, R., Sauter, W., and Schiestl, F. P. (2005). Floral scent emission and pollinator attraction in two species of *Gymnadenia* (Orchidaceae). *Oecologia* 142, 564–575. doi: 10.1007/s00442-004-1750-9

Husband, B. C. (2000). Constraints on polyploid evolution: a test of the minority cytotype exclusion principle. *Proc. R. Soc. Lond. B Biol. Sci.* 267, 217–223. doi: 10.1098/rspb.2000.0990

Husband, B. C., and Sabara, H. A. (2004). Reproductive isolation between autotetraploids and their diploid progenitors in fireweed, *Chamerion angustifolium* (Onagraceae). *New Phytol.* 161, 703–713. doi: 10.1046/j.1469-8137.2004.00998.x

Husband, B. C., and Schemske, D. W. (1998). Cytotype distribution at a diploid–tetraploid contact zone in *Chamerion* (Epilobium) *angustifolium* (Onagraceae). *Am. J. Bot.* 85, 1688–1694.

Husband, B. C., and Schemske, D. W. (2000). Ecological mechanisms of reproductive isolation between diploid and tetraploid *Chamerion angustifolium*. *J. Ecol.* 88, 689–701. doi: 10.1046/j.1365-2745.2000.00481.x

Husband, B. C., Ozimec, B., Martin, S. L., and Pollock, L. (2008). Mating consequences of polyploid evolution in flowering plants: current trends and insights from synthetic polyploids. *Int. J. Plant Sci.* 169, 195–206. doi: 10.1086/523367

Jersáková, J., Castro, S., Sonk, N., Milchreit, K., Schödelbauerová, I., Tolasch, T., et al. (2010). Absence of pollinator-mediated premating barriers in mixed-ploidy populations of *Gymnadenia conopsea* s.l. (Orchidaceae). *Evol. Ecol.* 24, 1199–1218. doi: 10.1007/s10682-010-9356-7

Junker, R. R., and Parachnowitsch, A. L. (2015). Working towards a holistic view on flower traits – How floral scents mediate plant–animal interactions in concert with other floral characters. *J. Indian Inst. Sci.* 95, 43–68.

Karban, R. (2020). The ecology and evolution of induced responses to herbivory and how plants perceive risk. *Ecol. Entomol.* 45, 1–9. doi: 10.1111/een.12771

Katzenberger, T., Lunau, K., and Junker, R. (2013). Salience of multimodal flower cues manipulates initial responses and facilitates learning performance of bumblebees. *Behav. Ecol. Sociobiol.* 67, 1587–1599. doi: 10.1007/s00265-013-1570-1

Kennedy, B. F., Sabara, H. A., Haydon, D., and Husband, B. C. (2006). Pollinator-mediated assortative mating in mixed ploidy populations of *Chamerion angustifolium* (Onagraceae). *Oecologia* 150, 398–408. doi: 10.1007/s00442-006-0536-7

Kessler, A., and Baldwin, I. T. (2001). Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291, 2141–2144.

Knauer, A. C., and Schiestl, F. P. (2015). Bees use honest floral signals as indicators of reward when visiting flowers. *Ecol. Lett.* 18, 135–143. doi: 10.1111/ele.12386

Knauer, A. C., and Schiestl, F. P. (2017). The effect of pollinators and herbivores on selection for floral signals: a case study in *Brassica rapa*. *Evol. Ecol.* 31, 285–304. doi: 10.1007/s10682-016-9878-8

Knudsen, J. T., Eriksson, R., Gershenzon, J., and Ståhl, B. (2006). Diversity and distribution of floral scent. *Bot. Rev.* 72, 1–120.

Kolář, F., Čertner, M., Suda, J., Schönswetter, P., and Husband, B. C. (2017). Mixed-ploidy species: progress and opportunities in polyploid research. *Trends Plant Sci.* 22, 1041–1055. doi: 10.1016/j.tplants.2017.09.011

Koski, M. H., and Ashman, T.-L. (2014). Dissecting pollinator responses to a ubiquitous ultraviolet floral pattern in the wild. *Funct. Ecol.* 28, 868–877. doi: 10.1111/1365-2435.12242

Krieger, G. M., Duchateau, M.-J., Van Doorn, A., Ibarra, F., Francke, W., and Ayasse, M. (2006). Identification of queen sex pheromone components of the bumblebee *Bombus terrestris*. *J. Chem. Ecol.* 32:453. doi: 10.1007/s10886-005-9013-8

Lack, A. J. (1982). The ecology of flowers of chalk grassland and their insect pollinators. *J. Ecol.* 70, 773–790. doi: 10.2307/2260104

Laport, R. G., Minckley, R. L., and Pilson, D. (2021). Pollinator assemblage and pollen load differences on sympatric diploid and tetraploid cytotypes of the desert-dominant *Larrea tridentata*. *Am. J. Bot.* 108, 297–308. doi: 10.1002/ajb2.1605

Levin, D. A. (1975). Minority cytotype exclusion in local plant populations. *TAXON* 24, 35–43. doi: 10.2307/1218997

Luizzi, V. J., Friberg, M., and Petrén, H. (2021). Phenotypic plasticity in floral scent in response to nutrient, but not water, availability in the perennial plant *Arabis alpina* (Brassicaceae). *Funct. Ecol.* 35, 1655–1665. doi: 10.1111/1365-2435.13866

Maherali, H., Walden, A. E., and Husband, B. C. (2009). Genome duplication and the evolution of physiological responses to water stress. *New Phytol.* 184, 721–731. doi: 10.1111/j.1469-8137.2009.02997.x

Maia, R., Gruson, H., Endler, J. A., and White, T. E. (2019). pavo 2: New tools for the spectral and spatial analysis of colour in R. *Methods Ecol. Evol.* 10, 1097–1107. doi: 10.1111/2041-210X.13174

Majetic, C. J., Fetters, A. M., Beck, O. M., Stachnik, E. F., and Beam, K. M. (2017). Petunia floral trait plasticity in response to soil nitrogen content and subsequent impacts on insect visitation. *Flora* 232, 183–193. doi: 10.1016/j.flora.2016.08.002

Martin, S. L., and Husband, B. C. (2013). Adaptation of diploid and tetraploid *Chamerion angustifolium* to elevation but not local environment. *Evolution* 67, 1780–1791. doi: 10.1111/evo.12065

Mosquin, T. (1966). A new taxonomy for *Epilobium angustifolium* L. (Onagraceae). *Brittonia* 18, 167–188. doi: 10.2307/2805200

Muñoz-Pajares, A. J., Perfectti, F., Loureiro, J., Abdelaziz, M., Biella, P., Castro, M., et al. (2018). Niche differences may explain the geographic distribution of

cytotypes in *Erysimum mediohispanicum*. *Plant Biol.* 20, 139–147. doi: 10.1111/plb.12605

Münzbergová, Z., and Skuhrovec, J. (2017). Contrasting effects of ploidy level on seed production in a diploid–tetraploid system. *AoB Plants* 9:lw077. doi: 10.1093/aobpla/plw077

Münzbergová, Z., Skuhrovec, J., and Maršík, P. (2015). Large differences in the composition of herbivore communities and seed damage in diploid and autotetraploid plant species. *Biol. J. Linn. Soc.* 115, 270–287. doi: 10.1111/bij.12482

Nuismer, S. L., and Cunningham, B. M. (2005). Selection for phenotypic divergence between diploid and autotetraploid Heuchera grossulariifolia. *Evolution* 59, 1928–1935. doi: 10.1111/j.0014-3820.2005.tb01062.x

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2019). *vegan: Community Ecology Package. R package version 2.5–6*. Available online at: https://CRAN.R-project.org/package=vegan

Ollerton, J., Killick, A., Lamborn, E., Watts, S., and Whiston, M. (2007). Multiple meanings and modes: on the many ways to be a generalist flower. *TAXON* 56, 717–728. doi: 10.2307/25065855

Parachnowitsch, A. L., Manson, J. S., and Sletvold, N. (2019). Evolutionary ecology of nectar. *Ann. Bot.* 123, 247–261.

Parachnowitsch, A. L., Raguso, R. A., and Kessler, A. (2012). Phenotypic selection to increase floral scent emission, but not flower size or colour in bee-pollinated *Penstemon digitalis*. *New Phytol.* 195, 667–675. doi: 10.1111/j.1469-8137.2012.04188.x

Parisod, C., Holderegger, R., and Brochmann, C. (2010). Evolutionary consequences of autopolyploidy: research review. *New Phytol.* 186, 5–17. doi: 10.1111/j.1469-8137.2009.03142.x

Pegoraro, L., De Vos, J. M., Cozzolino, S., and Scopece, G. (2019). Shift in flowering time allows diploid and autotetraploid *Anacamptis pyramidalis* (*Orchidaceae*) to coexist by reducing competition for pollinators. *Bot. J. Linn. Soc.* 191, 274–284. doi: 10.1093/botlinnean/boz043

Pinno, B. D., Landhäusser, S. M., Chow, P. S., Quideau, S. A., and MacKenzie, M. D. (2013). Nutrient uptake and growth of fireweed (*Chamerion angustifolium*) on reclamation soils. *Can. J. For. Res.* 44, 1–7. doi: 10.1139/cjfr-2013-0091

Porturas, L. D., Anneberg, T. J., Curé, A. E., Wang, S., Althoff, D. M., and Segraves, K. A. (2019). A meta-analysis of whole genome duplication and the effects on flowering traits in plants. *Am. J. Bot.* 106, 469–476. doi: 10.1002/ajb2.1258

R Core Team (2021). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at: https://www.R-project.org/.

Raabová, J., Fischer, M., and Münzbergová, Z. (2008). Niche differentiation between diploid and hexaploid *Aster amellus*. *Oecologia* 158, 463–472. doi: 10.1007/s00442-008-1156-1

Raguso, R. A. (2008). Wake up and smell the roses: the ecology and evolution of floral scent. *Annu. Rev. Ecol. Evol. Syst.* 39, 549–569. doi: 10.1146/annurev.ecolsys.38.091206.095601

Raguso, R. A., and Pellmyr, O. (1998). Dynamic headspace analysis of floral volatiles: a comparison of methods. *Oikos* 81:238. doi: 10.2307/3547045

Ramsey, J., and Ramsey, T. S. (2014). Ecological studies of polyploidy in the 100 years following its discovery. *Philos. Trans. R. Soc. B Biol. Sci.* 369:20130352. doi: 10.1098/rstb.2013.0352

Rering, C. C., Beck, J. J., Vannette, R. L., and Willms, S. D. (2018). Quantitative assessment of nectar microbe-produced volatiles. *ACS Symp. Ser.* 1294, 127–142. doi: 10.1021/bk-2018-1294.ch010

Rieseberg, L. H., and Willis, J. H. (2007). Plant speciation. *Science* 317, 910–914. doi: 10.1126/science.1137729

Roccaforte, K., Russo, S. E., and Pilson, D. (2015). Hybridization and reproductive isolation between diploid *Erythronium mesochoreum* and its tetraploid congener *E. albidum* (Liliaceae). *Evolution* 69, 1375–1389. doi: 10.1111/evo.12666

Sabara, H. A., Kron, P., and Husband, B. C. (2013). Cytotype coexistence leads to triploid hybrid production in a diploid–tetraploid contact zone of *Chamerion angustifolium* (Onagraceae). *Am. J. Bot.* 100, 962–970. doi: 10.3732/ajb.1200583

Schepper, S., Leus, L., Mertens, M., Debergh, P., Van Bockstaele, E., and Loose, M. (2001). Somatic polyploidy and its consequences for flower coloration and flower morphology in azalea. *Plant Cell Rep.* 20, 583–590. doi: 10.1007/s002990100372

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Segraves, K. A. (2017). The effects of genome duplications in a community context. *New Phytol.* 215, 57–69. doi: 10.1111/nph.14564

Segraves, K. A., and Anneberg, T. J. (2016). Species interactions and plant polyploidy. *Am. J. Bot.* 103, 1326–1335. doi: 10.3732/ajb.1500529

Segraves, K. A., and Thompson, J. N. (1999). Plant polyploidy and pollination: floral traits and insect visits to diploid and tetraploid *Heuchera grossulariifolia*. *Evolution* 53, 1114–1127. doi: 10.1111/j.1558-5646.1999.tb04526.x

Skorupski, P., and Chittka, L. (2010). Photoreceptor spectral sensitivity in the Bumblebee, Bombus impatiens (Hymenoptera: Apidae). *PLoS One* 5:e12049. doi: 10.1371/journal.pone.0012049

Soltis, D. E., Albert, V. A., Leebens-Mack, J., Bell, C. D., Paterson, A. H., Zheng, C., et al. (2009). Polyploidy and angiosperm diversification. *Am. J. Bot.* 96, 336–348. doi: 10.3732/ajb.0800079

Soltis, D. E., Soltis, P. S., Schemske, D. W., Hancock, J. F., Thompson, J. N., Husband, B. C., et al. (2007). Autopolyploidy in angiosperms: have we grossly underestimated the number of species? *Taxon* 56, 13–30. doi: 10.2307/25065732

Spoelhof, J. P., Soltis, P. S., and Soltis, D. E. (2017). Pure polyploidy: closing the gaps in autopolyploid research. *J. Syst. Evol.* 55, 340–352. doi: 10.1111/jse.12253

Sutherland, B. L., Miranda-Katz, T., and Galloway, L. F. (2020). Strength in numbers? Cytotype frequency mediates effect of reproductive barriers in mixed-ploidy arrays. *Evolution* 74, 2281–2292. doi: 10.1111/evo.14077

Svensson, G. P., Hickman, M. O. Jr., Bartram, S., Boland, W., Pellmyr, O., and Raguso, R. A. (2005). Chemistry and geographic variation of floral scent in *Yucca filamentosa* (Agavaceae). *Am. J. Bot.* 92, 1624–1631.

Thompson, J. N., and Merg, K. F. (2008). Evolution of polyploidy and the diversification of plant-pollinator interactions. *Ecology* 89, 2197–2206.

Thompson, K. A., Husband, B. C., and Maherali, H. (2014). Climatic niche differences between diploid and tetraploid cytotypes of *Chamerion angustifolium* (Onagraceae). *Am. J. Bot.* 101, 1868–1875. doi: 10.3732/ajb.1400184

Thompson, K. A., Husband, B. C., and Maherali, H. (2015). No influence of water limitation on the outcome of competition between diploid and tetraploid *Chamerion angustifolium* (Onagraceae). *J. Ecol.* 103, 733–741. doi: 10.1111/1365-2745.12384

Trunschke, J., Lunau, K., Pyke, G. H., Ren, Z.-X., and Wang, H. (2021). Flower color evolution and the evidence of pollinator-mediated selection. *Front. Plant Sci.* 12:617851. doi: 10.3389/fpls.2021.617851

Vallejo-Marín, M., Cooley, A. M., Lee, M. Y., Folmer, M., McKain, M. R., and Puzey, J. R. (2016). Strongly asymmetric hybridization barriers shape the origin of a new polyploid species and its hybrid ancestor. *Am. J. Bot.* 103, 1272–1288. doi: 10.3732/ajb.1500471

van der Kooi, C. J., Dyer, A. G., Kevan, P. G., and Lunau, K. (2019). Functional significance of the optical properties of flowers for visual signalling. *Ann. Bot.* 123, 263–276. doi: 10.1093/aob/mcy119

van der Kooi, C. J., Stavenga, D. G., Arikawa, K., Belušič, G., and Kelber, A. (2021). Evolution of insect color vision: from spectral sensitivity to visual ecology. *Annu. Rev. Entomol.* 66, 435–461. doi: 10.1146/annurev-ento-061720-071644

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 05 October 2021
doi: 10.3389/fevo.2021.743850

Major Flower Pigments Originate Different Colour Signals to Pollinators

Eduardo Narbona[1]†, José Carlos del Valle[2]†, Montserrat Arista[2], María Luisa Buide[1] and Pedro Luis Ortiz[2]*

[1] Departamento de Biología Molecular e Ingeniería Bioquímica, Universidad Pablo de Olavide, Seville, Spain, [2] Departamento de Biología Vegetal y Ecología, Facultad de Biología, Universidad de Sevilla, Seville, Spain

Flower colour is mainly due to the presence and type of pigments. Pollinator preferences impose selection on flower colour that ultimately acts on flower pigments. Knowing how pollinators perceive flowers with different pigments becomes crucial for a comprehensive understanding of plant-pollinator communication and flower colour evolution. Based on colour space models, we studied whether main groups of pollinators, specifically hymenopterans, dipterans, lepidopterans and birds, differentially perceive flower colours generated by major pigment groups. We obtain reflectance data and conspicuousness to pollinators of flowers containing one of the pigment groups more frequent in flowers: chlorophylls, carotenoids and flavonoids. Flavonoids were subsequently classified in UV-absorbing flavonoids, aurones-chalcones and the anthocyanins cyanidin, pelargonidin, delphinidin, and malvidin derivatives. We found that flower colour loci of chlorophylls, carotenoids, UV-absorbing flavonoids, aurones-chalcones, and anthocyanins occupied different regions of the colour space models of these pollinators. The four groups of anthocyanins produced a unique cluster of colour loci. Interestingly, differences in colour conspicuousness among the pigment groups were almost similar in the bee, fly, butterfly, and bird visual space models. Aurones-chalcones showed the highest chromatic contrast values, carotenoids displayed intermediate values, and chlorophylls, UV-absorbing flavonoids and anthocyanins presented the lowest values. In the visual model of bees, flowers with UV-absorbing flavonoids (i.e., white flowers) generated the highest achromatic contrasts. Ours findings suggest that in spite of the almost omnipresence of floral anthocyanins in angiosperms, carotenoids and aurones-chalcones generates higher colour conspicuousness for main functional groups of pollinators.

Keywords: anthocyanins, carotenoids, chlorophylls, colour space models, flavonoids, flower colour, flower pigments, pollinators

OPEN ACCESS

Edited by:
Casper J. Van Der Kooi, Groningen Institute for Evolutionary Life Sciences, University of Groningen, Netherlands

Reviewed by:
Doekele G. Stavenga, University of Groningen, Netherlands
Adrian G. Dyer, RMIT University, Australia
Matthew Koski, Clemson University, United States

***Correspondence:**
Eduardo Narbona
enarfer@upo.es

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Coevolution, a section of the journal Frontiers in Ecology and Evolution

Received: *19 July 2021*
Accepted: *15 September 2021*
Published: *05 October 2021*

Citation:
Narbona E, del Valle JC, Arista M, Buide ML and Ortiz PL (2021) Major Flower Pigments Originate Different Colour Signals to Pollinators. Front. Ecol. Evol. 9:743850. doi: 10.3389/fevo.2021.743850

INTRODUCTION

The colours of flowers, usually those of petals, mainly act as a signal to attract pollinators by making flowers highly conspicuous against the vegetative background. Thus, flower colour largely affects floral advertising to pollinators, which has subsequent implications in plant reproduction (Fenster et al., 2004; Phillips et al., 2020). Although abiotic agents of selection may be involved

in driving flower colour evolution, this trait is mostly influenced by selection pressures exerted by biotic agents, specifically antagonistic florivores and most importantly mutualistic pollinators (Strauss and Whittall, 2006; Dalrymple et al., 2020; Sullivan and Koski, 2021). Hence, a deeper insight into how flowers produce their colours and how animals perceive them becomes crucial for a comprehensive understanding of both plant-pollinator interactions and flower colour evolution.

Flower colour is mainly produced through pigmentation, in which chemical compounds, i.e., pigments, can absorb certain wavelengths of light and reflect the remaining (van der Kooi et al., 2019). The vast range of flower colours relies on four major pigment classes: chlorophylls, carotenoids, flavonoids, and betalains (Lee, 2007; Narbona et al., 2021). Each pigment class has a distinctive chemical structure, which ultimately affects the specific wavelengths it absorbs and thereby the colour it generates (Grotewold, 2006; Glover, 2007; Tanaka et al., 2008). Chlorophylls absorb in the blue and red regions of the spectrum, generating green colourations for humans; carotenoids mainly absorb in the blue region, giving rise to yellow-orange colourations; and betalains absorb in either the blue or green regions, generating yellow or pink colourations, respectively (Grotewold, 2006; Narbona et al., 2021). Flavonoids are the most widespread and diverse class of pigments in angiosperms (Iwashina, 2015). Flavonoids include important groups such as aurones and chalcones absorbing in the blue region (hereafter, aurones-chalcones; yellow colouration), and flavonols, flavones and flavanones absorbing in the ultraviolet spectrum (hereafter, UV-absorbing flavonoids; white and pale-yellow colourations) (Harborne, 1984; Tanaka et al., 2008; Narbona et al., 2021). Yet, anthocyanins are the flavonoids that generate the most varied colouration to flowers; they absorb in different parts of the green region of the spectrum and produce shades of blue-pink-orange-red floral colours (Grotewold, 2006). Anthocyanins are classified depending on the hydroxylation level of the basic structures, the anthocyanidins, being the six more common in angiosperms (in this order): cyanidin (pink colourations), delphinidin (blue), pelargonidin (orange-red), peonidin (red), petunidin (purple), and malvidin (blue) (Castañeda-Ovando et al., 2009). Furthermore, each of the major classes of pigments and subcategories contains hundreds or thousands of different compounds that vary in the configuration of the core molecular structure (Davies, 2009), which generally affect the absorption of light and thereby the resulting colour (i.e., hue).

Petals may advertise with simple homogenous colours in some species, through to extremely complex colour patterns in other plant species. Our ability to interpret such complex signals is just starting to emerge (Lunau et al., 2021; Tunes et al., 2021). Even in petals or petal parts with homogeneous colour, one or several types of major pigment classes may be accumulated to generate a unique colour (Kay et al., 1981; Grotewold, 2006; Davies, 2009). For example, it is common to find flowers with different types of anthocyanins or with the presence of anthocyanins and carotenoids coexisting (Glover, 2007; Ng and Smith, 2016; Narbona et al., 2021). Only the accumulations of betalains and anthocyanins are mutually exclusive, being the production of the former restricted to some families of the order Caryophyllales (Moghe and Smith, 2018; Timoneda et al., 2019). Pigments are usually located in the epidermal and mesophyll cell layers of petals, and each single cell may accumulate one or several major pigment classes (Kay et al., 1981). In this way, the production of each major class of pigments within the cell is completely independent from each other due to the biosynthetic pathways are unrelated (Tanaka et al., 2008; Fattorini and Glover, 2020; Li et al., 2020). The evolutionary gain and loss of one or various major flower pigment classes in species of the same linage is common across the angiosperms (Rausher, 2008; Smith and Goldberg, 2015; Ng and Smith, 2016; Landis et al., 2018; Wessinger et al., 2019; Roguz et al., 2020; Berardi et al., 2021). Those shifts are often reversible, suggesting that the functionality of the underlying biochemical pathways is conserved (Ng et al., 2018). Evolutionary changes in major floral pigment groups are ubiquitous, and there are attempts in some lineages to assess how different pigment groups are perceived by their main pollinators (Muchhala et al., 2014; Ng and Smith, 2016; Kellenberger et al., 2019; Wessinger et al., 2019; Ogutcen et al., 2020). However, broad comparative studies directly linking pigment biochemistry with pollinator visual system models need to be done (see An et al., 2018; Stavenga et al., 2021).

The pollinator's visual system clearly determines how colour is perceived (Garcia et al., 2020; Dyer et al., 2021), differing strikingly among higher-level taxonomical groups but showing greater similarity within more closely related animal groups (Price et al., 2019). In this regards, insect colour vision has been thoroughly studied, namely in the honeybee *Apis mellifera*, and several butterflies, hawkmoths, flies and beetles, and marked differences have been found among orders and even among families (van der Kooi et al., 2021). Among insect pollinators, Lepidoptera species present receptor's visual systems particularly diverse with up to 15 different photoreceptors, but not all are equally involved in colour perception (van der Kooi et al., 2021). In contrast, the visual system of main hymenopteran pollinators (bees) is rather constant, and most species have three photoreceptors sensitive to UV, blue and green light (Goldsmith, 1990; Briscoe and Chittka, 2001); however, an additional receptor sensitive to red is found in some few species (Peitsch et al., 1992; van der Kooi et al., 2021). Likewise, main dipteran pollinators (Syrphidae, Muscoidae, and Bombyliidae) have similar visual systems with four types of photoreceptors involved in colour processing, with sensitivity peaks at approximately 330, 340, 460, and 540 nm (Lunau, 2014; An et al., 2018; Hannah et al., 2019). Birds, the main pollinator group aside insects, have four kinds of photoreceptors, but two types of visual systems occur depending on the sensitivity of receptor with peak at lowest wavelengths (UVS or VS types, that is ultraviolet or violet sensitive), the remaining three being sensitive to blue, green, and red wavelengths (Goldsmith, 1990; Vorobyev and Osorio, 1998; Hart and Hunt, 2007).

In order to represent colour stimuli into perceptual spaces of different pollinator groups, different colour vision models have been developed taking into consideration their photoreceptor spectral sensitivities and their mechanisms of neuronal processing. The colour vision system of bees has been extensively studied and several vision models have been

proposed (Backhaus, 1991; Chittka, 1992; Vorobyev and Brandt, 1997; Vorobyev and Osorio, 1998). The colour hexagon (Chittka, 1992) is the most widely used vision model for bees and is applicable to a wide range of bee species; it allows measurement of perceived chromatic differences and categorisation of bee colours, but it is unclear if and how bees might form colour categories (Renoult et al., 2017). Our understanding of colour vision system of flies is considerably lesser; yet, Troje (1993) developed a vision model for blowflies and found strong evidence of a categorical colour vision, and blowflies were unable to distinguish stimuli falling within a colour category. However, it has been proved that hoverflies can discriminate colours throughout a continuous variation, although neural mechanisms underlying their colour vision are similar to those proposed by Troje for blowflies; thus, Troje's model can also be used to measure chromatic differences as perceived by hoverflies (An et al., 2018; Hannah et al., 2019). Generating a colour space model for butterflies is more complex due to the diversity of their visual systems and their high number of photoreceptors (Arikawa, 2017; van der Kooi et al., 2021). Yet, in *Papilio xuthus* it has been proved that not all eight photoreceptors are involved in colour vision and a tetrachromatic vision systems has been proved (Koshitaka et al., 2008; Arikawa, 2017); from this, a tetrahedral colour space, similar to that of birds (see below), has been proposed to represent colours under *Papilio* vision and measure the perceived chromatic differences (Ohashi et al., 2015; Kantsa et al., 2017). To represent colour stimuli under bird vision, a tetrahedral colour space is used, this space being a representation of the stimulation of the four photoreceptors involved in avian colour perception (Endler and Mielke, 2005; Stoddard and Prum, 2008; Burd et al., 2014). As the previous ones, this model also allows to measure perceived chromatic differences among stimuli (Endler and Mielke, 2005; Stoddard and Prum, 2008; Camargo et al., 2019). A considerable number of studies show that functional groups of pollinators can perceive differently the same flower colour (e.g., Ohashi et al., 2015; Bergamo et al., 2018; Whitney et al., 2020). In fact, it has been demonstrated that flower colours have evolved in different regions of the world to match the visual capabilities and preferences of local pollinator fauna (Burd et al., 2014; Shrestha et al., 2016; Camargo et al., 2019; Coimbra et al., 2020). Recently, it has been found in species of the Gesneriaceae that the production of certain groups of anthocyanins may generate flower reflectance spectra that are adapted to the specific pollinator groups (Ogutcen et al., 2020). Thus, it is particularly relevant to assess if major pigment groups generate visual signals which conspicuousness differs for the main pollinator groups.

In this study, we analysed how flowers containing different pigments classes are perceived by the main functional groups of pollinators, specifically hymenopterans, dipterans, lepidopterans, and birds. We focused our research on flowers with petals homogenously coloured containing only one group of flower pigments. We used the flower reflectance spectra of 123 species with known pigment composition to address the following questions: How do main groups of pollinators perceive the colours generated by different major pigment classes? Is there any pigment class that is more conspicuous for a particular pollinator group? Theory of pollination syndromes hypothesised that some groups of pollinators such as bees, flies, butterflies or birds show preference for certain flower colours (Faegri and Van der Pijl, 1979). However, further studies showed that these predictions are only met in certain pollinator groups (Reverté et al., 2016). Flowers visited by bees are expected to be blue or violet, which is supported in laboratory and field studies (Giurfa et al., 1995; Dyer et al., 2016; Reverté et al., 2016). In addition, honeybees and bumblebees show inmate flower colour preferences for these two colours (Chittka and Wells, 2004). Therefore, we predict that flowers containing blue-violet pigments, i.e., anthocyanin derivatives (Harborne, 2014), may show higher conspicuousness for hymenopterans than those containing other pigment groups. In contrast, flowers visited by dipterans are mostly yellow and white (Reverté et al., 2016) and hoverflies show innate preferences to these colours (Lunau, 2014; Dunn et al., 2020); thus, we expect that flowers containing yellow carotenoids or aurones-chalcones and those containing white UV-absorbing flavonoids may show higher conspicuousness for dipterans. Lastly, reported colour preferences for butterflies are diverse, varying among species or even between sexes (Yoshida et al., 2015; Arikawa, 2017; Arikawa et al., 2021) and no clear preferences have been reported for birds (Lunau et al., 2011). However, given that both birds and many butterflies have red photoreceptors (Peitsch et al., 1992; van der Kooi et al., 2021), one would expect that pelargonidin-containing flowers would be more conspicuous to both groups of pollinators.

MATERIALS AND METHODS

Groups of Flower Pigments Considered

We chose the pigment classes or groups more frequent in flowers: chlorophylls, carotenoids, and flavonoids (**Figure 1A**). We intentionally excluded betalains because the number of species with biochemical information on the presence of these pigments in their flowers is limited (Strack et al., 1981, 2003; Sakuta, 2014; Polturak and Aharoni, 2018), and no reflectance data is available for these species. The details of biochemical information of flowers containing flavonoids are much more comprehensive than those for the chlorophylls and carotenoids (e.g., Harborne et al., 1975; Harborne and Williams, 2000; Andersen and Markham, 2006); thus, for this reason we only considered pigment subcategories in flavonoids. In addition, in most studied species containing carotenoids, flowers frequently show a mix of several subcategories of carotenoids (Ohmiya, 2011, 2013; Yuan et al., 2015; Wang et al., 2018). On the basis of their molecular and absorption characteristics, we chose the following groups of flavonoids: UV-absorbing flavonoids (mostly flavonols and flavones), aurones-chalcones, and anthocyanins (Mabry et al., 1970; Harborne, 1984; Andersen and Markham, 2006). Furthermore, we subsequently divided anthocyanins according to the type of anthocyanidins, namely, cyanidin, pelargonidin, delphinidin, and malvidin derivatives (**Figure 1A**). We excluded other anthocyanins, such as peonidin or petunidin derivatives, from the study because of the low number of known

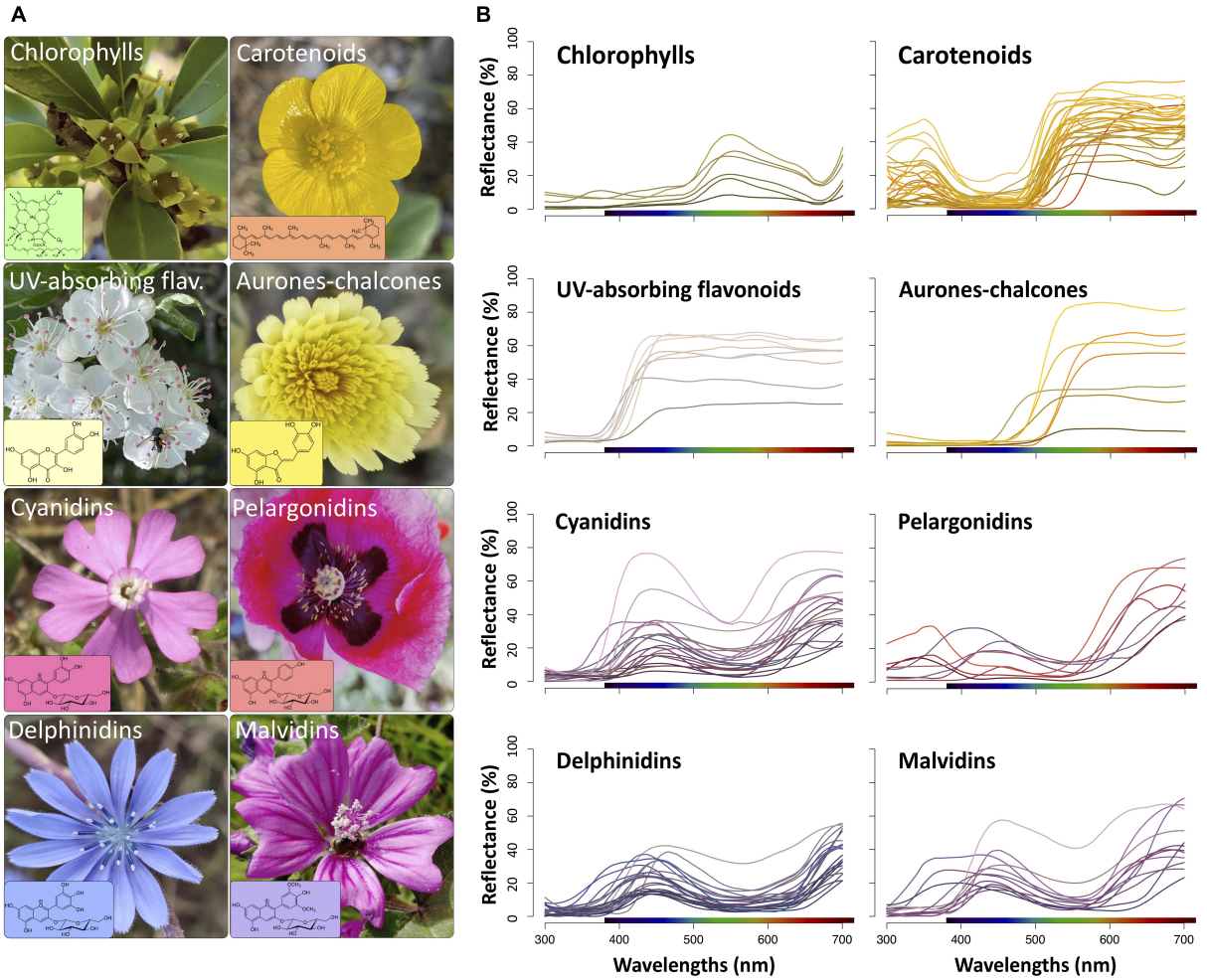

FIGURE 1 | (A) Examples of species with flowers containing one of the main groups of pigments considered in this study and its molecular structures (in the boxes). *Rhamnus lycioides* (Rhamnaceae), chlorophylls; *Ranunculus acris* (Ranunculaceae), carotenoids; *Crataegus monogyna* (Rosaceae), UV-absorbing flavonoids; *Andryala integrifolia* (Asteraceae), aurones-chalcones; *Silene littorea* (Caryophyllaceae), cyanidins; *P. somniferum* (Papaveraceae), pelargonidins; *Cichorium intybus* (Asteraceae), delphinidins; *Malva sylvestris* (Malvaceae), malvidins. The colour of the box represents the approximate colours producing in flowers based on human vision model. **(B)** Reflectance spectra of all species used in this study grouped by types of flower pigments. The colour of each spectral line represents the flower colour based on human vision model.

species containing these pigments (Harborne and Williams, 2000; Andersen and Markham, 2006).

Flower Pigment Composition

We mainly obtained biochemical data of flowers by literature review (N = 78), and additionally performed our own analyses (N = 45) to complete the sample of some pigment groups that are poorly represented in the bibliography, such as chlorophylls, carotenoids, and UV-absorbing flavonoids (see below). Regarding literature review, we mainly used the series "A survey of anthocyanins" published by G. M. Robison and R. Robison and their collaborator (Lawrence et al., 1938 and references therein) to obtain data of species with floral anthocyanins, although we also used other studies (**Supplementary Table 1**). For species with flowers containing either carotenoids or aurones-chalcones, we mainly used those listed in Camara et al. (1995) and Boucherle et al. (2017), respectively. In total, we obtained biochemical data of seven species containing chlorophylls, 33 species showing carotenoids and 83 species containing flavonoids (seven with aurones-chalcones, nine with UV-absorbing flavonoids, eight with pelargonidins, 13 with malvidins, 22 with cyanidins, and 24 with delphinidins; **Supplementary Table 1**). Species belong to 41 families of angiosperms, with a mean of three species per family and a maximum of 15 in Asteraceae and Fabaceae. We discarded species which flowers contain traces or mixtures of more than one group of anthocyanins or even mixtures with other pigments groups (mainly carotenoids).

For analytical identifications of major pigment groups, we performed differential extraction method followed by an analysis of absorbance spectra (Schoefs, 2004; Thrane et al., 2015). Briefly, we used two solvents to extract and separate the mayor pigments

classes in each sample: methanol with 1% HCl and pure acetone. Methanol solution is particularly effective to extract flavonoids, whereas acetone mainly extracts carotenoids and chlorophylls (Harborne, 1984; Schoefs, 2004). We placed the same quantity of floral tissue (6–25 mg of fresh weight) in two microtubes containing 1.5 mL of each solvent, which overnighted at 4°C in the dark and kept in the freezer at −80°C until further analysis. We measured absorbance spectra of the samples in each solvent using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., MA, United States) with polypropylene 96-well microplates. We set the scan mode from 280 to 700 nm with 1 nm steps at 22°C constant temperature. We performed identification of mayor pigment classes by means of spectrophotometric analysis. The fact that each pigment group have a particular absorbance spectrum with distinguishable peaks (Mabry et al., 1970; Ritchie, 2006; Narbona et al., 2021) allowed us to identify such pigments over the absorbance spectra of floral extracts (Harborne, 1984; Thrane et al., 2015).

Reflectance Data and Spectral Analysis

For 80 out of the total 123 species used in this study we downloaded floral reflectance data from the Floral Reflectance Database (hereafter "FReD") (Arnold et al., 2010), whereas for the remaining 43 we obtained them from direct measurements performed from wild flowers (**Supplementary Table 1**). For direct measurements, we used a Jaz portable spectrometer equipped with a deuterium–tungsten light source (200–800 nm; Ocean Optics, Dunedin, FL, United States), calibrated with a white standard (WS-1-SL, Ocean Optics; see more details in Del Valle et al., 2018). During measurements, light was incident on the adaxial surface of the petals at an angle of 45°. For species with multiple floral colours, we considered only the predominant colour occupying most of the petal area. Similarly, for species with UV or visible petal colour patterns (i.e., bull's-eyes), we studied the outer part of the petal (see specific methods in Heuschen et al., 2005 and Ortiz et al., 2021).

For the spectral analyses, we considered wavelengths between 300 and 700 nm (Briscoe and Chittka, 2001; Endler and Mielke, 2005). We processed reflectance curves prior to conduct further analysis and plotting. We first used the "*procspec*" function of the *pavo* R-package (Maia et al., 2019) to handle negative values, setting the minimum value to zero, but scaling other values accordingly. Then, for noise removal, we used the same function to smooth reflectance curves with a smoothness parameter of 0.20.

To describe the colour produced by each pigment, we calculated hue from its reflectance spectrum (Renoult et al., 2017; van der Kooi et al., 2019). Hue, is usually defined as the wavelength of maximum reflectance, which raises problems when processing reflectance spectra with more than one maximum (Grill and Rush, 2000; Maia et al., 2019), as is common in flowers (Chittka et al., 1994). Thus, we used hue from segment classification analysis (Endler and Mielke, 2005), which is particularly suitable for analysis of all data distributions and for detecting broad trends (Kemp et al., 2015), and we used the modification of Smith (2014) to express hue in degrees. Although more accurate methods exist to calculate hue as specifically perceived by some pollinators like bees (Chittka and Wells, 2004; Dyer et al., 2016) the method used here is suitable when considering a broader pollinator range.

Flower Colour Conspicuousness to Pollinators

To assess colour perception of flowers by hymenopterans, dipterans, lepidopterans and birds, we translated the reflectance spectra to a position in four widely used colour space models. We used the colour hexagon model for the trichromatic vision of bees (Chittka et al., 1992), the categorical space model for the tetravariant visual system of flies (Troje, 1993) and tetrahedral colour space models for the tetrachromatic visual systems of butterflies (Ohashi et al., 2015) and birds (Goldsmith, 1990; Briscoe and Chittka, 2001). We plotted the processed reflectance curves as loci in these colour spaces using the function "colspace" in the *pavo* R-package (Maia et al., 2019). For bee vision model, we used photoreceptor sensitivities of the honeybee *A. mellifera* (Chittka et al., 1992), for fly vision model those of housefly *Musca domestica* (Troje, 1993), for butterfly vision model those of swallowtail *P. xuthus* (Koshitaka et al., 2008), and for bird vision model we used the average photoreceptor sensitivities of the VS vision system of Trochilidae and Meliphagidae (Ödeen and Håstad, 2010; Burd et al., 2014), the two largest groups of bird pollinators (Krauss et al., 2017). All four models incorporate von Kries adaptation, which assumes that receptors adapt to light environment (Renoult et al., 2017). In all cases, we used the standard daylight function (D65 irradiance function) as illuminant, and the average spectrum of green foliage proposed by Chittka (1992) as background. We calculated the chromatic contrast against the background (i.e., the Euclidean distance between the colour loci of the flower and the achromatic centre; Rohde et al., 2013) according to the photoreceptor spectral sensitivities of the four colour space models. The stronger is flower contrast against the green foliage background, the easier will be the flower detection by pollinators. We also calculated the achromatic contrast for bees (i.e., green contrast) as the difference between the excitation value generated by the stimulus in the green photoreceptor and 0.5, which is the excitation value generated by the background in that receptor (Spaethe et al., 2001). This parameter is important because it is known that bees use this contrast to detect distant flowers under small visual angles (Spaethe et al., 2001). We did not calculate the achromatic contrast for flies, butterflies and birds because no behavioural information was available in this regard (Ohashi et al., 2015).

Statistical Analysis

We used phylogenetic ANOVAs (phylANOVAs) to control the potential influence of phylogeny when analysing differences among flowers containing main groups of pigments in hue, chromatic and achromatic contrasts. To perform phylANOVAs, we used the "*phytools*" R-package (Revell, 2012) with 10,000 simulations for each test and Holm-adjusted *P*-values for *post hoc* comparisons. To construct the phylogenetic tree used in the analyses, we used the "*phylo.maker*" function implemented in the "*V.PhyloMaker*" R-package (Jin and Qian, 2019), which uses

a mega-tree GBOTB.extended derived from the combination of GBOTB for seed plants (Smith and Brown, 2018) and the phylogeny for pteridophytes published in Zanne et al. (2014) as a backbone. We standardised species names according to The Plant List[1] since these are the ones used by V.PhyloMaker. We set the options "nodes = nodes.info.1" (the genus- and family-level node information was extracted from the mega-tree) and "scenarios = S3" (species tips absent from the mega-tree are bound to genus- or family-level following specific rules described in Jin and Qian, 2019). All statistical analyses were conducted in R 4.0.3 (R Core Team, 2021).

RESULTS

Spectral Properties of Flowers Containing Main Groups of Pigments

Chlorophyll-containing flowers were characterised by a low reflection, a peak in the green region and a shoulder in yellow and red regions (**Figure 1B**). Most flowers with carotenoids displayed a steep slope before the green region of the spectrum (∼560–600 nm) and a secondary peak in the UV region (300–400 nm). Flowers containing either UV-absorbing flavonoids or aurones-chalcones lacked reflectance in the UV wavelengths, but differed in the range they reflect: while UV-absorbing flavonoids reflected all visible light (400–700 nm), aurones-chalcones mostly reflected at wavelengths higher than 500–550 nm. Flowers with the four groups of anthocyanins showed reflectance spectra with a generalised low reflectance in the UV and green regions, but displayed some differences that may affect the resulting flower colour (**Figure 1B**). Three species with flowers containing pelargonidins (*Papaver somniferum, P. rhoeas, and Lysimachia arvensis* orange morph) had spectra with low reflection in the blue region producing human orange or red colourations. In most flowers containing delphinidins, reflection was also low in the yellow region, which confers the distinctive blue colourations, but this characteristic is shared with other flowers containing cyanidins, pelargonidins, or malvidins. Flowers containing anthocyanins showed the highest variability in reflectance spectra within each group. On the other hand, the groups of pigments showed significant differences in hue ($F = 1484.0$, $P < 0.001$), calculated following the segment classification method. Hue values were structured in three significantly different groups: chlorophylls; carotenoids, UV-absorbing flavonoids and aurones-chalcones; and anthocyanins (**Supplementary Figure 1** and **Supplementary Table 2**).

Flower Pigment Groups as Perceived by Pollinators

In general, floral colour loci of main pigment groups occupied different regions in the bee, fly, butterfly, and bird colour space models (**Figure 2**; see **Supplementary Figures 2–5** for individual representations of each pigment group in each colour space). In the hexagonal colour model for bee vision, colour loci of flowers containing chlorophylls were located in the green region. Most flowers with carotenoids were concentrated in the UV-green zone, whereas aurones-chalcones were mainly located in the green region; only a few flowers containing carotenoids also occupied the green region, indeed these flowers showed spectra with no reflectance in the UV region (**Figure 1B**). Flowers with UV-absorbing flavonoids mostly occupied the blue-green region of the hexagon, and those with anthocyanins are mainly perceived as blue or UV-blue, with the exception of three species containing orange-red pelargonidins previously described, which are perceived as UV (**Figure 2A** and **Supplementary Figure 2**).

In the fly model, colour loci of pigment groups occupied the four quadrants of the colour space (**Figure 2B** and **Supplementary Figure 3**). Flowers containing chlorophylls, UV-absorbing flavonoids, or aurones-chalcones were mostly located in the green category. Carotenoids were widespread across the green, purple, and UV categories of the colour space, being more predominant in the purple region. Anthocyanins predominantly occupied the blue category of the colour space, except for the same three pelargonidin loci previously mentioned, as well as one cyanidin and three malvidin loci, which occupy the UV region.

In the tetrahedron colour space of butterflies, flowers containing aurones-chalcones were mainly located towards the red region, whereas those containing carotenoids showed a more dispersed distribution between the red and UV regions (**Figure 2C** and **Supplementary Figure 4**). Anthocyanins were dispersed between the UV and blue regions, apart from some pelargonidins located towards the UV-red edge. UV-absorbing flavonoids occupied positions between blue, green, and red regions of butterfly colour space. Finally, chlorophylls were located between carotenoids and anthocyanins, near the centre of tetrahedron.

In the tetrahedron colour space of birds, flowers containing different groups of pigment showed a similar distribution pattern that in the butterfly colour space (**Figure 2D** and **Supplementary Figure 5**). Exceptions occurred with carotenoids that were more clustered towards the red region, and with some pelargonidins appearing near carotenoids.

Colour Conspicuousness of Pigment Groups to Pollinators

We found a significant effect of main pigment groups in the chromatic contrast using the four colour space models ($F = 11.72$, $P < 0.001$ for the bee model, $F = 8.08$, $P < 0.001$ for the fly model, $F = 11.90$, $P < 0.001$ for the butterfly model, and $F = 14.31$, $P < 0.001$ for the bird model). In general, flowers containing chlorophylls showed the lowest values of chromatic contrast, and UV-absorbing flavonoids and the four groups of anthocyanins also showed low values of chromatic contrast statistically similar to those of chlorophylls (**Figure 3** and **Supplementary Table 3**). In contrast, flowers containing aurones-chalcones showed the highest chromatic contrast, and carotenoids showed intermediate values. An exception of this general pattern occurred in the fly colour space, where UV-absorbing flavonoids reached intermediate values, similar to those of carotenoids. Moreover, in all visual models, pelargonidins tended to show higher values

[1] www.theplantlist.org

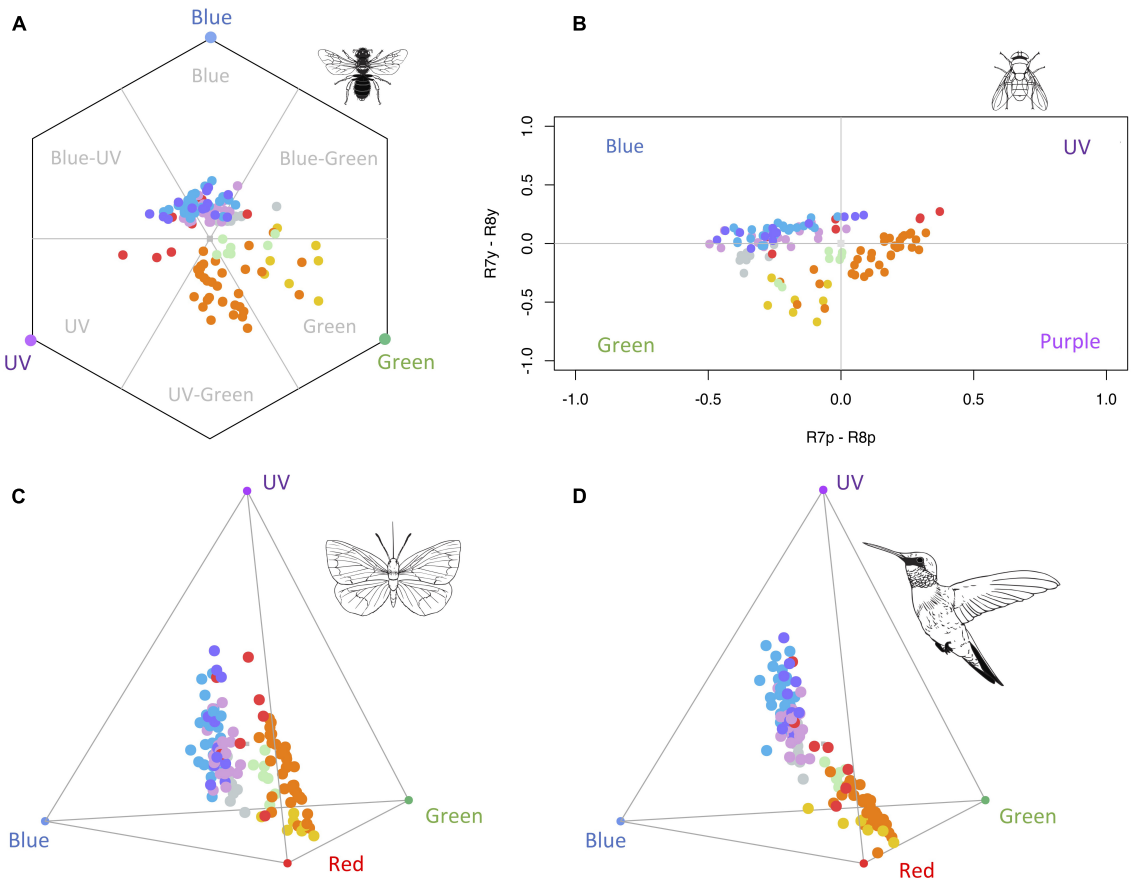

FIGURE 2 | Colour loci of species used in the study according to different colour vision models. The 123 colour loci are represented in the hexagon colour space for the trichromatic vision of bees **(A)**, the categorical colour space for the tetravariant visual system of flies **(B)**, the tetrachromatic colour space model for butterflies **(C)**, and the tetrahedral colour space model for birds with VS vision system **(D)**. Coloured blue, green, purple, and red circles in each vertex represent the maximum signals in the blue, green, UV, and red photoreceptors. Colour loci correspond to each pigment group: green (chlorophylls), orange (carotenoids), grey (UV-absorbing flavonoids), yellow (aurones-chalcones), pink (cyanidins), red (pelargonidins), blue (delphinidins), and purple (malvidins). See **Supplementary Figures 2–5** for individual representations of each pigment group in the four colour vision models. Animal silhouettes taken from Divulgare (www.divulgare.net) under a Creative Common licence.

than the other groups of anthocyanins, especially in bird model and relative to cyanidins, but differences were only marginally significant due to the high variation within pelargonidin.

With respect the achromatic contrast using the bee colour space model (i.e., green contrast), we found a significant effect of main pigment groups as well ($F = 16.59$, $P < 0.001$; **Figure 4** and **Supplementary Table 4**). The four groups of anthocyanins and chlorophylls produced the lowest values while UV-absorbing flavonoids produced the highest ones. Carotenoids and aurones-chalcones showed intermediate values that were statistically similar to those of UV-absorbing flavonoids.

DISCUSSION

The overall goal of this study was to know whether flowers containing different major groups of pigments generate different colour signals for pollinators. Our study demonstrated that flowers containing chlorophylls, carotenoids, UV-absorbing flavonoids, aurones-chalcones and anthocyanins showed distinctive reflectance spectra, which differences and similarities were mostly maintained when the spectral information was translated into the pollinator's visual system models. In general, flower colour loci of these pigment groups occupied different regions of the bee, fly, butterfly, and bird colour space models. Within the flavonoids, the four groups of anthocyanins produced a unique cluster of colour loci in all the visual colour spaces, UV-absorbing flavonoids were located within or close to anthocyanins, but aurones-chalcones were located in an independent region sometimes shared with some carotenoids samples. The different pigment groups generated differences in colour conspicuousness in all the four visual space models of pollinators. Most of these differences showed high similarity among colour space models, as we found that aurones-chalcones always showed the highest chromatic contrast values, carotenoids displayed intermediate values, and chlorophylls, UV-absorbing flavonoids and anthocyanins presented the lowest values. Yet, some differences among visual models

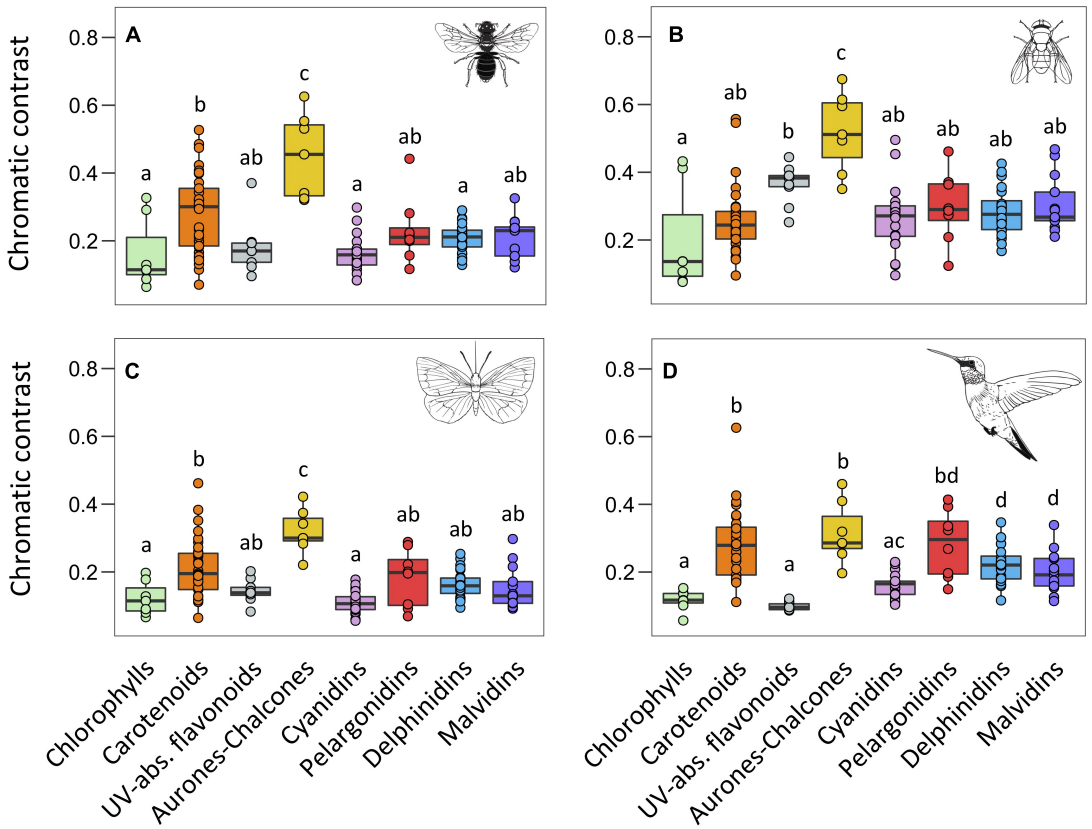

FIGURE 3 | Boxplots representing the distribution of chromatic contrast values for each type of pigment obtained from the vision models of bees **(A)**, flies **(B)**, butterflies **(C)**, and birds **(D)**. The central line displays the median, the bottom and top of the box are the first and third quartiles, and dots represent sample values. We performed phylANOVAs with 10,000 simulations and Holm-adjusted *P*-value. Different letters represent significant differences at 0.05 level. Animal silhouettes taken from Divulgare (www.divulgare.net) under a Creative Common licence.

emerged. The implications of these findings for the evolution of flower pigmentation and the ecological consequences for plant-pollinator interaction are discussed.

In general, the clustering patterns of loci observed in the colour space of bees, flies, butterflies and birds were similar. Loci of flowers with chlorophylls occupied central position in all models and showed low chromatic contrast, which suggest that these flowers are difficult to perceive by the four groups of pollinators (Chittka et al., 1994; Endler and Mielke, 2005; Hannah et al., 2019), at least in environments where dominant background is green foliage (Endler, 2012; Bukovac et al., 2017; Martins et al., 2021). This fact may explain the low frequency of green flowers in local floras (Weevers, 1952; Dyer, 1996; Warren and Mackenzie, 2001). However, the distinct colour signal and low chromatic contrast of chlorophylls may help to explain why these pigments are frequently combined with UV-absorbing flavonoids, anthocyanins, or carotenoids to form visible floral guides and patterned flowers (Lunau, 1992; An et al., 2018; Koski, 2020a; Narbona et al., 2021) and thus, to generate highly perceptible colour patterns for pollinators (van den Berg et al., 2020; Roguz et al., 2021).

Flowers with aurones-chalcones generated the highest chromatic contrast in the fly model, thus matching our predictions, but those with carotenoids did not so. In fact, flowers with aurones-chalcones exhibited high contrast in the three colour space models for insects, higher than that of flowers with carotenoids. The higher colour contrast of aurones-chalcones in flowers could lead to a higher attraction for bees, flies and butterflies and, consequently, represent a selective advantage for this group of pigments over carotenoids. Nevertheless, the occurrence of aurones-chalcones in flowers is anecdotic in comparison with carotenoids, being found in certain lineages of Asteraceae, Fabaceae, Plantaginaceae, Oxalidaceae, Gesneriaceae, Rosaceae, and Hamamelidaceae (Bohm, 1988; Iwashina, 2015; Boucherle et al., 2017). The biosynthesis of aurones requires complex enzymatic machinery, particularly the enzyme aurone synthase, with an unusual polyphenol oxidase activity (Molitor et al., 2016; Boucherle et al., 2017), which would explain the rarity of aurones in flowers. It is worth mentioning that in flowers, aurones mostly appear in combination with carotenoids, and to a lesser extend with anthocyanins or UV-absorbing flavonoids, to form floral guides; only a few species are known to contain aurones alone (Valdés, 1970; Iwashina, 2015; Boucherle et al., 2017). Our results support that colour properties of aurones-chalcones are suitable for shaping spatial patterns in flowers (see below).

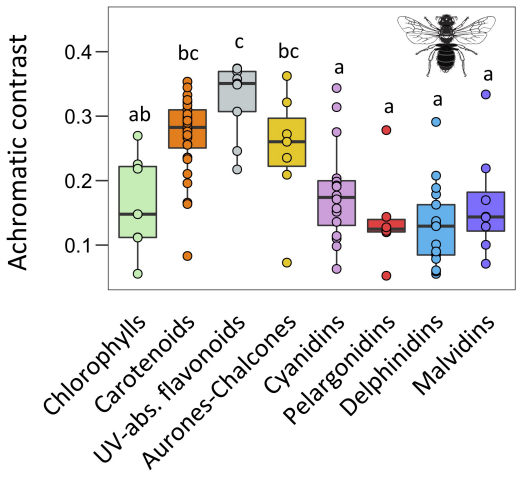

FIGURE 4 | Boxplots representing the distribution of achromatic contrast values for each type of pigment calculated in the hexagonal colour model for bee vision. The central line displays the median, the bottom and top of the box are the first and third quartiles, and dots represent sample values. We performed phylANOVAs with 10,000 simulations and Holm-adjusted *P*-value. Different letters represent significant differences at 0.05 level. Bee silhouette taken from Divulgare (www.divulgare.net) under a Creative Common licence.

The conspicuousness of white flowers with UV-absorbing flavonoids was intriguing for two reasons. On the one hand, they showed low chromatic contrast values in all visual models, excepting the fly model where contrast values were intermediate and only lower than those of flowers with aurones-chalcones, which matches our predictions of both groups of flowers generating the highest chromatic contrasts for dipterans. On the other hand, these flowers showed the highest achromatic contrast in the bee visual model, as reported in previous studies with white flowers (Chittka et al., 1994; Lunau et al., 2011; Coimbra et al., 2020). This fact might be relevant because honeybees use such achromatic contrast to find small flowers and flowers at long distance (Giurfa et al., 1997; Spaethe et al., 2001). Besides that, flowers with UV-absorbing flavonoids are defined by reflectance spectra with a steep slope around 400 nm (Chittka et al., 1994; Kevan et al., 2001; Reverté et al., 2016). Insects, and particularly hymenopterans show a maximum of discrimination capacity at 400 nm (Chittka, 1996) and can act as selective agents on UV-absorbing white flowers as has been proposed (Dyer et al., 2012). This suggests selective pressures for accumulating specific types or concentrations of these compounds to generate such UV-absorbing white flowers.

A clear trend is observed for carotenoids, UV-absorbing flavonoids and aurones-chalcones to be separately clustered in all four colour space models, which means that these pigment groups generate contrasting colour signals for all these pollinators and has important consequences in flowers with floral guides. A common pattern in insect-pollinated yellow flowers is an UV-absorbing centre and UV-reflecting periphery (Papiorek et al., 2016; Lunau et al., 2021). This pattern may be produced in two ways: by accumulating aurones-chalcones in the centre and carotenoids in the periphery (e.g., species of the Asteraceae and Plantaginaceae; Harborne and Smith, 1978; Boucherle et al., 2017), or by accumulating carotenoids plus UV-absorbing flavonoids in the centre and only carotenoids in the periphery (e.g., species of the Asteraceae and Fabaceae; Harbone and Grayer, 1994; Bohm and Stuessy, 2001). This intrafloral pattern in yellow flowers could have behavioural consequences for flies and bees. Hoverflies accept UV-reflecting as well as UV-absorbing yellow for landing, but they extend their proboscis preferably to UV-absorbing yellow colours (An et al., 2018). A similar behaviour is found in bees, which made the first antennal contact preferably in UV-absorbing areas (Papiorek et al., 2013). Our results suggest that spatial segregation of aurones-chalcones and carotenoids may produce an UV pattern in yellow flowers that would be highly different in colour category but also in chromatic contrast. It has been found that flowers displaying multiple colours that maximise contrast are more attractive for bees than flowers with homogeneous colours (Heuschen et al., 2005; Leonard et al., 2011; Ma et al., 2016).

Finally, it is worth noting that most flowers containing any of the four groups of anthocyanins here studied clustered close together in all four colour spaces, and in general showed no differentiation among them in terms of chromatic and achromatic contrasts. The case of flowers containing delphinidins or malvidins deserves special mention. They produce human blue colourations, which agree with previous studies (Harborne and Williams, 2000; Yoshida et al., 2009), and also generated loci that mostly occupied the blue category in both bee and fly colour spaces. This colour is the preferred for bees and bumblebees (reviewed in Dyer et al., 2021) but, contrary to our predictions, flowers with delphinidins or malvidins did not generate the highest chromatic contrasts in the bee colour space model. To produce delphinidins or malvidins it is necessary the enzyme flavonoid 3′5′hydroxylase (*F3′5′H*), which is proposed as the most recent addition to the anthocyanin biosynthetic pathway (Campanella et al., 2014). Our results show that similar blue loci in both bee and fly colour space may result from flowers with cyanidin and pelargonidins through a variety of biochemical or cellular modifications (Harborne and Williams, 2000; Okitsu et al., 2018). Thus, blue colour in flowers can be produced through the three main biosynthetic branches of the anthocyanin pathway (i.e., cyanidin, delphinidin, pelargonidin branches; Grotewold, 2006; Tanaka et al., 2008), which originates an interesting case of phenotypic convergence (Larter et al., 2018). It is also worth noting that the highest chromatic contracts for flowers with pelargonidins were generated in the bird colour space, which matches our predictions. In fact, flowers with pelargonidins were the most contrasting to birds along with those with carotenoids and aurones-chalcones. The role of pelargonidins in bird-pollinated flowers was previously reported in species of the Solanaceae and Gesnerioideae (Ng and Smith, 2016, 2018; Ogutcen et al., 2020). Although birds do not show preferences for a particular colour, they have an excellent colour discrimination (Lunau et al., 2011; Stoddard et al., 2020; Whitney et al., 2020). Our results suggest that both carotenoids and pelargonidins pigments generate a higher conspicuousness to birds than other pigment groups, which would favour their accumulation in flowers of bird-pollinated species. In fact, an

important characteristic of this pollination syndrome is the presence of red flowers (Chen et al., 2020), which is typically produced by pelargonidins or by other pigment combinations (Sakuta, 2014; Ng and Smith, 2016; Ogutcen et al., 2020).

In conclusion, we have found that main groups of flower pigments generate distinct flower colours with differential conspicuousness, and each group of pigment showed nearly similar chromatic contrast values in the four visual models of pollinators. As expected, yellow aurones-chalcones showed a high chromatic contrast in the fly visual model, but white UV-absorbing flavonoids showed intermediate contrast and carotenoids showed lower values, similar to anthocyanins. As predicted, pelargonidins generated a higher chromatic contrast in the bird model. Yet, in contrast to our predictions, anthocyanins showed low chromatic and achromatic contrasts in the visual model of bees. Our results suggest that to explain the success of anthocyanins as the most frequent floral pigment in angiosperms (Warren and Mackenzie, 2001; Narbona et al., 2018), other causes than their role in attracting pollinators should be taken into account. Indeed, an increased body of knowledge indicate a protective function of anthocyanin against environmental stressors, which may play an important role in the success and distribution of anthocyanins in flowers (Strauss and Whittall, 2006; Del Valle et al., 2019; Dalrymple et al., 2020). Our results must be taken with caution because the visual systems of the main groups of pollinators are only approximations to their colour vision and further validation with behavioural tests will be needed (Renoult et al., 2017; Lunau and Gerten, 2020; Garcia et al., 2021). Furthermore, our study used specific illumination and background conditions, and colour signals may notably vary in different habitats or environmental conditions (Endler, 2012; Bukovac et al., 2017; Koski, 2020b).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author. The raw data supporting the conclusions of this study are included as **Supplementary Table 5**.

AUTHOR CONTRIBUTIONS

EN and JV obtained the data. JV analysed the data and performed the statistical analyses. EN, JV, and PO drafted the manuscript. All authors conceived the study, contributed to the final manuscript, and approved the submitted version.

FUNDING

This work was supported by Research by the European Regional Development Fund (ERDF) and grants from the Spanish Government (PID2020-116222GB-I00 and CGL2015-63827) and the Andalusian Regional Ministry of Economy, Knowledge, Business and University (PY18-3651, US-1265280, and UPO-1261687).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.743850/full#supplementary-material

Supplementary Figure 1 | Boxplot representing the distribution of hue values for each group of pigments. The central line displays the median, the bottom, and top of the box are the first and third quartiles, and dots represent sample values. We performed phylANOVAs with 10,000 simulations and Holm-adjusted *P*-value. Different letters represent significant differences at 0.05 level.

Supplementary Figure 2 | Individual representation of each pigment type in the hexagon colour space for the trichromatic vision of bees. Coloured blue, green, and purple circles in each vertex represent the maximum signals in the blue, green, and UV photoreceptors. Colour loci correspond to each pigment group: green (chlorophylls), orange (carotenoids), grey (UV-absorbing flavonoids), yellow (aurones-chalcones), pink (cyanidins), red (pelargonidins), blue (delphinidins), and purple (malvidins).

Supplementary Figure 3 | Individual representation of each pigment type in the categorical colour space for the tetravariant visual system of flies. Colour loci correspond to each pigment group: green (chlorophylls), orange (carotenoids), grey (UV-absorbing flavonoids), yellow (aurones-chalcones), pink (cyanidins), red (pelargonidins), blue (delphinidins), and purple (malvidins).

Supplementary Figure 4 | Individual representation of each pigment type in the tetrahedral colour space for butterflies. Coloured blue, green, purple, and red circles in each vertex represent the maximum signals in the blue, green, UV, and red photoreceptors. Colour loci correspond to each pigment group: green (chlorophylls), orange (carotenoids), grey (UV-absorbing flavonoids), yellow (aurones-chalcones), pink (cyanidins), red (pelargonidins), blue (delphinidins), and purple (malvidins).

Supplementary Figure 5 | Individual representation of each pigment type in the tetrahedral colour space for birds with VS vision system. Coloured blue, green, purple, and red circles in each vertex represent the maximum signals in the blue, green, UV, and red photoreceptors. Colour loci correspond to each pigment group: green (chlorophylls), orange (carotenoids), grey (UV-absorbing flavonoids), yellow (aurones-chalcones), pink (cyanidins), red (pelargonidins), blue (delphinidins), and purple (malvidins).

REFERENCES

An, L., Neimann, A., Eberling, E., Algora, H., Brings, S., and Lunau, K. (2018). The yellow specialist: Dronefly Eristalis tenax prefers different yellow colours for landing and proboscis extension. *J. Exp. Biol.* 221:jeb184788. doi: 10.1242/jeb.184788

Andersen, ØM., and Markham, K. R. (2006). *Flavonoids: chemistry, biochemistry and applications*. Boca Raton, FL: CRC Press. doi: 10.1201/9781420039443

Arikawa, K. (2017). The eyes and vision of butterflies. *J. Physiol.* 595, 5457–5464. doi: 10.1113/jp273917

Arikawa, K., Nakatani, Y., Koshitaka, H., and Kinoshita, M. (2021). Foraging small white butterflies, Pieris rapae, search flowers using color vision. *Front. Ecol. Evol.* 9:650069. doi: 10.3389/fevo.2021.650069

Arnold, S. E. J., Faruq, S., Savolainen, V., McOwan, P. W., and Chittka, L. (2010). FReD: The floral reflectance database – A web portal for analyses of flower colour. *PLoS ONE* 5:e14287. doi: 10.1371/journal.pone.0014287

Backhaus, W. (1991). Color opponent coding in the visual system of the honeybee. *Vision Res.* 31, 1381–1397. doi: 10.1016/0042-6989(91)90059-E

Berardi, A. E., Esfeld, K., Jäggi, L. V., Mandel, T., Cannarozzi, G. M., and Kuhlemeier, C. (2021). Complex evolution of novel red floral color in Petunia. *Plant Cell* 33, 2273–2295. doi: 10.1093/plcell/koab114

Bergamo, P. J., Telles, F. J., Arnold, S. E. J., and Brito, V. L. G. (2018). Flower colour within communities shifts from overdispersed to clustered along an alpine altitudinal gradient. *Oecologia* 188, 223–235. doi: 10.1007/s00442-018-4204-5

Bohm, B. A. (1988). "The minor flavonoids," in *The Flavonoids: Advances in Research since 1980*, ed. J. B. Harborne (Dordrecht: Springer Science), 329–388. doi: 10.1007/978-1-4899-2913-6_9

Bohm, B. A., and Stuessy, T. F. (2001). *Flavonoids of the sunflower family (Asteraceae)*. New York, NY: Springer-Verlag Wien.

Boucherle, B., Peuchmaur, M., Boumendjel, A., and Haudecoeur, R. (2017). Occurrences, biosynthesis and properties of aurones as high-end evolutionary products. *Phytochemistry* 142, 92–111. doi: 10.1016/j.phytochem.2017.06.017

Briscoe, A. D., and Chittka, L. (2001). The evolution of color vision in insects. *Annu. Rev. Entomol.* 46, 471–510. doi: 10.1146/annurev.ento.46.1.471

Bukovac, Z., Shrestha, M., Garcia, J. E., Burd, M., Dorin, A., and Dyer, A. G. (2017). Why background colour matters to bees and flowers. *J. Comp. Physiol. A* 203, 369–380. doi: 10.1007/s00359-017-1175-7

Burd, M., Tristan Stayton, C., Shrestha, M., and Dyer, A. G. (2014). Distinctive convergence in Australian floral colours seen through the eyes of Australian birds. *Proc. R. Soc. B Biol. Sci.* 281:20132862. doi: 10.1098/rspb.2013.2862

Camara, B., Hugueney, P., Bouvier, F., Kuntz, M., and Monéger, R. (1995). Biochemistry and molecular biology of chromoplast development. *Int. Rev. Cytol.* 163, 175–247. doi: 10.1016/S0074-7696(08)62211-1

Camargo, M. G. G., Lunau, K., Batalha, M. A., Brings, S., Brito, V. L. G., and Morellato, L. P. C. (2019). How flower colour signals allure bees and hummingbirds: a community-level test of the bee avoidance hypothesis. *New Phytol.* 222, 1112–1122. doi: 10.1111/nph.15594

Campanella, J. J., Smalley, J. V., and Dempsey, M. E. (2014). A phylogenetic examination of the primary anthocyanin production pathway of the Plantae. *Bot. Stud.* 55, 1–10. doi: 10.1186/1999-3110-55-10

Castañeda-Ovando, A., Pacheco-Hernández, M., de, L., Páez-Hernández, M. E., Rodríguez, J. A., and Galán-Vidal, C. A. (2009). Chemical studies of anthocyanins: a review. *Food Chem.* 113, 859–871. doi: 10.1016/j.foodchem.2008.09.001

Chen, Z., Niu, Y., Liu, C. Q., and Sun, H. (2020). Red flowers differ in shades between pollination systems and across continents. *Ann. Bot.* 126, 837–848. doi: 10.1093/AOB/MCAA103

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543. doi: 10.1007/BF00199331

Chittka, L. (1996). Optimal sets of color receptors and color opponent systems for coding of natural objects in insect vision. *J. Theor. Biol.* 181, 179–196. doi: 10.1006/jtbi.1996.0124

Chittka, L., and Wells, H. (2004). "Color vision in bees: mechanisms, ecology and evolution," in *How simple nervous systems create complex perceptual worlds*, ed. F. Prete (Boston: MIT Press), 165–191.

Chittka, L., Beier, W., Hertel, H., Steinmann, E., and Menzel, R. (1992). Opponent colour coding is a universal strategy to evaluate the photoreceptor inputs in Hymenoptera. *J. Comp. Physiol. A* 170, 545–563. doi: 10.1007/BF00199332

Chittka, L., Troje, N., and Menzel, R. (1994). Ultraviolet as a component of flower reflections, and the color perception of Hymenoptera. *Vision Res.* 34, 1489–1508. doi: 10.1016/0042-6989(94)90151-1

Coimbra, G., Araujo, C., Bergamo, P. J., Freitas, L., and Rodríguez-Gironés, M. A. (2020). Flower conspicuousness to bees across pollination systems: A generalized test of the bee-avoidance hypothesis. *Front. Plant Sci.* 11:558684. doi: 10.3389/fpls.2020.558684

Dalrymple, R. L., Kemp, D. J., Flores-Moreno, H., Laffan, S. W., White, T. E., Hemmings, F. A., et al. (2020). Macroecological patterns in flower colour are shaped by both biotic and abiotic factors. *New Phytol.* 228, 1972–1985. doi: 10.1111/nph.16737

Davies, K. M. (2009). "Modifying anthocyanin production in flowers," in *Anthocyanins*, eds K. S. Gould, K. M. Davies, and C. Winefield (New York, NY: Springer), 49–83. doi: 10.1007/978-0-387-77335-3_3

Del Valle, J. C., Alcalde-Eon, C., Escribano-Bailón, M. T., Buide, M. L., Whittall, J. B., and Narbona, E. (2019). Stability of petal color polymorphism: the significance of anthocyanin accumulation in photosynthetic tissues. *BMC Plant Biol.* 19:496. doi: 10.1186/s12870-019-2082-6

Del Valle, J. C., Gallardo-López, A., Buide, M. L., Whittall, J. B., and Narbona, E. (2018). Digital photography provides a fast, reliable, and noninvasive method to estimate anthocyanin pigment concentration in reproductive and vegetative plant tissues. *Ecol. Evol.* 8, 3064–3076. doi: 10.1002/ece3.3804

Dunn, L., Lequerica, M., Reid, C. R., and Latty, T. (2020). Dual ecosystem services of syrphid flies (Diptera: Syrphidae): pollinators and biological control agents. *Pest Manag. Sci.* 76, 1973–1979. doi: 10.1002/ps.5807

Dyer, A. G. (1996). Reflection of near-ultraviolet radiation from flowers of Australian native plants. *Aust. J. Bot.* 44, 473–488. doi: 10.1071/BT9960473

Dyer, A. G., Boyd-Gerny, S., Mcloughlin, S., Rosa, M. G. P., Simonov, V., and Wong, B. B. M. (2012). Parallel evolution of angiosperm colour signals: Common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. B Biol. Sci.* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Dyer, A. G., Boyd-Gerny, S., Shrestha, M., Lunau, K., Garcia, J. E., Koethe, S., et al. (2016). Innate colour preferences of the Australian native stingless bee Tetragonula carbonaria Sm. *J. Comp. Physiol. A* 202, 603–613. doi: 10.1007/s00359-016-1101-4

Dyer, A. G., Jentsch, A., Burd, M., Garcia, J. E., Giejsztowt, J., Camargo, M. G. G., et al. (2021). Fragmentary blue: Resolving the rarity paradox in flower colors. *Front. Plant Sci.* 11:618203. doi: 10.3389/fpls.2020.618203

Endler, J. A. (2012). A framework for analysing colour pattern geometry: adjacent colours. *Biol. J. Linn. Soc.* 107, 233–253. doi: 10.1111/j.1095-8312.2012.01937.x

Endler, J. A., and Mielke, P. W. (2005). Comparing entire colour patterns as birds see them. *Biol. J. Linn. Soc.* 86, 405–431. doi: 10.1111/j.1095-8312.2005.00540.x

Faegri, K., and Van der Pijl, L. (1979). *The principles of pollination ecology*. Oxford: Pergamon Press.

Fattorini, R., and Glover, B. J. (2020). Molecular mechanisms of pollination biology. *Annu. Rev. Plant Biol.* 71, 487–515. doi: 10.1146/annurev-arplant-081519-040003

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., and Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.* 35, 375–403. doi: 10.1146/annurev.ecolsys.34.011802.132347

Garcia, J. E., Phillips, R. D., Peter, C. I., and Dyer, A. G. (2020). Changing how biologists view flowers—color as a perception not a trait. *Front. Plant Sci.* 11:601700. doi: 10.3389/fpls.2020.601700

Garcia, J. E., Rohr, D. H., and Dyer, A. G. (2021). Colour discrimination from perceived differences by birds. *Front. Ecol. Evol.* 9:639513. doi: 10.3389/fevo.2021.639513

Giurfa, M., Nunez, J., Chittka, L., and Menzel, R. (1995). Colour preferences of flower-naive honeybees. *J. Comp. Physiol. A* 177, 247–259.

Giurfa, M., Vorobyev, M., Brandt, R., Posner, B., and Menzel, R. (1997). Discrimination of coloured stimuli by honeybees: Alternative use of achromatic and chromatic signals. *J. Comp. Physiol. A* 180, 235–243. doi: 10.1007/s003590050044

Glover, B. (2007). *Understanding flowers and flowering: an integrated approach*. New York, NY: Oxford University Press.

Goldsmith, T. H. (1990). Optimization, constraint, and history in the evolution of eyes. *Q. Rev. Biol.* 65, 281–322. doi: 10.1086/416840

Grill, C. P., and Rush, V. N. (2000). Analysing spectral data: Comparison and application of two techniques. *Biol. J. Linn. Soc.* 69, 121–138. doi: 10.1006/bijl.1999.0360

Grotewold, E. (2006). The genetics and biochemistry of floral pigments. *Annu. Rev. Plant Biol.* 57, 761–780. doi: 10.1146/annurev.arplant.57.032905.105248

Hannah, L., Dyer, A. G., Garcia, J. E., Dorin, A., and Burd, M. (2019). Psychophysics of the hoverfly: Categorical or continuous color discrimination? *Curr. Zool.* 65, 483–492. doi: 10.1093/cz/zoz008

Harbone, J. B., and Grayer, R. J. (1994). "Flavonoids and insects," in *The flavonoids. Advances in research since 1986*, ed. J. B. Harborne (London, UK: Chapman & Hall), 589–618. doi: 10.1007/978-1-4899-2911-2_14

Harborne, J. B. (1984). *Phytochemical Methods. A guide to modern techniques of plant analysis*. London, UK: Chapman & Hall.

Harborne, J. B. (2014). *Introduction to ecological biochemistry*. Cambridge, UK: Academic Press.

Harborne, J. B., and Smith, D. M. (1978). Anthochlors and other flavonoids as honey guides in the Compositae. *Biochem. Syst. Ecol.* 6, 287–291. doi: 10.1016/0305-1978(78)90047-9

Harborne, J. B., and Williams, C. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry* 55, 481–504. doi: 10.1016/S0031-9422(00)00235-1

Harborne, J. B., Marby, H., and Marby, T. J. (1975). *The flavonoids. Advances in research*. London, UK: Chapman & Hall.

Hart, N. S., and Hunt, D. M. (2007). Avian visual pigments: Characteristics, spectral tuning, and evolution. *Am. Nat.* 169, S7–S26. doi: 10.1086/510141

Heuschen, B., Gumbert, A., and Lunau, K. (2005). A generalised mimicry system involving angiosperm flower colour, pollen and bumblebees' innate colour preferences. *Plant Syst. Evol.* 252, 121–137. doi: 10.1007/s00606-004-0249-5

Iwashina, T. (2015). Contribution to flower colors of flavonoids including anthocyanins: A review. *Nat. Prod. Commun.* 10, 529–544. doi: 10.1177/1934578x1501000335

Jin, Y., and Qian, H. (2019). V.PhyloMaker: an R package that can generate very large phylogenies for vascular plants. *Ecography (Cop.).* 42, 1353–1359. doi: 10.1111/ecog.04434

Kantsa, A., Raguso, R. A., Dyer, A. G., Sgardelis, S. P., Olesen, J. M., and Petanidou, T. (2017). Community-wide integration of floral colour and scent in a Mediterranean scrubland. *Nat. Ecol. Evol.* 1, 1502–1510. doi: 10.1038/s41559-017-0298-0

Kay, Q. O. N., Daoud, H. S., and Stirton, C. H. (1981). Pigment distribution, light reflection and cell structure in petals. *Bot. J. Linn. Soc.* 83, 57–83. doi: 10.1111/j.1095-8339.1981.tb00129.x

Kellenberger, R. T., Byers, K. J., Francisco, R. M. D. B., Staedler, Y. M., LaFountain, A. M., Schönenberger, J., et al. (2019). Emergence of a floral colour polymorphism by pollinator-mediated overdominance. *Nat. Commun.* 10:63.

Kemp, D. J., Herberstein, M. E., Fleishman, L. J., Endler, J. A., Bennett, A. T. D., Dyer, A. G., et al. (2015). An integrative framework for the appraisal of coloration in nature. *Am. Nat.* 185, 705–724. doi: 10.1086/681021

Kevan, P. G., Chittka, L., and Dyer, A. G. (2001). Limits to the salience of ultraviolet: Lessons from colour vision in bees and birds. *J. Exp. Biol.* 204, 2571–2580. doi: 10.1242/jeb.204.14.2571

Koshitaka, H., Kinoshita, M., Vorobyev, M., and Arikawa, K. (2008). Tetrachromacy in a butterfly that has eight varieties of spectral receptors. *Proc. R. Soc. B* 275, 947–954. doi: 10.1098/rspb.2007.1614

Koski, M. H. (2020a). Macroevolution of flower color patterning: biased transition rates and correlated evolution with flower size. *Front. Plant Sci.* 11:945. doi: 10.3389/fpls.2020.00945

Koski, M. H. (2020b). The role of sensory drive in floral evolution. *New Phytol.* 227, 1012–1024. doi: 10.1111/nph.16510

Krauss, S. L., Phillips, R. D., Karron, J. D., Johnson, S. D., Roberts, D. G., and Hopper, S. D. (2017). Novel consequences of bird pollination for plant mating. *Trends Plant Sci.* 22, 395–410. doi: 10.1016/j.tplants.2017.03.005

Landis, J. B., Bell, C. D., Hernandez, M., Zenil-Ferguson, R., McCarthy, E. W., Soltis, D. E., et al. (2018). Evolution of floral traits and impact of reproductive mode on diversification in the Phlox family (Polemoniaceae). *Mol. Phylogenet. Evol.* 127, 878–890. doi: 10.1016/j.ympev.2018.06.035

Larter, M., Dunbar-Wallis, A., Berardi, A. E., and Smith, S. D. (2018). Convergent evolution at the pathway level: Predictable regulatory changes during flower color transitions. *Mol. Biol. Evol.* 35, 2159–2169. doi: 10.1093/molbev/msy117

Lawrence, W. J. C., Price, J. R., Robinson, G. M., and Robinson, R. (1938). A survey of anthocyanins. *V. Biochem. J.* 32, 1661–1667. doi: 10.1042/bj0321661

Lee, D. W. (2007). *Nature's Palette*. Chicago, IL: University of Chicago Press.

Leonard, A. S., Dornhaus, A., and Papaj, D. R. (2011). Flowers help bees cope with uncertainty: signal detection and the function of floral complexity. *J. Exp. Biol.* 214, 113–121. doi: 10.1242/jeb.047407

Li, B. J., Zheng, B. Q., Wang, J. Y., Tsai, W. C., Lu, H. C., Zou, L. H., et al. (2020). New insight into the molecular mechanism of colour differentiation among floral segments in orchids. *Commun. Biol.* 3:89. doi: 10.1038/s42003-020-0821-8

Lunau, K. (1992). A new interpretation of flower guide colouration: Absorption of ultraviolet light enhances colour saturation. *Plant Syst. Evol.* 183, 51–65. doi: 10.1007/BF00937735

Lunau, K. (2014). Visual ecology of flies with particular reference to colour vision and colour preferences. *J. Comp. Physiol. A* 200, 497–512. doi: 10.1007/s00359-014-0895-1

Lunau, K., and Gerten, S. (2020). Selective attraction in bird-pollinated flowers. A commentary on:'Red flowers differ in shades between pollination systems and across continents'. *Ann. Bot.* 126, iv–v. doi: 10.1093/aob/mcaa144

Lunau, K., Papiorek, S., Eltz, T., and Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *J. Exp. Biol.* 214, 1607–1612. doi: 10.1242/jeb.052688

Lunau, K., Scaccabarozzi, D., Willing, L., and Dixon, K. (2021). A bee's eye view of remarkable floral colour patterns in the south-west Australian biodiversity hotspot revealed by false colour photography. *Ann. Bot.* 2021:mcab088. doi: 10.1093/aob/mcab088

Ma, X., Shi, J., Bänziger, H., Sun, Y., Guo, Y., Liu, Z., et al. (2016). The functional significance of complex floral colour pattern in a food-deceptive orchid. *Funct. Ecol.* 30, 721–732. doi: 10.1111/1365-2435.12571

Mabry, T. J., Markham, K. R., and Thomas, M. B. (1970). *The systematic identification of flavonoids*. New York, NY: Springer.

Maia, R., Gruson, H., Endler, J. A., and White, T. E. (2019). pavo 2: New tools for the spectral and spatial analysis of colour in R. *Methods Ecol. Evol.* 10, 1097–1107. doi: 10.1111/2041-210X.13174

Martins, A. E., Camargo, M. G. G., and Morellato, L. P. C. (2021). Flowering phenology and the influence of seasonality in flower conspicuousness for bees. *Front. Plant Sci.* 11:594538. doi: 10.3389/fpls.2020.594538

Moghe, G. D., and Smith, S. D. (2018). The push and pull of plant specialized metabolism underlies a long-standing, colorful mystery. *New Phytol.* 217, 471–473. doi: 10.1111/nph.14914

Molitor, C., Mauracher, S. G., and Rompel, A. (2016). Aurone synthase is a catechol oxidase with hydroxylase activity and provides insights into the mechanism of plant polyphenol oxidases. *Proc. Nat. Acad. Sci.* 113, E1806–E1815.

Muchhala, N., Johnsen, S., and Smith, S. D. (2014). Competition for hummingbird pollination shapes flower color variation in Andean Solanaceae. *Evolution*, 68, 2275–2286.

Narbona, E., Del Valle, J. C., and Whittall, J. B. (2021). Painting the green canvas: how pigments produce flower colours. *Biochemistry* 43, 6–12. doi: 10.1042/bio_2021_137

Narbona, E., Wang, H., Ortiz, P. L., Arista, M., and Imbert, E. (2018). Flower colour polymorphism in the Mediterranean Basin: occurrence, maintenance and implications for speciation. *Plant Biol.* 20, 8–20. doi: 10.1111/plb.12575

Ng, J., and Smith, S. D. (2016). How to make a red flower: the combinatorial effect of pigments. *AoB Plants* 8:lw013. doi: 10.1093/aobpla/plw013

Ng, J., and Smith, S. D. (2018). Why are red flowers so rare? Testing the macroevolutionary causes of tippiness. *J. Evol. Biol.* 31, 1863–1875. doi: 10.1111/jeb.13381

Ng, J., Freitas, L. B., and Smith, S. D. (2018). Stepwise evolution of floral pigmentation predicted by biochemical pathway structure. *Evolution (N. Y).* 72, 2792–2802. doi: 10.1111/evo.13589

Ödeen, A., and Håstad, O. (2010). Pollinating birds differ in spectral sensitivity. *J. Comp. Physiol. A* 196, 91–96. doi: 10.1007/s00359-009-0474-z

Ogutcen, E., Durand, K., Wolowski, M., Clavijo, L., Graham, C., Glauser, G., et al. (2020). Chemical basis of floral color signals in gesneriaceae: The effect of alternative anthocyanin pathways. *Front. Plant Sci.* 11:604389. doi: 10.3389/fpls.2020.604389

Ohashi, K., Makino, T. T., and Arikawa, K. (2015). Floral colour change in the eyes of pollinators: Testing possible constraints and correlated evolution. *Funct. Ecol.* 29, 1144–1155. doi: 10.1111/1365-2435.12420

Ohmiya, A. (2011). Diversity of carotenoid composition in flower petals. *Japan Agric. Res. Q.* 45, 163–171. doi: 10.6090/jarq.45.163

Ohmiya, A. (2013). Qualitative and quantitative control of carotenoid accumulation in flower petals. *Sci. Hortic. (Amsterdam).* 163, 10–19. doi: 10.1016/j.scienta.2013.06.018

Okitsu, N., Noda, N., Chandler, S., and Tanaka, Y. (2018). "Flower color and its engineering by genetic modification," in *Ornamental Crops*, ed. J. Van Huylenbroeck (Cham: Springer), 29–62. doi: 10.1007/978-3-319-90698-0_3

Ortiz, P. L., Fernández-Díaz, P., Pareja, D., Escudero, M., and Arista, M. (2021). Do visual traits honestly signal floral rewards at community level? *Funct. Ecol.* 35, 369–383. doi: 10.1111/1365-2435.13709

Papiorek, S., Junker, R. R., Alves-dos-Santos, I., Melo, G. A. R., Amaral-Neto, L. P., Sazima, M., et al. (2016). Bees, birds and yellow flowers: Pollinator-dependent convergent evolution of UV patterns. *Plant Biol.* 18, 46–55. doi: 10.1111/plb.12322

Papiorek, S., Rohde, K., and Lunau, K. (2013). Bees' subtle colour preferences: How bees respond to small changes in pigment concentration. *Naturwissenschaften* 100, 633–643. doi: 10.1007/s00114-013-1060-3

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* 170, 23–40. doi: 10.1007/bf00190398

Phillips, R. D., Peakall, R., van der Niet, T., and Johnson, S. D. (2020). Niche perspectives on plant–pollinator interactions. *Trends Plant Sci.* 25, 779–793. doi: 10.1016/j.tplants.2020.03.009

Polturak, G., and Aharoni, A. (2018). "La Vie en Rose": Biosynthesis, sources, and applications of betalain pigments. *Mol. Plant* 11, 7–22. doi: 10.1016/j.molp.2017.10.008

Price, T. D., Stoddard, M. C., Shevell, S. K., and Bloch, N. I. (2019). Understanding how neural responses contribute to the diversity of avian colour vision. *Anim. Behav.* 155, 297–305. doi: 10.1016/j.anbehav.2019.05.009

R Core Team (2021). *R: A Language and Environment for Statistical Computing.* Available online at: http://www.R-project.org

Rausher, M. D. (2008). Evolutionary transitions in floral color. *Inter. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Renoult, J. P., Kelber, A., and Schaefer, H. M. (2017). Colour spaces in ecology and evolutionary biology. *Biol. Rev.* 92, 292–315. doi: 10.1111/brv.12230

Revell, L. J. (2012). phytools: An R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* 3, 217–223. doi: 10.1111/j.2041-210X.2011.00169.x

Reverté, S., Retana, J., Gómez, J. M., and Bosch, J. (2016). Pollinators show flower colour preferences but flowers with similar colours do not attract similar pollinators. *Ann. Bot.* 118, 249–257. doi: 10.1093/aob/mcw103

Ritchie, R. J. (2006). Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. *Photosynth. Res.* 89, 27–41. doi: 10.1007/s11120-006-9065-9

Roguz, K., Gallagher, M. K., Senden, E., Bar-Lev, Y., Lebel, M., Heliczer, R., et al. (2020). All the colors of the rainbow: Diversification of Flower color and intraspecific color variation in the genus Iris. *Front. Plant Sci.* 11:569811. doi: 10.3389/fpls.2020.569811

Roguz, K., Hill, L., Koethe, S., Lunau, K., Roguz, A., and Zych, M. (2021). Visibility and attractiveness of Fritillaria (Liliaceae) flowers to potential pollinators. *Sci. Rep.* 11:11006.

Rohde, K., Papiorek, S., and Lunau, K. (2013). Bumblebees (Bombus terrestris) and honeybees (Apis mellifera) prefer similar colours of higher spectral purity over trained colours. *J. Comp. Physiol. A* 199, 197–210. doi: 10.1007/s00359-012-0783-5

Sakuta, M. (2014). Diversity in plant red pigments: anthocyanins and betacyanins. *Plant Biotechnol. Rep.* 8, 37–48. doi: 10.1007/s11816-013-0294-z

Schoefs, B. (2004). Determination of pigments in vegetables. *J. Chromatogr. A* 1054, 217–226. doi: 10.1016/j.chroma.2004.05.105

Shrestha, M., Lunau, K., Dorin, A., Schulze, B., Bischoff, M., Burd, M., et al. (2016). Floral colours in a world without birds and bees: the plants of Macquarie Island. *Plant Biol. (Stuttg).* 18, 842–850. doi: 10.1111/plb.12456

Smith, S. A., and Brown, J. W. (2018). Constructing a broadly inclusive seed plant phylogeny. *Am. J. Bot.* 105, 302–314. doi: 10.1002/ajb2.1019

Smith, S. D. (2014). Quantifying color variation: Improved formulas for calculating hue with segment classification. *Appl. Plant Sci.* 2:1300088. doi: 10.3732/apps.1300088

Smith, S. D., and Goldberg, E. E. (2015). Tempo and mode of flower color evolution. *Am. J. Bot.* 102, 1014–1025. doi: 10.3732/ajb.1500163

Spaethe, J., Tautz, J., and Chittka, L. (2001). Visual constraints in foraging bumblebees: Flower size and color affect search time and flight behavior. *Proc. Natl. Acad. Sci.* 98, 3898–3903. doi: 10.1073/pnas.071053098

Stavenga, D. G., Leertouwer, H. L., Dudek, B., and van der Kooi, C. J. (2021). Coloration of flowers by flavonoids and consequences of pH dependent absorption. *Front. Plant Sci.* 11:600124. doi: 10.3389/fpls.2020.600124

Stoddard, M. C., and Prum, R. O. (2008). Evolution of avian plumage color in a tetrahedral color space: A phylogenetic analysis of new world buntings. *Am. Nat.* 171, 755–776. doi: 10.1086/587526

Stoddard, M. C., Eyster, H. N., Hogan, B. G., Morris, D. H., Soucy, E. R., and Inouye, D. W. (2020). Wild hummingbirds discriminate nonspectral colors. *Proc. Natl. Acad. Sci.* 117, 15112–15122.

Strack, D., Engel, U., and Reznik, H. (1981). High performance liquid chromatography of betalains and its application to pigment analysis in Aizoaceae and Cactaceae. *Zeitschrift für Pflanzenphysiol.* 101, 215–222. doi: 10.1016/s0044-328x(81)80027-x

Strack, D., Vogt, T., and Schliemann, W. (2003). Recent advances in betalain research. *Phytochemistry* 62, 247–269. doi: 10.1016/S0031-9422(02)00564-2

Strauss, S. Y., and Whittall, J. B. (2006). "Non-pollinator agents of selection on floral traits," in *Ecology and evolution of flowers*, eds L. D. Harder and S. C. H. Barret (Oxford, U.K: Oxford University Press), 120–138.

Sullivan, C. N., and Koski, M. H. (2021). The effects of climate change on floral anthocyanin polymorphisms. *Proc. R. Soc. B Biol. Sci.* 288:20202693. doi: 10.1098/rspb.2020.2693

Tanaka, Y., Sasaki, N., and Ohmiya, A. (2008). Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant J.* 54, 733–749. doi: 10.1111/j.1365-313X.2008.03447.x

Thrane, J. E., Kyle, M., Striebel, M., Haande, S., Grung, M., Rohrlack, T., et al. (2015). Spectrophotometric analysis of pigments: A critical assessment of a high-throughput method for analysis of algal pigment mixtures by spectral deconvolution. *PLoS One* 10:e0137645. doi: 10.1371/journal.pone.0137645

Timoneda, A., Feng, T., Sheehan, H., Walker-Hale, N., Pucker, B., Lopez-Nieves, S., et al. (2019). The evolution of betalain biosynthesis in Caryophyllales. *New Phytol.* 224, 71–85. doi: 10.1111/nph.15980

Troje, N. (1993). Spectral categories in the learning behaviour of blowflies. *Zeitschrift fur Naturforsch. C J. Biosci.* 48, 96–104. doi: 10.1515/znc-1993-1-218

Tunes, P., Camargo, M. G. G., and Guimarães, E. (2021). Floral UV features of plant species from a Neotropical savanna. *Front. Plant Sci.* 12:618028. doi: 10.3389/fpls.2021.618028

Valdés, B. (1970). Flavonoid pigments in flower and leaf of the genus Linaria (Scrophulariaceae). *Phytochemistry* 9, 1253–1260.

van den Berg, C. P., Troscianko, J., Endler, J. A., Marshall, N. J., and Cheney, K. L. (2020). Quantitative Colour Pattern Analysis (QCPA): A comprehensive framework for the analysis of colour patterns in nature. *Methods Ecol. Evol.* 11, 316–332. doi: 10.1111/2041-210X.13328

van der Kooi, C. J., Dyer, A. G., Kevan, P. G., and Lunau, K. (2019). Functional significance of the optical properties of flowers for visual signalling. *Ann. Bot.* 123, 263–276. doi: 10.1093/aob/mcy119

van der Kooi, C. J., Stavenga, D. G., Arikawa, K., Belušič, G., and Kelber, A. (2021). Evolution of insect color vision: From spectral sensitivity to visual ecology. *Annu. Rev. Entomol.* 66, 435–461. doi: 10.1146/annurev-ento-061720-071644

Vorobyev, M., and Brandt, R. (1997). How do insect pollinators discriminate colors? *Israel J. Plant Sci.* 45, 103–113.

Vorobyev, M., and Osorio, D. (1998). Receptor noise as a determinant of colour threshoIds. *Proc. R. Soc. B Biol. Sci.* 265, 351–358. doi: 10.1098/rspb.1998.0302

Wang, Y., Zhang, C., Dong, B., Fu, J., Hu, S., and Zhao, H. (2018). Carotenoid accumulation and its contribution to flower coloration of Osmanthus fragrans. *Front. Plant Sci.* 871:1499. doi: 10.3389/fpls.2018.01499

Warren, J., and Mackenzie, S. (2001). Why are all colour combinations not equally represented as flower-colour polymorphisms? *New Phytol.* 151, 237–241. doi: 10.1046/j.1469-8137.2001.00159.x

Weevers, T. H. (1952). Flower colours and their frequency. *Acta Bot. Neerl.* 1, 81–92.

Wessinger, C. A., Rausher, M. D., and Hileman, L. C. (2019). Adaptation to hummingbird pollination is associated with reduced diversification in Penstemon. *Evol. Lett.* 3, 521–533.

Whitney, K. D., Smith, A. K., White, T. E., and Williams, C. F. (2020). Birds perceive more intraspecific color variation in bird-pollinated than bee-pollinated flowers. *Front. Plant Sci.* 11:590347. doi: 10.3389/fpls.2020.590347

Yoshida, K., Mori, M., and Kondo, T. (2009). Blue flower color development by anthocyanins: From chemical structure to cell physiology. *Nat. Prod. Rep.* 26, 884–915. doi: 10.1039/b800165k

Yoshida, M., Itoh, Y., Ômura, H., Arikawa, K., and Kinoshita, M. (2015). Plant scents modify innate colour preference in foraging swallowtail butterflies. *Biol. Lett.* 11:20150390.

Yuan, H., Zhang, J., Nageswaran, D., and Li, L. (2015). Carotenoid metabolism and regulation in horticultural crops. *Hortic. Res.* 2:15036. doi: 10.1038/hortres.2015.36

Zanne, A. E., Tank, D. C., Cornwell, W. K., Eastman, J. M., Smith, S. A., Fitzjohn, R. G., et al. (2014). Three keys to the radiation of angiosperms into freezing environments. *Nature* 506, 89–92. doi: 10.1038/nature12872

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Ecology and Evolution

REVIEW
published: 15 October 2021
doi: 10.3389/fevo.2021.698137

The Sensory and Cognitive Ecology of Nectar Robbing

Sarah K. Richman[1,2], Jessica L. Barker[3,4], Minjung Baek[5], Daniel R. Papaj[5], Rebecca E. Irwin[6] and Judith L. Bronstein[5]*

[1] Department of Biology, University of Nevada, Reno, Reno, NV, United States, [2] Department of Environmental Systems Science, ETH Zürich, Zurich, Switzerland, [3] Interacting Minds Centre, Aarhus University, Aarhus, Denmark, [4] Division of Public Health, Alaska Department of Health and Social Services, Juneau, AK, United States, [5] Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, United States, [6] Department of Applied Ecology, North Carolina State University, Raleigh, NC, United States

Animals foraging from flowers must assess their environment and make critical decisions about which patches, plants, and flowers to exploit to obtain limiting resources. The cognitive ecology of plant-pollinator interactions explores not only the complex nature of pollinator foraging behavior and decision making, but also how cognition shapes pollination and plant fitness. Floral visitors sometimes depart from what we think of as typical pollinator behavior and instead exploit floral resources by robbing nectar (bypassing the floral opening and instead consuming nectar through holes or perforations made in floral tissue). The impacts of nectar robbing on plant fitness are well-studied; however, there is considerably less understanding, from the animal's perspective, about the cognitive processes underlying nectar robbing. Examining nectar robbing from the standpoint of animal cognition is important for understanding the evolution of this behavior and its ecological and evolutionary consequences. In this review, we draw on central concepts of foraging ecology and animal cognition to consider nectar robbing behavior either when individuals use robbing as their only foraging strategy or when they switch between robbing and legitimate foraging. We discuss sensory and cognitive biases, learning, and the role of a variable environment in making decisions about robbing vs. foraging legitimately. We also discuss ways in which an understanding of the cognitive processes involved in nectar robbing can address questions about how plant-robber interactions affect patterns of natural selection and floral evolution. We conclude by highlighting future research directions on the sensory and cognitive ecology of nectar robbing.

Keywords: cognition, foraging, nectar robbing, pollination, plant reproduction, sensory ecology

OPEN ACCESS

Edited by:
Casper J. Van Der Kooi, University of Groningen, Netherlands

Reviewed by:
David Pritchard, University of Stirling, United Kingdom
Nigel E. Raine, University of Guelph, Canada

****Correspondence:***
Sarah K. Richman
sarahkrichman@gmail.com

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *20 April 2021*
Accepted: *23 September 2021*
Published: *15 October 2021*

Citation:
Richman SK, Barker JL, Baek M, Papaj DR, Irwin RE and Bronstein JL (2021) The Sensory and Cognitive Ecology of Nectar Robbing. Front. Ecol. Evol. 9:698137. doi: 10.3389/fevo.2021.698137

INTRODUCTION

Plant-pollinator mutualisms involve cooperation by each partner but are rife with conflict as well. Although plants and pollinators rely upon one another for reproduction and food resources, respectively, the strategies used by each to maximize fitness are often at odds. Floral nectar, for example, is a carbohydrate-rich reward for pollinators, but functions to attract foragers and direct their activities in a way that benefits plants but not necessarily foragers (Pyke, 2016; van der Kooi et al., 2021). For instance, plants may produce many flowers, each with a small amount of nectar,

to promote multiple visits, even though this may be less efficient for the pollinator (Belsare et al., 2009; Lichtenberg et al., 2020a). This conflict has made plant-pollinator interactions an ideal system to investigate mutualism, with many studies quantifying the relative costs and benefits for the pollinator and the plant.

A subset of the pollination literature focuses on nectar robbing, a floral foraging behavior with wide-ranging reproductive effects on plants (Inouye, 1980; Irwin et al., 2010). While many floral visitors insert their mouthparts through the floral opening to access nectar, often pollinating in the process (a behavior referred to hereafter as "legitimate" foraging), nectar robbers handle flowers in ways that make pollination less likely. Primary robbers make holes or slits in flowers through which they consume nectar, whereas secondary robbers consume nectar through existing holes (Inouye, 1980). The majority of nectar robbing studies focus on bees, but a wide variety of taxa, including birds and mammals, have been reported as nectar robbers (Irwin et al., 2010). Almost all plant species with hidden or recessed nectar in tubular corollas or spurs experience nectar robbing, with up to 100% of flowers robbed per plant (Irwin and Maloof, 2002). Given the ubiquity of the behavior, as well as the breadth in taxonomy and breeding systems of plants that are robbed, nectar robbing has the potential to strongly influence the ecology and evolution of pollination mutualisms.

Both plant ecologists and behavioral ecologists have studied nectar robbing; however, their respective foci and research questions have overlapped relatively little. The majority of nectar-robbing studies have taken the plant's perspective, testing the effects of nectar-robbing on plant individuals and populations, exploring the direct and pollinator-mediated indirect mechanisms by which those effects occur, and investigating the community contexts that affect robbing frequencies (Irwin et al., 2010; Rojas-Nossa et al., 2016; Fitch and Vandermeer, 2020). In contrast, a small but growing body of research on nectar robbing has taken the animal's perspective (Bronstein et al., 2017; **Table 1**). This work focuses primarily on the costs and benefits of robbing behavior to the forager itself, quantified in terms of energetics (Dedej and Delaplane, 2005; Zhang et al., 2011; Hazlehurst and Karubian, 2018). Findings point to some floral visitor-plant combinations in which robbing is more energetically profitable for the visitor than is legitimate visitation, and to other visitor-plant combinations that show the reverse (Lichtenberg et al., 2018).

Studies of nectar robbing that have taken the floral visitor's perspective have generally not considered the cognitive mechanisms that underlie visitors' foraging decisions (but see, e.g., Leadbeater and Chittka, 2008; Barker et al., 2018; **Table 1**). For example, while there are many reports in the literature of bees using a mix of robbing and legitimate foraging tactics (**Figure 1**), and while work has investigated the role of competition in tactic choice (Lichtenberg et al., 2020b), the cognitive mechanisms underlying these behaviors are largely unknown. We argue here that a deeper exploration into the sensory and cognitive processes involved in nectar robbing is needed to gain insight into the ecological and evolutionary causes of the behavior, as well as its consequences for plants. Toward this end, we can appeal to a growing literature on sensory and cognitive ecology, including a substantial body of work focusing on pollinator behavior (Chittka and Thomson, 2001; Schiestl and Johnson, 2013; Baracchi, 2019; Lihoreau et al., 2019; van der Kooi et al., 2021). This work has made significant inroads into characterizing the mechanisms that govern decision-making by floral visitors. We argue that it can be applied to nectar robbing as well as legitimate visitation.

Here, we extend the pollinator behavior literature to consider the sensory and cognitive processes that underlie nectar robbing. In doing so, we highlight ways in which variation across the floral resource landscape, such as in nectar standing crop, may promote or discourage nectar robbing behavior. We also discuss how incorporating sensory and cognitive biology into theoretical and empirical research frameworks can benefit plant evolutionary ecologists studying nectar robbing. Throughout this review, we highlight critical gaps in knowledge awaiting exploration, and we conclude by featuring two promising directions for future research.

WHAT SENSORY AND COGNITIVE PROCESSES ARE INVOLVED IN NECTAR ROBBING?

Floral visitors seeking nectar are bombarded by sensory information about their foraging environment. An individual visitor is presented with an array of floral traits that vary both within and among plant species and must choose a rewarding option. The challenge for foragers, given the onslaught of information they receive, is to integrate across sensory modalities to perceive and process their environment, and to make decisions about how and where to forage (i.e., perform cognitive functions; Webb, 2012). The task of finding floral rewards is complicated by the fact that floral traits can vary with the environment. For example, abiotic conditions such as water availability may alter a flower's scent profile or the volume or concentration of nectar (Parachnowitsch et al., 2019). Further, over the course of the day, flowers are asynchronously drained of nectar by other foragers, erasing nectar standing crop differences that might have existed among flowers earlier in the day (Lichtenberg et al., 2020a). A fluctuating environment requires foragers to rely on innate sensory biases, and ultimately make decisions based on experience and learning (Lichtenberg et al., 2020a). We first discuss such biases. Second, we discuss learning by floral visitors, and how primary and secondary nectar robbers might learn these foraging behaviors. Finally, we explore how innate and learned behaviors ultimately affect decision-making, highlighting ways that environmental variation can complicate the decision-making process and/or require more complicated exercises, such as task switching. We focus here primarily on bees, as their behavior in the context of decisions to nectar-rob has been studied in the greatest detail.

Sensory Biases of Floral Visitors

Naïve animals possess evolved, innate sensory responses that form a foundation for higher-level cognitive functioning (Webb, 2012). The pollination literature has extensively documented sensory biases of floral foragers. For example, naïve *Bombus*

TABLE 1 | Sensory and cognitive processes involved in nectar robbing, including predictions, key findings and citations.

Sensory/cognitive process	Factors that might affect process	Predictions	Exemplar studies	Citations
Sensory bias (innate preference)	Floral traits, Forager evolutionary history	Floral signals will not match sensory biases of nectar robbers.	Floral nectar guides discouraged secondary nectar robbing in bees.	Leonard and Papaj, 2011
Learning				
Location learning	Floral traits	Nectar robbers will learn floral traits associated with the location of robbing damage.	Bumble bees primary-robbed flowers consistently on their left or right sides; behavior attributed to visual cue of robbing holes in other flowers (although social demonstration was not ruled out).	Goulson et al., 2013
Instrumental learning	Complexity of motor routines	Robbing behavior will be learned through trial and error	Exposure to an artificial robbing hole facilitated primary robbing behavior in bumble bees.	Leadbeater and Chittka, 2008
Transfer and interference	Plant community assemblage	Handling tactics that increase transfer and decrease interference in a given environment will be preferred.	Not studied explicitly in nectar robbing context.	n/a
Decision making				
Previous experience	Flowering phenology, Plant community assemblage	Pollinators will choose tactics based on their prior experience.	Bumble bees randomly assigned to legitimate visitation or nectar robbing tended to choose their previously experienced tactics later.	Barker et al., 2018
Risk assessment	Resource quality, Resource variability, Hunger state	Pollinators will choose tactics based on their relative benefit.	Visitors robbed flowers in the wild when nectar robbing was more efficient than legitimate visitation.	Lichtenberg et al., 2018
Switching between two tactics	Investment in learning, Working memory capacity	If nectar robbing is easier to learn or affected less by working memory capacity than foraging legitimately, robbers will tend to switch more between plant species.	Not studied in the context of tactic constancy, but floral constancy literature exists.	n/a

Cognitive processes are listed in the order discussed in the review; we consider multiple levels of analysis non-exhaustively.

FIGURE 1 | A honey bee (*Apis mellifera*) using different foraging tactics to consume nectar from a pointleaf manzanita (*Arctostaphylous pungens*) flower. The bee first uses a legitimate foraging tactic (left) before switching to secondary nectar robbing (right). Photos by Carl Hutter.

terrestris bumble bees consistently show a preference for colors at the blue-violet end of the visual light spectrum, and *Bombus* spp. are commonly associated with blue-violet flowers in nature (Eidesen et al., 2017). The association can improve bee foraging efficiency, perhaps driving selection for innate biases (Raine and Chittka, 2007b). Bees show a propensity to orient toward divided patterns. The star-like form of a daisy, generated by its ray flowers, is particularly attractive (Howard et al., 2019). It is not currently known whether nectar robbing differs from legitimate visitation in terms of how individuals express innate

preferences for floral traits, or whether sensory biases (for color, odor, etc.) drive their foraging behavior in the same way. Drawing from the literature on pollinator sensory ecology, however, we are able to generate hypotheses about the extent to which nectar robbing stems from a different set of sensory responses.

Evaluating sensory biases in the context of nectar robbing requires an understanding of how they develop, function, and evolve. We expect floral signals mediating legitimate visitation to be matched to pollinators' sensory biases, since plants benefit by soliciting visits (Schiestl, 2017). However, except in the case in which robbing is beneficial to the plant (e.g., Maloof and Inouye, 2000), no such match would be expected between biases and the cues used to rob a flower. In fact, we might expect evolution to favor plant traits that conceal robbing sites or that make them less conspicuous to the robber, such as shorter corollas or larger calyces (Irwin et al., 2010). It is therefore reasonable to surmise that nectar robbing emerges in response to a different set of sensory biases than legitimate foraging, and/or that it has evolved from biological processes unrelated to foraging for floral rewards. One such process is mate choice (Schiestl and Johnson, 2013). Bees show innate preferences for dots or small circles contrasted against a background, possibly because these resemble individual mates or aggregations of mates (Van Kleunen et al., 2007; Ellis and Johnson, 2010). Secondary-robbing bees may be innately attracted to robber holes or slits because they resemble such markings. Sensory biases have long been thought to play a role in sexual selection (Dawkins and Guilford, 1996; Fuller et al., 2005), and more recently have been argued to influence foraging decisions (Parachnowitsch et al., 2019), including floral visitation (Schiestl and Johnson, 2013; Schiestl, 2017). A major question, moving forward, is the degree to which non-foraging and foraging sensory biases influence each other, and how selection for sensory biases may act in pollinator populations with different foraging behaviors.

Learning to Rob: Floral Cues and Motor Routines

Floral visitation, like any behavior, has learned as well as innate components. Learned behavior is generally functional, meaning in this case that, through experience, pollinators improve their ability to collect floral rewards. We expect learning to be an important component of nectar robbing behavior as well. Two basic forms of learning during nectar robbing are particularly pertinent: (1) Learning cues that identify places on flowers suitable for robbing, and (2) learning motor routines that mediate nectar extraction through robbing holes. We first briefly address learning of suitable robbing cues. We then discuss in more detail how motor routines used in robbing might be learned.

Nectar robbers generally perforate flowers, or feed from pre-existing perforations, at the base of a flower, usually near the nectary or where nectar accumulates in flower spurs (Irwin et al., 2010). Therefore, robbers might learn to identify cues associated with the base of the corolla close to where nectar is located through visual, chemical, and/or tactile means. Studies aiming to uncover how individuals learn to rob, although limited, generally point to discrimination learning, defined here as "the formation of associations between different stimuli and corresponding outcomes or behaviors" (Rose and Schmidt, 2012). One interesting implication of this work is that individual foragers may use discrimination learning to associate certain cues with legitimate foraging and others with nectar robbing. This would explain the widespread intraspecific (and sometimes intraindividual) variation in robbing behavior across taxa observed in nature (Richardson and Bronstein, 2012). Such choice discrimination may enable foragers to rob flowers when robbing is beneficial to them but visit flowers legitimately when that behavior carries the higher benefit; for example, when a flower has not completely opened and therefore nectar is better accessed through robbing.

Learning can also account for the use of different motor routines in different contexts, by linking specific stimuli with specific routines. Such learning is directly relevant to the expression of nectar robbing and legitimate visitation, as nectar robbing requires a different set of motor capabilities than legitimate foraging. For example, primary robbing involves exerting enough force on a flower to puncture petal tissue, and both primary and secondary robbing require foragers to orient themselves on the outside of flowers rather than at the opening of the flower. The visitor might learn simply that piercing the corolla with its mouthparts yields a sugar reward. Such learning would be indicative of instrumental learning, in which an action is acquired and shaped by a contingency between the action and the reward (Dickinson, 1994). Many studies of instrumental learning focus on operant conditioning, a procedure in which the experimenter makes the presentation of a reward contingent upon an animal's actions (Chittka and Thomson, 1997). However, these studies generally focus only on legitimate foraging; the motor routines involved in robbing may not be elucidated from them. Therefore, we advocate for more studies that compare and contrast learning legitimate foraging, primary robbing, and secondary robbing.

It is easy to imagine that successfully acquiring nectar via robbing reinforces robbing movements; however, it is also possible that for visitors that can both rob and visit flowers legitimately, robbing reflects in part learning to *not* visit legitimately if attempts to do so yield little or no nectar. Initial experience with one behavior instead of another may arise by chance and can influence the extent to which a visitor will sample and learn the second behavior (Barker et al., 2018). Innovation (Tebbich et al., 2016) may be involved, wherein bees try out novel motor movements that might facilitate robbing. For secondary robbers, inserting their mouthparts into the already existing hole might be sufficient to initiate robbing; in contrast, primary robbers have to find the right place to make a hole, and then chew/cut that hole. Initiating the additional motor action required for primary robbing has been shown to be hastened by exposure to robber holes: in a lab study, primary robbing behavior took less time to initiate for naïve bees that were exposed to artificial holes compared to those that were not, suggesting a role of social transmission in learning nectar robbing motor routines (Leadbeater and Chittka, 2008).

Decision-Making Based on Learning and Memory

Both innate and learned processes give floral visitors the ability to extract food from flowers. Given the array of signals from different flowers that they experience in nature, visitors must then decide which flowers to extract food from, and how. As floral signals vary considerably over space and/or time, decision-making can be cumbersome and expensive. In the early stage of learning about a novel plant species, foragers might have to spend time and energy to assess its quality (Grüter and Ratnieks, 2011) and be less efficient in exploiting its nectar (Laverty, 1994). After they have learned to find and use different plant species, limitations on short-term memory capacity might prevent recall of many search images (Goulson, 2000; Raine and Chittka, 2007a; Ishii and Masuda, 2014) or retrieval of many handling routines from long-term memory (Woodward and Laverty, 1992; Dukas, 1995). Therefore, it would benefit foragers to develop cognitive strategies allowing them to cope with an abundance of information.

Many animals use cognitive heuristics and shortcuts to make decisions in a variable environment, which, although they can seem irrational in the traditional economic sense (when considering short-term costs and benefits), likely evolved to allow animals to make sense of a world with too much information to process (Johnson et al., 2013; Marshall et al., 2013; Vasconcelos et al., 2015; Lichtenberg et al., 2020a). Floral visitors assess the available options not just on their absolute costs and benefits, but relative to the other options that are currently available. For example, honey bees' choices between two foraging options have been shown to be influenced by the introduction of a third option, even if it is less rewarding than the first two (Shafir et al., 2002). Similarly, bumble bees that underperformed in two-color discrimination task were more flexible in sampling a third, novel color (Evans and Raine, 2014). As the nectar-foraging landscape is constantly changing (Lichtenberg et al., 2020a)—open flowers are visited by robbing and legitimate foragers, depleting nectar, while flowers continue to open—we would expect foragers to use such cognitive heuristics and shortcuts to decide which flowers to visit. For example, evaluating flowers on their relative, rather than absolute, rewards may result in foragers choosing options that seem to violate economic cost-benefit analysis (Biernaskie et al., 2009).

Experience and risk assessment are two further examples of how cognitive heuristics influence floral foragers' decisions (Chittka et al., 2003). For example, bumble bees' initial experience with either robbing or legitimate visitation, even if serendipitous, discourages them from attempting the tactic they have not experienced (Barker et al., 2018). We can liken this decision to that of a forager choosing between two resource types, one that it has experienced and one that it has not. Furthermore, a forager's decision threshold can change depending on the current availability of the resources (Hodges, 1985), or their variability in quality and/or quantity (Keasar et al., 2013). Floral visitors' decisions are also affected by how risky each option is likely to be, and how the animal assesses the uncertainty of other options (Kacelnik and El Mouden, 2013). The extent to which a forager is risk averse depends on its current hunger state, as well as its perception of fitness consequences of each choice (Chittka et al., 2003; Houston et al., 2014). These physiological and perception factors would affect nectar-robbing decisions if robbing and legitimate visitation do not reliably provide nectar rewards of the same volume or concentration. For example, robbed flowers often have lower nectar volumes and/or higher nectar concentrations than unrobbed flowers (Pleasants, 1983; Newman et al., 2005). When this is the case, a forager might encounter different rewards when secondary robbing vs. when foraging legitimately on a previously unrobbed flower. How the relative risks and rewards of robbing vs. visiting legitimately affect the decision to adopt these behaviors is not known for any system of which we are aware. It is an important area for future research.

For floral visitors potentially able to use either tactic, the conundrum they face over whether and when to adopt legitimate foraging vs. robbing is a problem of task switching. Pollinators that switch frequently between two tasks have been reported to suffer reduced performance on both tasks compared to pollinators conducting only one of the two tasks (Monsell, 2003; Kiesel et al., 2010; Caselli and Chelazzi, 2011). The cost of task switching by insect pollinators has been evaluated both with respect to foraging on flowers with different morphologies (Lewis, 1986; Woodward and Laverty, 1992; Laverty, 1994; Chittka et al., 1999; Goulson, 2000) and to switching between nectar foraging and egg-laying, usually in butterflies (Stanton, 1984; Weiss and Papaj, 2003). We propose that the same considerations of costs can be extended to switching between legitimate foraging and nectar robbing.

Recent studies of tactic constancy of nectar robbers—akin to floral constancy—offer an example of this approach. Floral constancy, the consistent visitation to one species or floral morph even if other equally or more rewarding options are available, is often interpreted as a consequence of costs associated with switching from one option to another (Waser, 1979; Chittka et al., 1999). Costs of switching may pertain to nectar robbing and legitimate visitation. Do floral visitors show tactic constancy (i.e., consistent use of one tactic over another), suggestive of the possibility of costs of task switching? Use of a combination of tactics has been documented at the species level (Johnson et al., 2013; Marshall et al., 2013; Lichtenberg et al., 2020b). Switching between tactics at the individual level has also been reported, although at least sometimes at low frequencies (Richardson and Bronstein, 2012; Richman et al., 2017a). At present, the frequency of switching at the individual level is too poorly explored to draw generalizations. Similarly, we currently know too little about the factors predicting when switching would take place (Bronstein et al., 2017). It is logical to suppose that tactic choice will be governed by the relative gains an individual receives from each food handling tactic (Biernaskie et al., 2009; Lichtenberg et al., 2018). This hypothesis was addressed in a recent study on competition for nectar when standing crop is highly variable. That study found no connection between competition intensity and the probability that an individual would switch tactics (Lichtenberg et al., 2020b).

Since assessing the relative gains of each food handling tactic may require time investment, another hypothesis is that

organisms exhibit an ontogenetic shift in tactic constancy. Younger individuals may be more likely to switch as they seek and acquire information about different tactics, whereas older individuals may be more likely to settle in on one tactic as they become more efficient over time in one handling tactic, leading to an ontogenetic pattern of shift in tactic constancy. To our knowledge, this hypothesis has not been tested.

Limitation on memory capacities could also play a role in tactic constancy. When animals must learn two motor routines in succession, memory of one sometimes inhibits learning and performance of the other, which is called an interference effect (Bouton, 1993; Dukas, 1995; Bond and Kamil, 1999). For example, even if pollinators can successfully store more than two flower handling motor routines in their long-term memory, retrieving those memories for behavioral execution might be interfered with switching from one motor routine to another (Chittka et al., 1999). Although little or no interference has been shown when pollinators switch among flowers with simple morphologies during foraging (Laverty, 1994; Raine and Chittka, 2007a), switching among flowers with complex/different morphologies yielded increased flower handling time (Woodward and Laverty, 1992; Laverty, 1994; Raine and Chittka, 2007a), suggesting the similarities of flower handling motor routines may affect the degree of interference. In contrast to the interference effect, learning in one context may improve performance in another context, indicative of a transfer effect (Perkins and Salomon, 1992). For example, foragers can learn an appropriate motor routine to exploit nectar from a specific flower type (Laverty, 1980, 1994), which then may be generalized to other, similar flower types, helping to exploit new flower types.

CONSIDERING COGNITION IN PLANT-FOCUSED STUDIES

Floral visitors express foraging preferences and make decisions, including whether to legitimately forage or to rob nectar, underlaid by a complex suite of sensory and cognitive mechanisms, as discussed above. These behaviors can have profound consequences for plants. While many studies have tested how robbing affects plant fitness, few have considered how the cognitive processes underpinning nectar robbers' decisions may be relevant to their experimental designs. There are some situations in which ignoring robber cognition would have little impact on interpretation of results. For example, if robbers select plants at random to rob and if robbers damage flower reproductive organs when they rob, assigning robbing treatments to plants at random and mimicking robbing damage at realistic levels (e.g., Zhang et al., 2007; Castro et al., 2008; Richman et al., 2017b) should provide an accurate assessment of robbing effects on plant fitness. However, in other cases, a lack of consideration of robber cognition could lead to misinterpretation of findings. For instance, using a randomized experimental approach removes any covariance between plant vigor and robbing levels, for example if robbers select larger or more fecund plants (e.g., Irwin, 2006). Therefore, an experimenter using an artificial robbing approach would need to consider whether they should also try to mimic robbing damage based on behavioral patterns instead of at random. Below, we provide two additional scenarios in which an understanding of the sensory and cognitive processes involved in nectar robbing can inform studies of plant reproduction.

First, it seems unlikely that robbers select flowers and plants to rob at random. Instead, like legitimate floral visitors, robbers likely use floral traits to select flowers and plants to rob (Gélvez-Zúñiga et al., 2018; Wu et al., 2019), and therefore would (1) exhibit sensory and cognitive biases and (2) rely on previous experience, as discussed above. In scenarios where robbers also act as legitimate visitors, either at the individual or population level, it is plausible that similar cognitive processes and constraints mediate how visitors select among flowers in legitimate and robbing visits. This scenario could play out either when foragers use both tactics on the same plant species or if their experience with one plant species carries over to affect behavior at another plant species. In either scenario, plants must contend with visits from both robbers and legitimate visitors based upon the same set of cues, so when robbing reduces plant fitness, they may experience tradeoffs in the attraction of legitimate vs. robbing visits (Gélvez-Zúñiga et al., 2018). Alternatively, if floral signals used by visitors differ when they use legitimate vs. robbing tactics (as may be the case, as discussed above), plants may have a greater opportunity to discourage robbing and encourage legitimate visits, in scenarios where robbing reduces fitness. For instance, legitimate visitation could involve responses to cues at the front of flowers, while robbing tactics may involve greater responses to cues at the side or base of flowers. These differences in acquired stimuli could affect the decision to visit particular flowers and ultimately flower choice depending on the tactic used. Thus, the results of cognitive processes with different stimuli could lead to a scenario in which robbing visits occur on some flowers or plants, but legitimate visits on others, such that robbing could reinforce patterns of pollinator-mediated selection. No study to our knowledge has characterized the underlying cognitive mechanisms that underlie flower choice when visitors rob vs. visit legitimately, and the subsequent implications for plant fitness and patterns of natural selection on floral traits.

Second, the extent to which floral foragers are tactic-constant should determine plant reproductive success. While still not entirely understood, tactic constancy is driven by cognition, and so an understanding of robber cognition will help generate hypotheses about how the behavior is expressed and how it affects plants. As discussed above, evidence is accumulating that floral visitors exhibit tactic constancy, i.e., constancy to one foraging tactic, either legitimate or robbing visits (Bronstein et al., 2017; Lichtenberg et al., 2020b). Consider a forager on a plant species whose flowers it can legitimately visit and pollinate, or else rob and fail to pollinate (reviewed in Irwin et al., 2010; Bronstein et al., 2017). If foragers rob a series of flowers (short-term tactic constancy), punctuated by periodic legitimate (pollinating) visits, such behavior could serve to reduce geitonogamy (within-plant pollen transfer) and increase pollen flow distances and outcrossing as the number of flowers between

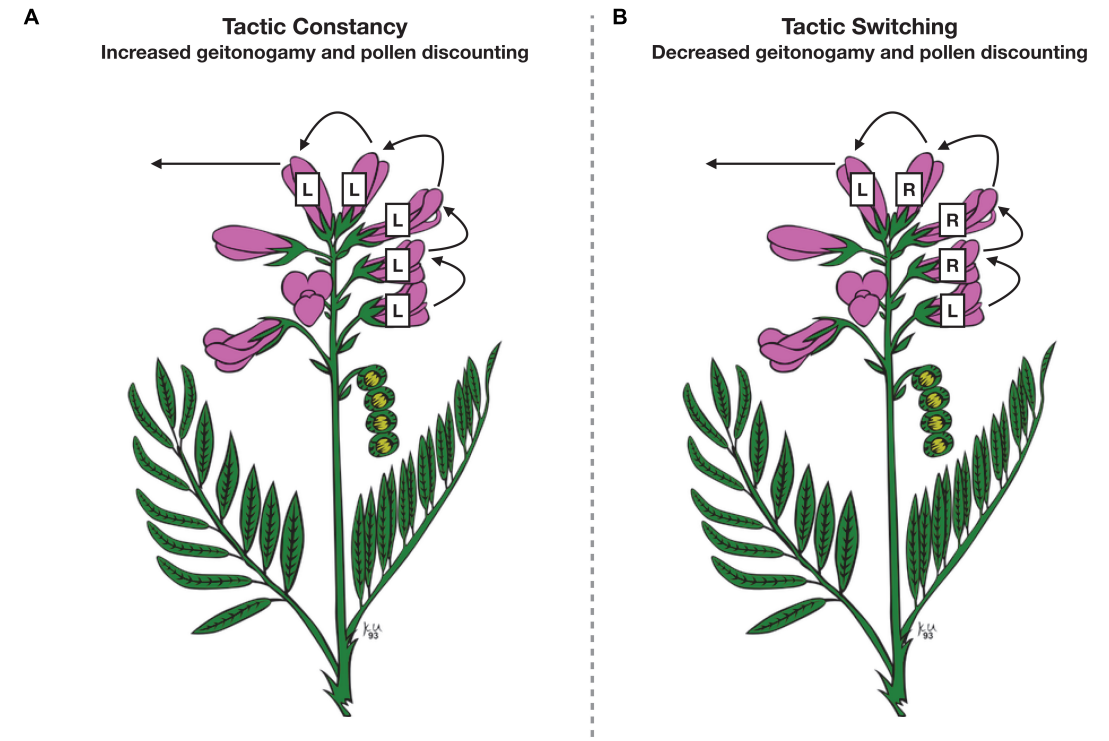

FIGURE 2 | Hypothesized effects of tactic constancy vs. tactic switching on pollen flow. Each panel shows a visitation pattern of a floral forager; flowers labeled "L" receive legitimate foraging visits and flowers labeled "R" receive robbing visits (primary or secondary). Floral visitors that remain constant to legitimate foraging **(A)** have a higher potential of depositing geitonogamous self-pollen, which can lead to increased pollen discounting. Floral visitors that switch between legitimate foraging and nectar robbing **(B)** bypass the stigmas of more flowers per plant than a constant legitimate forager, reducing the potential of depositing geitonogamous self-pollen and of pollen discounting. Flower drawing by K. Urban, available under Creative Commons licensing.

two legitimate foraging visits increases (**Figure 2**). Increased outcrossing has previously been hypothesized to be a potential benefit to plants of being robbed (e.g., Zimmerman and Cook, 1985; Maloof, 2001). However, the proposed mechanism was completely different: it was hypothesized that pollinators would fly further after visiting a robbed flower with lower nectar reward, relative to an unrobbed flower with higher nectar reward, in an effort to escape an unrewarding flower patch. This behavioral pattern would presumably increase pollen flow and outcrossing. In our scenario, tactic constancy, and its underlying cognitive mechanism, indirectly affects pollen flow and outcrossing, a result that would not have been captured by experimentally robbing flowers and recording legitimate visitation. While, as we point out above, tactic constancy has been observed (Bronstein et al., 2017; Lichtenberg et al., 2020b), the degree to which it affects pollen flow and outcrossing remains to be explored.

CONCLUSION

In many ways, nectar robbing resembles any other floral foraging tactic. Floral visitors use signals and cues provided by flowers, coupled with information about their foraging environment, to make decisions about which flowers to visit and how to extract their rewards. Following this logic, we would expect the basic sensory and cognitive processes underlying nectar robbing to overlap substantially with those underlying legitimate foraging. However, as we have pointed out in this review, the ways in which nectar robbers and legitimate foragers react to stimuli may differ. Furthermore, because the motor routines for each tactic differ, nectar robbers and legitimate foragers may also differ in how they learn flower handling.

Many issues central to our understanding of the cognitive ecology of nectar-robbing remain unexplored. One open question is the extent to which the decision to rob is economically "rational," resulting in a higher net benefit to the forager than other foraging options. As accumulating research, discussed above, reveals that foragers make decisions that violate traditional "rationality" (e.g., context-dependent preferences), we would expect to see this reflected in robbing behavior. Another question is the extent to which robbing motor routines are innate vs. learned. The literature on cognitive ecology and pollination has provided a rich body of work describing innate preferences (sensory biases) for flower color, shape, scent, and reward properties (Schiestl and Johnson, 2013; Schiestl, 2017). Although nectar robbers likely show many of the same preferences in deciding which flowers to visit, how they develop flower handling tactics after deciding on a flower remains largely unknown. Controlled experiments in the laboratory using naïve foragers are one way to begin answering this question. For instance,

experiments could be designed to test the hypothesis that a primary robbing motor routine develops when a forager encounters flowers that are not fully open or too narrow to enter (Rivera et al., 2006), predicting a higher probability of primary robbing when bees are presented with a high frequency of closed, partially open, or narrow flowers. This experiment could be conducted using captive, naïve bees provided with arrays of closed vs. open flowers or of flowers with narrow vs. broad corollas. In order to distinguish whether development of a motor routine was indicative of learning to rob vs. choosing between two tactics, researchers could compare the degree of trial and error in naïve and experienced bees; if learning to rob, the difference in trial and error between naïve and experienced bees should be greater than if bees are choosing between tactics.

The overwhelming majority of studies on the cognitive ecology of floral visitation, whether in the context of pollination or nectar robbing, have been conducted using bumble bees (*Bombus* spp.) and honey bees (*Apis mellifera*), both social species, as study subjects. As we have pointed out, however, the taxonomic breadth of organisms reported to be nectar robbers is high, spanning multiple phyla and classes within phyla. We advocate for broadening the representation of taxa in experimental studies of robbing to match the breadth of species exhibiting robbing behaviors. Recent advances in the laboratory rearing of solitary bee species such as *Xylocopa virginica* (the Eastern carpenter bee) for experiments in lab, field, or semi-field settings allow researchers to compare and contrast nectar robbing in bees with solitary vs. social life histories (that are likely to have evolved different cognitive processes). Hummingbirds have been well studied in terms of context-dependent decision-making and risk sensitivity (e.g., Hurly and Oseen, 1999; Morgan et al., 2014), which could be used as the foundation for studies of nectar robbing. In addition, manipulative studies using captive or semi-captive nectar robbing birds, such as those in the genus *Diglossa* (flowerpiercers, e.g., Schondube and Del Rio, 2003), will be particularly important for expanding beyond insects our understanding of cognition as it relates to nectar robbing. By doing so, we will be able to have a stronger understanding of the generality of nectar robbing behavior, as well as begin to comprehend its evolutionary origins.

AUTHOR CONTRIBUTIONS

JBa conceived of the idea for the article. All authors contributed to providing content, which was organized and curated by SR.

FUNDING

Some of the work reported here was supported by grants from the U.S. National Science Foundation (DEB-1354061/1641243 to RI and JBr).

ACKNOWLEDGMENTS

A. Dornhaus and F. Muth provided key ideas that shaped this article. We thank two reviewers for their valuable feedback on an earlier version of this manuscript.

REFERENCES

Baracchi, D. (2019). Cognitive ecology of pollinators and the main determinants of foraging plasticity. *Curr. Zool.* 65, 421–424. doi: 10.1093/cz/zoz036

Barker, J. L., Dornhaus, A., Bronstein, J. L., and Muth, F. (2018). Learning about larceny: experience can bias bumble bees to rob nectar. *Behav. Ecol. Sociobiol.* 72:68. doi: 10.1007/s00265-018-2478-6

Belsare, P. V., Sriram, B., and Watve, M. G. (2009). The co-optimization of floral display and nectar reward. *J. Biosci.* 34, 963–967. doi: 10.1007/s12038-009-0110-7

Biernaskie, J. M., Walker, S. C., and Gegear, R. J. (2009). Bumblebees learn to forage like Bayesians. *Am. Nat.* 174, 413–423. doi: 10.1086/603629

Bond, A. B., and Kamil, A. C. (1999). Searching image in blue jays: facilitation and interference in sequential priming. *Anim. Learn. Behav.* 27, 461–471. doi: 10.3758/BF03209981

Bouton, M. E. (1993). Context, time, and memory retrieval in the interference paradigms of Pavlovian learning. *Psychol. Bull.* 114, 80–99. doi: 10.1037/0033-2909.114.1.80

Bronstein, J. L., Barker, J. L., Lichtenberg, E. M., Richardson, L. L., and Irwin, R. E. (2017). The behavioral ecology of nectar robbing: why be tactic constant? *Curr. Opin. Insect Sci.* 21, 14–18. doi: 10.1016/j.cois.2017.05.013

Caselli, L., and Chelazzi, L. (2011). Does the macaque monkey provide a good model for studying human executive control? A comparative behavioral study of task switching. *PLoS One* 6:e21489. doi: 10.1371/journal.pone.0021489

Castro, S., Silveira, P., and Navarro, L. (2008). Consequences of nectar robbing for the fitness of a threatened plant species. *Plant Ecol.* 199, 201–208. doi: 10.1007/s11258-008-9424-z

Chittka, L., and Thomson, J. D. (1997). Sensori-motor learning and its relevance for task specialization in bumble bees. *Behav. Ecol. Sociobiol.* 41, 385–398. doi: 10.1007/s002650050400

Chittka, L., and Thomson, J. D. (eds) (2001). *Cognitive Ecology of Pollination: Animal Behaviour and Floral Evolution*. Cambridge: Cambridge University Press. doi: 10.1017/CBO9780511542268

Chittka, L., Dyer, A. G., Bock, F., and Dornhaus, A. (2003). Bees trade off foraging speed for accuracy. *Nature* 424:388. doi: 10.1038/424388a

Chittka, L., Thomson, J. D., and Waser, N. M. (1999). Flower constancy, insect psychology, and plant evolution. *Naturwissenschaften* 86, 361–377. doi: 10.1007/s001140050636

Dawkins, M. S., and Guilford, T. (1996). Sensory bias and the adaptiveness of female choice. *Am. Nat.* 148, 937–942. doi: 10.1086/285964

Dedej, S., and Delaplane, K. (2005). Net energetic advantage drives honey bees (*Apis mellifera* L) to nectar larceny in *Vaccinium ashei* Reade. *Behav. Ecol. Sociobiol.* 57, 398–403. doi: 10.1007/s00265-004-0852-z

Dickinson, A. (1994). "Instrumental conditioning," in *Animal Learning and Cognition*, ed. N. J. Mackintosh (Amsterdam: Elsevier), 45–79. doi: 10.1016/B978-0-08-057169-0.50009-7

Dukas, R. (1995). Transfer and interference in bumblebee learning. *Anim. Behav.* 49, 1481–1490. doi: 10.1016/0003-3472(95)90069-1

Eidesen, P. R., Little, L., Müller, E., Dickenson, K. J. M., and Lord, J. M. (2017). Plant-pollinator interactions affect colonization efficiency: abundance of blue-purple flowers is associated with species richness of bumblebees in the Arctic. *Biol. J. Linn. Soc.* 121, 150–162.

Ellis, A. G., and Johnson, S. D. (2010). Floral mimicry enhances pollen export: the evolution of pollination by sexual deceit outside of the Orchidaceae. *Am. Nat.* 176, E143–E151. doi: 10.1086/656487

Evans, L. J., and Raine, N. E. (2014). Foraging errors play a role in resource exploration by bumble bees (*Bombus terrrestris*). *J. Comp. Physiol. A* 200, 475–484. doi: 10.1007/s00359-014-0905-3

Fitch, G., and Vandermeer, J. H. (2020). Light availability influences the intensity of nectar robbery and its effects on reproduction in a tropical shrub via multiple pathways. *Am. J. Bot.* 107, 1635–1644. doi: 10.1002/ajb2.1559

Fuller, R. C., Houle, D., and Travis, J. (2005). Sensory bias as an explanation for the evolution of mate preferences. *Am. Nat.* 166, 437–446. doi: 10.1086/444443

Gélvez-Zúñiga, I., Teixido, A. L., Neves, A. C. O., and Fernandes, G. W. (2018). Floral antagonists counteract pollinator-mediated selection on attractiveness traits in the hummingbird-pollinated *Collaea cipoensis* (Fabaceae). *Biotropica* 50, 797–804. doi: 10.1111/btp.12574

Goulson, D. (2000). Are insects flower constant because they use search images to find flowers? *Oikos* 88, 547–552.

Goulson, D., Park, K. J., Tinsley, M. C., Bussière, L. F., and Vallejo-Marin, M. (2013). Social learning drives handedness in nectar-robbing bumblebees. *Behav. Ecol. Sociobiol.* 67, 1141–1150.

Grüter, C., and Ratnieks, F. L. W. (2011). Flower constancy in insect pollinators: adaptive foraging behaviour or cognitive limitation? *Commun. Integr. Biol.* 4, 633–636. doi: 10.4161/cib.16972

Hazlehurst, J. A., and Karubian, J. O. (2018). Impacts of nectar robbing on the foraging ecology of a territorial hummingbird. *Behav. Process.* 149, 27–34. doi: 10.1016/j.beproc.2018.01.001

Hodges, C. M. (1985). Bumble bee foraging: the threshold departure rule. *Ecology* 66, 179–187. doi: 10.2307/1941318

Houston, A. I., Fawcett, T. W., Mallpress, D. E. W., and McNamara, J. M. (2014). Clarifying the relationship between prospect theory and risk-sensitive foraging theory. *Evol. Hum. Behav.* 35, 502–507. doi: 10.1016/j.evolhumbehav.2014.06.010

Howard, S. R., Shrestha, M., Schramme, J., Garcia, J. F., Avarguès-Weber, A., Greentree, A. D., et al. (2019). Honeybees prefer novel insect-pollinated flower shapes over bird-pollinated flower shapes. *Curr. Zool.* 65, 457–465.

Hurly, T. A., and Oseen, M. D. (1999). Context-dependent, risk-sensitive foraging preferences in wild rufous hummingbirds. *Anim. Behav.* 58, 59–66. doi: 10.1006/anbe.1999.1130

Inouye, D. W. (1980). The terminology of floral larceny. *Ecology* 61, 1251–1253. doi: 10.2307/1936841

Irwin, R. E. (2006). The consequences of direct versus indirect species interactions to selection on traits: pollination and nectar robbing in *Ipomopsis aggregata*. *Am. Nat.* 167, 315–328. doi: 10.1086/499377

Irwin, R. E., and Maloof, J. E. (2002). Variation in nectar robbing over time, space, and species. *Oecologia* 133, 525–533. doi: 10.1007/s00442-002-1060-z

Irwin, R. E., Bronstein, J. L., Manson, J. S., and Richardson, L. (2010). Nectar robbing: ecological and evolutionary perspectives. *Annu. Rev. Ecol. Evol. Syst.* 41, 271–292. doi: 10.1146/annurev.ecolsys.110308.120330

Ishii, H. S., and Masuda, H. (2014). Effect of flower visual angle on flower constancy: a test of the search image hypothesis. *Behav. Ecol.* 25, 933–944. doi: 10.1093/beheco/aru071

Johnson, D. D. P., Blumstein, D. T., Fowler, J. H., and Haselton, M. G. (2013). The evolution of error: error management, cognitive constraints, and adaptive decision-making biases. *Trends Ecol. Evol.* 28, 474–481. doi: 10.1016/j.tree.2013.05.014

Kacelnik, A., and El Mouden, C. (2013). Triumphs and trials of the risk paradigm. *Anim. Behav.* 86, 1117–1129. doi: 10.1016/j.anbehav.2013.09.034

Keasar, T., Motro, U., and Shmida, A. (2013). Temporal reward variability promotes sampling of a new flower type by bumblebees. *Anim. Behav.* 86, 747–753. doi: 10.1016/j.anbehav.2013.07.010

Kiesel, A., Steinhauser, M., Wendt, M., Falkenstein, M., Jost, K., Philipp, A. M., et al. (2010). Control and interference in task switching—a review. *Psychol. Bull.* 136, 849–874. doi: 10.1037/a0019842

Laverty, T. M. (1980). The flower-visiting behaviour of bumble bees: floral complexity and learning. *Can. J. Zool.* 58, 1324–1335. doi: 10.1139/z80-184

Laverty, T. M. (1994). Bumble bee learning and flower morphology. *Anim. Behav.* 47, 531–545. doi: 10.1006/anbe.1994.1077

Leadbeater, E., and Chittka, L. (2008). Social transmission of nectar-robbing behaviour in bumble-bees. *Proc. Biol. Sci.* 275, 1669–1674. doi: 10.1098/rspb.2008.0270

Leonard, A. S., and Papaj, D. R. (2011). 'X' marks the spot: the possible benefit of nectar guides to bees and plants. *Funct. Ecol.* 25, 1–9.

Lewis, A. C. (1986). Memory constraints and flower choice in *Pieris rapae*. *Science* 232:863. doi: 10.1126/science.232.4752.863

Lichtenberg, E. M., Irwin, R. E., and Bronstein, J. L. (2018). Costs and benefits of alternative food handling tactics help explain facultative exploitation of pollination mutualisms. *Ecology* 99, 1815–1824. doi: 10.1002/ecy.2395

Lichtenberg, E. M., Heiling, J. M., Bronstein, J. L., and Barker, J. L. (2020a). Noisy communities and signal detection: why do foragers visit rewardless flowers? *Philos. Trans. R. Soc. B* 375:20190486. doi: 10.1098/rstb.2019.0486

Lichtenberg, E. M., Richman, S. K., Irwin, R. E., and Bronstein, J. L. (2020b). Competition for nectar resources does not affect bee foraging tactic constancy. *Ecol. Entomol.* 45, 904–909. doi: 10.1111/een.12866

Lihoreau, M., Dubois, T., Gomez-Moracho, T., Kraus, S., Monchanin, C., and Pasquaretta, C. (2019). "Putting the ecology back into insect cognition research," in *Advances in Insect Physiology*, ed. R. Jurenka (Amsterdam: Elsevier), 1–25. doi: 10.1016/bs.aiip.2019.08.002

Maloof, J. E. (2001). The effects of a bumble bee nectar robber on plant reproductive success and pollinator behavior. *Am. J. Bot.* 88, 1960–1965. doi: 10.2307/3558423

Maloof, J. E., and Inouye, D. W. (2000). Are nectar robbers cheaters or mutualists? *Ecology* 81, 2651–2661. doi: 10.1890/0012-96582000081[2651:ANRCOM]2.0.CO;2

Marshall, J. A. R., Trimmer, P. C., Houston, A. I., and McNamara, J. M. (2013). On evolutionary explanations of cognitive biases. *Trends Ecol. Evol.* 28, 469–473. doi: 10.1016/j.tree.2013.05.013

Monsell, S. (2003). Task switching. *Trends Cogn. Sci.* 7, 134–140. doi: 10.1016/S1364-6613(03)00028-7

Morgan, K. V., Hurly, T. A., and Healy, S. D. (2014). Individual differences in decision making by foraging hummingbirds. *Behav. Process.* 109, 195–200. doi: 10.1016/j.beproc.2014.08.015

Newman, D. A., Thomson, J. D., and Ranta, E. (2005). Effects of nectar robbing on nectar dynamics and bumblebee foraging strategies in *Linaria vulgaris* (Scrophulariaceae). *Oikos* 110, 309–320.

Parachnowitsch, A. L., Manson, J. S., and Sletvold, N. (2019). Evolutionary ecology of nectar. *Ann. Bot.* 123, 247–261. doi: 10.1093/aob/mcy132

Perkins, D. N., and Salomon, G. (1992). "Transfer of learning," in *International Encyclopedia of Eduction*, Eds T. N. P. Torsten Husen (Oxford: Pergamon Press), 6452–6457.

Pleasants, J. M. (1983). Nectar production patterns in *Ipomopsis aggregata* Polemoniaceae). *Am. J. Bot.* 70, 1468–1475. doi: 10.1002/j.1537-2197.1983.tb10850.x

Pyke, G. H. (2016). Floral nectar: pollinator attraction or manipulation? *Trends Ecol. Evol.* 31, 339–341. doi: 10.1016/j.tree.2016.02.013

Raine, N. E., and Chittka, L. (2007b). The adaptive significance of sensory bias in a foraging context: floral colour preferences in the bumblebee *Bombus terrestris*. *PLoS One* 2:e556. doi: 10.1371/journal.pone.0000556

Raine, N. E., and Chittka, L. (2007a). Flower constancy and memory dynamics in bumblebees (Hymenoptera: Apidae: *Bombus*). *Entomol. Gen.* 29, 179–199. doi: 10.1127/entom.gen/29/2007/179

Richardson, L., and Bronstein, J. L. (2012). Reproductive biology of pointleaf manzanita (*Arctostaphylos pungens*) and the pollinator-nectar robber spectrum. *J. Pollinat. Ecol.* 9, 115–123.

Richman, S. K., Irwin, R. E., and Bronstein, J. L. (2017a). Foraging strategy predicts foraging economy in a facultative secondary nectar robber. *Oikos* 126, 1250–1257. doi: 10.1111/oik.04229

Richman, S. K., Irwin, R. E., Nelson, C. J., and Bronstein, J. L. (2017b). Facilitated exploitation of pollination mutualisms: fitness consequences for plants. *J. Ecol.* 105, 188–196. doi: 10.1111/1365-2745.12657

Rivera, A. J., Hernandez, E. J., and Nates-Parra, G. (2006). Morphological constraints and nectar robbing in three Andean bumble bee species (Hymenoptera, Apidae, Bombini). *Caldasia* 28, 111–114.

Rojas-Nossa, S. V., Sánchez, J. M., and Navarro, L. (2016). Nectar robbing: a common phenomenon mainly determined by accessibility constraints, nectar volume and density of energy rewards. *Oikos* 125, 1044–1055. doi: 10.1111/oik.02685

Rose, J., and Schmidt, R. (2012). "Discrimination learning model," in *Encyclopedia of the Sciences of Learning*, ed. N. M. Seel (Boston, MA: Springer US), 1013–1015. doi: 10.1007/978-1-4419-1428-6_343

Schiestl, F. P. (2017). Innate receiver bias: its role in the ecology and evolution of plant–animal interactions. *Annu. Rev. Ecol. Evol. Syst.* 48, 585–603. doi: 10.1146/annurev-ecolsys-110316-023039

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Schondube, J. E., and Del Rio, C. M. (2003). Concentration-dependent sugar preferences in nectar-feeding birds: mechanisms and consequences: concentration-dependent sugar preferences. *Funct. Ecol.* 17, 445–453. doi: 10.1046/j.1365-2435.2003.00749.x

Shafir, S., Waite, T. A., and Smith, B. H. (2002). Context-dependent violations of rational choice in honeybees (*Apis mellifera*) and gray jays (*Perisoreus canadensis*). *Behav. Ecol. Sociobiol.* 51, 180–187. doi: 10.1007/s00265-001-0420-8

Stanton, M. L. (1984). Short-term learning and the searching accuracy of egg-laying butterflies. *Anim. Behav.* 32, 33–40. doi: 10.1016/S0003-3472(84)80321-8

Tebbich, S., Griffin, A. S., Peschl, M. F., and Sterelny, K. (2016). From mechanisms to function: an integrated framework of animal innovation. *Philos. Trans. R. Soc. B* 371:20150195. doi: 10.1098/rstb.2015.0195

van der Kooi, C. J., Vallejo-Marín, M., and Leonhardt, S. D. (2021). Mutualisms and (a)symmetry in plant–pollinator interactions. *Curr. Biol.* 31, R91–R99. doi: 10.1016/j.cub.2020.11.020

Van Kleunen, M., Nänni, I., Donaldson, J. S., and Manning, J. C. (2007). The Role of beetle marks and flower colour on visitation by monkey beetles (Hopliini) in the Greater Cape Floral Region, South Africa. *Ann. Bot.* 100, 1483–1489. doi: 10.1093/aob/mcm256

Vasconcelos, M., Monteiro, T., and Kacelnik, A. (2015). Irrational choice and the value of information. *Sci. Rep.* 5:13874. doi: 10.1038/srep13874

Waser, N. M. (1979). Pollinator availability as a determinant of flowering time in ocotillo (*Fouquieria splendens*). *Oecologia* 39, 107–121. doi: 10.1007/BF00346001

Webb, B. (2012). Cognition in insects. *Philos. Trans. R. Soc. B* 367, 2715–2722. doi: 10.1098/rstb.2012.0218

Weiss, M. R., and Papaj, D. R. (2003). Colour learning in two behavioural contexts: how much can a butterfly keep in mind? *Anim. Behav.* 65, 425–434. doi: 10.1006/anbe.2003.2084

Woodward, G. L., and Laverty, T. M. (1992). Recall of flower handling skills by bumble bees: a test of Darwin's interference hypothesis. *Anim. Behav.* 44, 1045–1051. doi: 10.1016/S0003-3472(05)80316-1

Wu, Y., Zhang, Z. Q., and Li, Q. J. (2019). Nectar robbers influence the trait–fitness relationship of *Primula secundiflora*. *Plant Biol. J.* 21, 967–974. doi: 10.1111/plb.13000

Zhang, Y.-W., Robert, G. W., Wang, Y., and Guo, Y.-H. (2007). Nectar robbing of a carpenter bee and its effects on the reproductive fitness of *Glechoma longituba* (Lamiaceae). *Plant Ecol.* 193, 1–13.

Zhang, Y.-W., Zhao, J.-M., Yang, C.-F., and Gituru, W. R. (2011). Behavioural differences between male and female carpenter bees in nectar robbing and its effect on reproductive success in *Glechoma longituba* (Lamiaceae). *Plant Biol.* 13, 25–32. doi: 10.1111/j.1438-8677.2009.00279.x

Zimmerman, M., and Cook, S. (1985). Pollinator foraging, experimental nectar-robbing and plant fitness in *Impatiens capensis*. *Am. Midl. Nat.* 113, 84–91. doi: 10.2307/2425350

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 15 October 2021
doi: 10.3389/fevo.2021.727471

Unraveling the Olfactory Biases of Male Euglossine Bees: Species-Specific Antennal Responses and Their Evolutionary Significance for Perfume Flowers

Katharina Brandt[1], Stefan Dötterl[2], Santiago R. Ramírez[3], Florian Etl[4], Isabel Cristina Machado[5], Daniela Maria do Amaral Ferraz Navarro[6], Daniel Dobler[7], Oliver Reiser[7], Manfred Ayasse[1] and Paulo Milet-Pinheiro[5,8]*

[1] Institute of Evolutionary Ecology and Conservation Genomics, Ulm University, Ulm, Germany, [2] Department of Biosciences, Paris Lodron University Salzburg, Salzburg, Austria, [3] Department of Evolution and Ecology, University of California, Davis, Davis, CA, United States, [4] Division of Structural and Functional Botany, University of Vienna, Vienna, Austria, [5] Departamento de Botânica, Universidade Federal de Pernambuco, Recife, Brazil, [6] Departamento de Química Fundamental, Universidade Federal de Pernambuco, Recife, Brazil, [7] Institute of Organic Chemistry, University of Regensburg, Regensburg, Germany, [8] Laboratório de Ecologia Ciências Biológicas, Universidade de Pernambuco, Petrolina, Brazil

OPEN ACCESS

Edited by:
Johannes Spaethe, Julius Maximilian University of Würzburg, Germany

Reviewed by:
David Roubik, Smithsonian Tropical Research Institute, Panama
Johannes Stökl, University of Bayreuth, Germany
Bjorn Bohman, Swedish University of Agricultural Sciences, Sweden

***Correspondence:**
Katharina Brandt
katjbrandt@gmail.com

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *18 June 2021*
Accepted: *16 September 2021*
Published: *15 October 2021*

Citation:
Brandt K, Dötterl S, Ramírez SR, Etl F, Machado IC, Navarro DMdAF, Dobler D, Reiser O, Ayasse M and Milet-Pinheiro P (2021) Unraveling the Olfactory Biases of Male Euglossine Bees: Species-Specific Antennal Responses and Their Evolutionary Significance for Perfume Flowers. Front. Ecol. Evol. 9:727471. doi: 10.3389/fevo.2021.727471

Male euglossine bees exhibit unique adaptations for the acquisition and accumulation of chemical compounds from "perfume flowers" and other sources. During courtship display, male bees expose perfume mixtures, presumably to convey species-specific recognition and/or mate choice signals to females. Because olfaction regulates both signal production (in males) and signal detection (in females) in this communication system, strong selective pressures are expected to act on the olfactory system, which could lead to sensory specialization in favor of an increased sensitivity to specific chemical compounds. The floral scents of euglossine-pollinated plants are hypothesized to have evolved in response to the preexisting sensory biases of their male euglossine bee pollinators. However, this has never been investigated at the peripheral olfactory circuitry of distinct pollinating genera. Here, we present a comparative analysis using electroantennography (EAG) of males across the phylogeny of 29 euglossine bee species, among them *Euglossa* and *Eulaema* species. First, we tested whether antennal responses differ among different euglossine genera, subgenera and species. Secondly, we conducted a comparative phylogenetic analysis to investigate the macroevolutionary patterns of antennal responses across the euglossine bee phylogeny. We found that antennal response profiles are very unique on the species level and differ on the subgenus and the genus level. The differences can be explained by chemical compounds typically found in the floral scent bouquets of perfume flowers and specific compounds of species either pollinated by *Euglossa* (e.g., ipsdienol) or *Eulaema* bees (e.g., (−)-(*E*)-carvone epoxide). Also, we detected a phylogenetic signal in mean antennal responses and found that especially at the species level of our simulation the overall antennal responses exhibit greater disparity relative to a null model of pure Brownian-motion across the phylogeny. Altogether, our results suggest

that (1) euglossine bee species exhibit species-specific antennal responses that differ among euglossine genera and subgenera, (2) antennal responses diverge early after speciation events, and (3) scent composition of perfume flowers evolved in response to pollinator-mediated selection imposed by preexisting sensory biases in euglossine bees.

Keywords: antennal responses, electroantennography (EAG), *Eufriesea*, *Euglossa*, *Eulaema*, *Exaerete*, euglossine bees, perfume flowers

INTRODUCTION

For most insects, just like for the majority of animals across phyla, the ability to detect a diversity of airborne molecules in their environment is critically important for survival (Hildebrand and Shepherd, 1997; Hansson and Stensmyr, 2011). Olfaction plays a pivotal role in the detection of food, hosts, predators, and kin (Olsson and Hansson, 2013), as well as in the attraction, location and identification of potential mates (Birch and Haynes, 1982; Cardé and Baker, 1984; Roelofs, 1984; Ayasse et al., 2001). The importance of olfaction in insects is apparent by looking at the elaborate antennal structures that exist in a diversity of shapes (Hansson and Stensmyr, 2011). Insect antennae are covered with different types of olfactory sensilla (Schneider and Steinbrecht, 1968), which contain the sensitive dendrites of the olfactory sensory neurons (Zacharuk, 1980; Couto et al., 2005). Olfactory stimulation occurs when odor molecules enter through pores or slits on the antenna surface (Steinbrecht, 1997) and are directed by odorant binding proteins (OBPs) that bind together with the volatile to olfactory receptors (ORs) situated in the membrane of these dendrites (Hallem and Carlson, 2006; Robertson and Wanner, 2006). These olfactory receptors vary in the type of molecules that activate them, their chemical tuning spectrum and the molecular receptive ranges (Hallem and Carlson, 2006; Getahun et al., 2013). Therefore, the olfactory periphery plays an important role in compound discrimination and represents the first step of specificity in olfactory sensitivity (Shields and Hildebrand, 2001; Hallem and Carlson, 2006; Brand et al., 2015) prior higher-level neural processing in the insects brain (see e.g., Renou, 2014).

Specificity in olfactory signals can be achieved either through complex molecules that are rare in nature (Chow and Wang, 1981; Ayasse et al., 2003; Schäffler et al., 2015) or by specific blends of relatively simple and ubiquitous compounds (Knudsen et al., 2006; Ayasse et al., 2011; Ayasse and Dötterl, 2014). While most insects synthesize such specific olfactory signals (e.g., pheromones) *de novo* or modify precursors found in their diet (Roelofs, 1984), male euglossine bees (Apidae, Euglossini) are known to harvest volatile compounds directly from flowers (Vogel, 1966; Dodson et al., 1969) as well as from non-floral sources (e.g., rotting plant material, bark, leaves and feces; Whitten et al., 1993). A set of morphological, biochemical and behavioral adaptations thereby enable the location, collection and storage of volatile compounds (Eltz et al., 2005b) forming complex species-specific blends that are stored in tibial organs on the hindlegs and exposed by male euglossine bees during courtship in the forest understory (Eltz et al., 2005a,b). The blends are presumedly used to communicate species affiliation (Eltz et al., 2006; Zimmermann et al., 2006) and/or to demonstrate genetic fitness to conspecific females (Zimmermann et al., 2009b). However, the precise function of perfume blends in mediating mating decision by females awaits experimental support.

The perfume collection behavior of male euglossine bees has the unique feature that the olfactory system is involved in determining both signal production (i.e., the collection of volatile compounds) and signal detection (e.g., during mating) by female bees. Therefore, a strong selection pressure is expected to act on the olfactory system which could lead to sensory specialization in favor of an increased sensitivity to specific single volatiles or volatile blends in different species of euglossine bees (Eltz et al., 2006). In addition to the higher-level neural processing that takes place in the insect brain (see e.g., Renou, 2014), olfactory specialization can be achieved through changes in the peripheral sensory system, for example, by the presence/absence and abundance of specific types of ORs or by divergent chemical tuning of individual ORs (Hallem and Carlson, 2006; Renou, 2014). So far, more than 40 different chemical compounds are known to attract male euglossine bees (Williams and Whitten, 1983; Ramírez et al., 2002; Roubik and Hanson, 2004). Although there is a broad overlap in the range of compounds collected by different species, subgenera or genera of euglossine bees (Ackerman, 1983; Pearson and Dressler, 1985), several studies support a scenario of high species-specific preferences (Ackerman, 1989) as illustrated by the species-specific chemical blends stored in the hind-legs (Eltz et al., 2003, 2005a; Zimmermann et al., 2006; Weber et al., 2016).

This behavior evolved at least 38 million years ago (Engel, 1999; Ramírez et al., 2011) and various neotropical plants, mainly orchids, have adapted to attract male euglossine bees as pollinators by offering volatile compounds as floral reward (i.e., perfume-rewarding plants; Vogel, 1966; Dressler, 1982; Williams and Whitten, 1983; Ramírez et al., 2002). The mutualistic system between euglossine males and perfume-rewarding flowers involves diverse bee genera, which differ considerably in size/morphology, olfactory preferences and behavior (Dressler, 1982; Ramírez et al., 2002). Some of the plants pollinated by male euglossine bees attract many distinct species from all genera, irrespective of their body size (e.g., *Anthurium* spp. and *Spathiphyllum* spp.; Montalvo and Ackerman, 1986; Hentrich et al., 2010). However, mutualistic interactions can also be very specific if pollinator size is essential to ensure successful pollinarium removal and subsequent deposition. This is often the case in perfume-producing orchids (e.g., Dodson, 1962, 1978; Dressler, 1968; Meeuse and Morris, 1984).

The orchid genus *Catasetum* is mainly pollinated by species of *Euglossa* and *Eulaema*, but for a few species also pollination by *Eufriesea* (Hills et al., 1972; Peruquetti et al., 1999; Milet-Pinheiro et al., 2018) and *Exaerete* (Cancino and Damon, 2007) is reported. Species that are pollinated by *Euglossa* are usually visited by two or more congeneric pollinator species, but rarely by species of *Eulaema*, and vice versa (Frankie et al., 1983; Whitten et al., 1986, 1988). Chemical analysis of floral scents emitted by *Catasetum* orchids suggest that they differ among pollinator genera and subgenera (i.e., Eufriesea, Euglossa or Eulaema; Milet-Pinheiro and Gerlach, 2017; Brandt et al., 2019) but are also highly specific on the species level. Based on these findings, together with the fact that perfume as floral reward has evolved after perfume-gathering behavior, it has been hypothesized that preexisting sensory biases of each euglossine genus and the resulting behavioral preferences for distinct compounds among euglossine bees shaped the evolution of floral scent of perfume-rewarding plants (Ramírez et al., 2011). Experimental evidence for the possible influence of sensory biases on the evolution of floral scents of perfume-rewarding plants from the pollinator perspective, however, is missing. In the present study, we used electroantennography (EAG) to investigate, in a comparative approach, whether bees of the distinct genera *Eufriesea*, *Euglossa*, *Eulaema*, and *Exaerete* respond differently to chemical compounds that are most representative in the floral perfumes of euglossinophilous plants, particularly in the genus *Catasetum* (Milet-Pinheiro and Gerlach, 2017). We expect the antennal response profiles of euglossine species to differ among distinct genera thereby reflecting differences in the olfactory periphery of euglossine species that could have influenced the evolution of the floral scents in perfume flowers. Moreover, we conducted a comparative phylogenetic analysis to test whether antennal responses can be explained by bee phylogeny.

MATERIALS AND METHODS

Tested Bee Species

In total, we tested the antennal sensitivity in males of all 29 euglossine bee species we were able to attract in the field, 19 occurring in Costa Rica and 10 in NE-Brazil, among them three species of *Eufriesea* ($N = 12$ individuals), 16 species of *Euglossa* ($N = 154$), eight species of *Eulaema* ($N = 80$), and two species of *Exaerete* ($N = 16$; **Figure 1**). The tested species of *Eulaema* belong to the subgenus *Apeulaema* and *Eulaema* s. st. (Nemésio, 2009; Melo, 2014; **Table 1**), whereas those of *Euglossa* belong to the subgenera *Euglossa* s. st., *Glossura* and *Glossurella* (Nemésio, 2009; Ramírez et al., 2010b; **Table 1**).

In Costa Rica bees were collected at the surroundings of Piedras Blancas National Park (320 m a.s.l; 8°41′37.6″N 83°12′51.7″W) and the Tropical Field Station La Gamba (76 m a.s.l; 8°42′03.6″N 83°12′05.7″W). Sampling of bees in Costa Rica was authorized by the Ministerio de Ambiente y Energía Sistema Nacional de Áreas de Conservacíon (permit numbers SINAC-ACOSTA-PI-PC-001-19 and SINAC-ACOSTA-PI-PC-002-19). In Brazil, bees were either collected at the surroundings of the "Mata do Curado" (10 m a.s.l; 8°02′30.5″S, 34°57′54.1″W), municipality of Recife (Pernambuco), or at the surroundings of the farm "Agua Fria" (600 m a.s.l; 8°11′19.0″S, 35°28′13.6″W), located in the municipality of Chã-Grande (Pernambuco). Sampling of bees in Brazil was authorized by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) of the Ministério Brasileiro do Meio Ambiente (permit number 53545–1).

Bees were collect using entomological nets at scent baits (Gruber et al., 2008), i.e., filter papers (10 × 10 cm) impregnated with 100 μL of the following pure synthetic compounds: eucalyptol (99%; Merck), benzyl acetate (≥99%; Merck), eugenol (≥98%; Merck), methyl salicylate (≥99%; Merck), skatole (98%; Merck), veratrole (99%; Sigma-Aldrich). After analyses (see below), bees were mounted with entomological pins and deposited either at the collection of the Tropical Field Station La Gamba (Costa Rica) or at the UFPE (Brazil).

Electroantennographic Measurements (EAGs)

The physiological measurements were performed either at the facilities of the Tropical Field Station La Gamba or the Departamento de Química Fundamental (DQF) of the Universidade Federal of Pernambuco (UFPE). For the measurements, we used micro-scissors (Castroviejo, Fine Science tools; 69121 Heidelberg, Germany) to excise one antenna of each tested bee at the scape. Using a stereomicroscope (Stemi 2000-CS, ZEISS, Oberkochen, Germany) and a razor blade, the excised antenna was cut at the tip (last segment of flagellum) and at the base (first segment of flagellum). The antenna was mounted between two glass capillaries filled with insect Ringer solution (1 L demineralized water containing 5 g of NaCl, 0.42 g of KCl and 0.19 g of CaCl), which were connected to gold-electrodes. The electrode connected with the base of the antenna was grounded, while the electrode connected to the tip transmitted changes of the potential within the antenna to a signal acquisition controller (IDAC-2 Signal acquisition controller; Syntech, Hilversum, Netherlands). The preparation was placed in front of a glass tube, through which a constant humidified airflow (25 mL/s) was blown.

We tested the antennal sensitivity of the different species to compounds that are typically found in perfume-rewarding plants pollinated by different genera of euglossine bees. Based on a data set on floral scent chemistry of 60 euglossinophilous species (Milet-Pinheiro and Gerlach, 2017; Milet-Pinheiro, unpublished) we prepared testing solutions for 23 compounds (**Table 2**) in a concentration of 10 μL/mL using n-hexane as the solvent (**Table 2**). Testing solutions were applied to each antennal preparation in a randomized order using the Android App "Who's Next?!" (v.0.8.0; Martin Philippi 2017) starting and ending with the negative control n-hexane. To avoid decreased antennal responses as a result of prolonged or repetitive stimulation (Strausfeld and Kaissling, 1986), we allowed a resting phase of 60 s between stimuli. For each stimulus, we added 5 μL of testing solution onto a v-shaped strip of filter paper (ca. 0.5 × 1 cm) located inside a Pasteur pipette (15 cm,

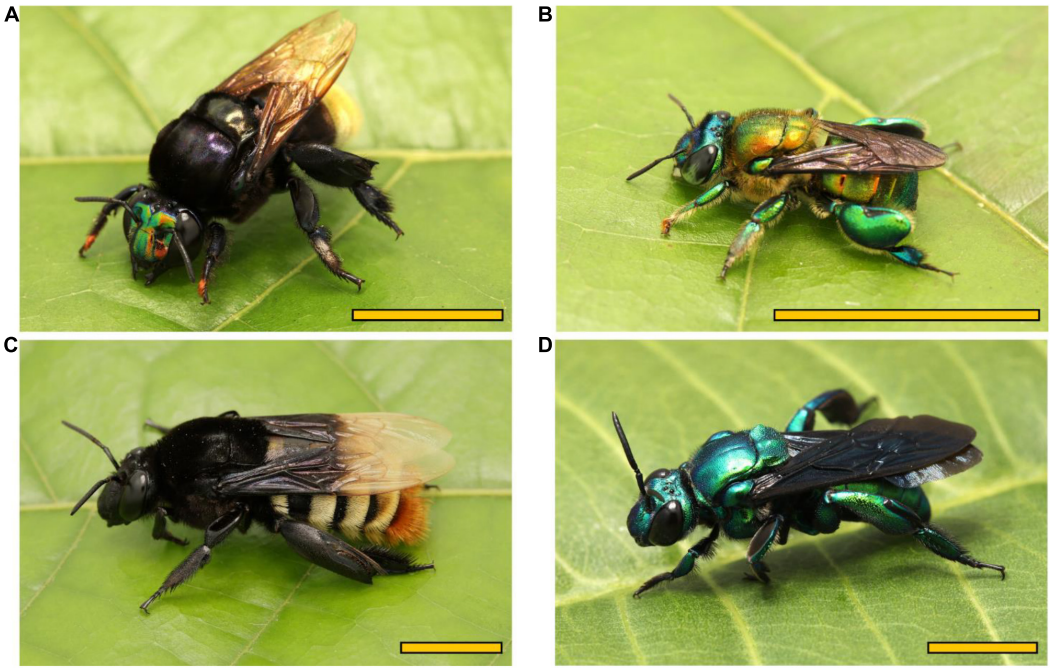

FIGURE 1 | Overview of the four tested euglossine genera **(A)** *Eufriesea*, **(B)** *Euglossa*, **(C)** *Eulaema*, and **(D)** *Exaerete*. Scale bar: 1 cm. Photos by Paulo Milet-Pinheiro.

VWR International, Darmstadt, Germany). After the solvent was allowed to evaporate for 1 min, the Pasteur pipettes were connected to a stimulus controller (CS-05; Syntech, Hilversum, Netherlands) that delivered an air-puff to the antenna for 0.3 s with a pulse flow of 25 ml/s. Antennal responses were analyzed by Syntech EAG software (EAG Pro, v. 2.2; Hilversum, Netherlands). Responses to n-hexane were used to normalize the data (using the option provided the software), and thus, to correct for a change in antennal sensitivity during measurements.

For the statistical analyses we used a different standardization of antennal responses to compare the different species and genera. The strongest antennal response of each tested bee individual was set as 100%, and the responses to all other stimuli were expressed as percentages in relation to this reference. To test for differences in these multivariate standardized antennal responses to the compounds (excluding the negative control) among genera, subgenera and species of euglossine bees, we used a multivariate three-level nested PERMANOVA analysis [factors: genus, subgenus (nested in genus), and species (nested in subgenus)] with subsequent pair-wise comparisons based on fourth-root transformed Bray Curtis similarities. Further, we used non-metric multidimensional scaling (nMDS; Clarke and Gorley, 2006), based on the Bray Curtis similarities, to graphically depict variation in antennal responses among genera, subgenera and species (species-means were used for analyses), and SIMPER was used to determine the compounds to which the genera responded most differently. We performed PERMDISP (factor: genus or subgenus) to test for differences in variability (dispersion) among antennal responses. Results of these analyses provided information about the variation of antennal responses *per se* and indicated potential influences of dispersion on the PERMANOVA results (see Anderson et al., 2008).

Absolute antennal responses were used to test, separately for each species and floral scent compound, whether responses were stronger than to the negative control, n-hexane. Therefore, we performed two-factorial PERMANOVA analyses [factors: bee individual and compound] with subsequent pair-wise comparisons (adjusted via Bonferroni correction) based on univariate (using single compounds) Euclidean distance matrices.

The PERMANOVA analyses were ran using the software PRIMER 6 (version 6.1.15; PRIMER-E Ltd., 2012) in combination with the add-on PERMANOVA + (version 1.0.5; PRIMER-E Ltd., 2012). We used (1) sums of squares type III (partial), (2) fixed effects sum to zero for mixed terms, (3) a permutation of residuals under a reduced model, and (4) 9,999 permutations for all analyses. The level of significance was defined at $\alpha \leq 0.05$.

Phylogenetic Analyses

In order to investigate the evolutionary patterns of antennal responses across the euglossine bee phylogeny, we used the species-level phylogenetic tree estimated by Ramírez et al. (2010b). Briefly, the species-level phylogeny was built using ∼ 4.0 kb of nuclear (*EF1-a*, *ArgK*, and *Pol-II*) and mitochondrial (*CO1*) DNA available for 26 of our 29 tested euglossine species (no data available for *El. atleticana*, *El. Marcii*, and *El. niveofasciata*). Phylogenetic tree searches and fossil calibrated molecular clock analyses were estimated as described in Ramírez et al. (2010b).

TABLE 1 | Tested euglossine species of Brazil (BR) and Costa Rica (CRC) belonging to the genera *Eufriesea*, *Euglossa* (subgenera: *Euglossa* s. st., *Glossura*, and *Glossurella*), *Eulaema* (subgenera: *Apeulaema* and *Eulaema* s. st.), and *Exaerete* and known chemical compounds used in this study attracting male bees of these species.

Species	*N* of individuals	Area	Known attractants	References
Eufriesea				
Ef. chrysopyga (Mocsáry, 1898)	$N = 1$	CRC	C*	1, 10, 15
Ef. lucifera Kimsey, 1977	$N = 1$	CRC	C, E, G, I, MB, MS*	1, 10, 15
Ef. pulchra (Smith, 1854)	$N = 10$	CRC	C, E*, G, L, MB, MS*, T	1, 10, 15
Euglossa				
***Euglossa* s. st.**				
Eg. carolina Nemésio, 2009	$N = 10$	BR	BA, C, DB, E, G, MS, TB, VT	Brandt pers. obs.
Eg. championi Cheesman, 1929	$N = 10$	CRC	C*, M, MS*	1, 8, 9, 15
Eg. cognata Moure, 1970	$N = 11$	CRC	C*, BA, E, MB, MS*	1, 9, 13, 15
Eg. erythrochlora Moure, 1968	$N = 10$	CRC	C, E, MS*	9, 15
Eg. hansoni Moure, 1965	$N = 10$	CRC	C*, E	1, 9, 15
Eg. mixta Friese, 1899	$N = 10$	CRC	BA, C*, E, L, MB, MS*	1, 12, 13, 15, 19
Eg. nanomelanotricha Nemésio, 2009	$N = 10$	BR	BA, C, DB, E, G, MS, TB, VT	Brandt pers. obs.
Eg. securigera Dressler, 1982	$N = 2$	BR	C, E	15, 16, 17
Eg. tridentata Moure, 1970	$N = 10$	CRC	APH, BA, C*, E*, I, IP, L, M, MB, MS, T	1, 9, 15, 18, 19, 20
Eg. villosiventris Moure, 1968	$N = 10$	CRC	C, MS*	9, 15
Glossura				
Eg. flammea Moure, 1969	$N = 10$	CRC	BA, C*, COX, E, MS, IP, VT	5, 9, 15, 18
Eg. ignita Smith, 1874	$N = 10$	BR	BA, BH, C*, COX, E, IP, M, MS*	13, 15, 18, 19, 20
Eg. imperialis Cockerell, 1922	$N = 10$	CRC	BA, C*, E, MB, MS*	1, 13, 15, 19, 20
Glossurella				
Eg. dodsoni Moure, 1965	$N = 11$	CRC	BA*, C*, E*, I, MS, T	1, 5, 9, 15
Eg. gorgonensis Cheesman, 1929	$N = 10$	CRC	C*, COX* E, I, MS	5, 9, 15, 18
Eg. sapphirina Moure, 1968	$N = 10$	CRC	BA, C*, E, I, L, MB, MS*	1, 9, 14, 15
Eulaema				
Apeulaema				
El. cingulata (Fabricius, 1804)	$N = 10$	CRC	BA*, C, COX, DB, E*, I, MB, MS	1, 9, 13, 15, 18, 19
El. marcii Nemésio, 2009	$N = 10$	BR	BA, C, E, MS	4
El. nigrita Lepeletier, 1841	$N = 10$	BR	BA, C*, COX, E, IP, L, MS	1, 4, 14, 15, 18, 19
El. polychroma (Mocsáry, 1899)	$N = 10$	CRC	BA, C*, COX, E, I*, T	1, 2, 7, 11, 15, 18
***Eulaema* s. st.**				
El. atleticana Nemésio, 2009	$N = 10$	BR	BA, C, COX*, E, MS*	4
El. bombiformis (Packard, 1869)	$N = 10$	CRC	BA*, C, COX, DB, E, G*, MB, MS*	1, 12, 13, 15, 18, 19
El. meriana (Olivier, 1789)	$N = 10$	CRC	BA*, BH, C* COX, E, I, G, MB, MS*, T	1, 6, 13, 15, 18, 19
El. niveofasciata (Friese, 1899)	$N = 10$	BR	BA, C*, COX, E, MS	4, 13, 15
Exaerete				
Ex. frontalis (Guérin-Méneville, 1845)	$N = 6$	BR	BA, C* E, MS	13, 15
Ex. smaragdina (Guérin-Méneville, 1845)	$N = 10$	BR	BA, C*, E, MB, MS, T	1, 3, 13,15, 19

Full names of compounds given in ***Table 2****. *Chemicals acting as strong attractants. References: [1]Ackerman (1983), [2]Armbruster and McCormick (1990), [3]Armbruster et al. (1989), [4]Brandt et al. (2019), [5]Dressler (1982), [6]Eltz et al. (1999), [7]González (1996), [8]Hills (1968), [9]Janzen et al. (1982), [10]Kimsey (1982), [11]López (1963), [12]Morato et al. (1992), [13]Pearson and Dressler (1985), [14]Peruquetti et al. (1999), [15]Ramírez et al. (2002), [16]Rebelo and Moure (1995), [17]Silva and Rebêlo (1999), [18]Whitten et al. (1988), [19]Williams and Dodson (1972), and [20]Williams and Whitten (1983).*

Comparative phylogenetic analyses were conducted in RStudio v.1.4.1103 (implemented R v.4.0.3) using the R packages "phytools" v.0.7-70 (Revell, 2012) and "geiger" v.2.0.7 (Pennell et al., 2014). For all phylogenetic analyses we used a Bray Curtis similarity matrix based on standardized mean antennal responses (in percent, see above). We computed a phylogenetic signal for continuous traits on multivariate antennal responses of tested euglossine species using Blomberg's *K*-statistic test (Blomberg et al., 2003) based on 1,000 randomizations ("phylosig" function). Blomberg's *K* measures phylogenetic signal by quantifying the amount of observed trait variance relative to trait variance expected under a Brownian motion model (simulating evolution conditions similar to genetic drift; Kamilar and Cooper, 2013).

We also examined the phylogenetic patterns of antennal responses across species when stimulated with individual compounds. To this end, we fitted and compared two different models of trait evolution. First, we fitted a single-rate multivariate Brownian Motion (BM) model that corresponds to a random

TABLE 2 | Tested compounds in the study.

Chemical compound	Abbreviation[#]	Purity	Provider
Alkanes			
n-Hexane*		≥99%	Sigma-Aldrich
Aromatics			
Benzyl acetate	BA	≥99%	Sigma-Aldrich
Benzyl alcohol	BH	≥99%	Alfa Aesar
1,4-Dimethoxy benzene	DB	≥99%	Sigma-Aldrich
Eugenol	E	≥98%	Merck
Methyl benzoate	MB	99%	Alfa Aesar
Methyl salicylate	MS	≥99%	Sigma-Aldrich
Methyl *o*-anisate		≥97%	Sigma-Aldrich
1,2,4-Trimethoxy benzene	TB	≥97%	Sigma-Aldrich
Veratrole	VT	99%	Sigma-Aldrich
Monoterpenes			
(−)-(*E*)-Carvone epoxide[a]	COX	98%	[b]
Eucalyptol	C	99%	Merck
Geraniol	G	≥97%	SAFC
Ipsdienol	IP	≥99%	Merck
Limonene	DL	≥99%	Fluka Analytical
Linalool	L	≥99%	Sigma-Aldrich
β-Myrcene	M	>75%	Sigma-Aldrich
Nerol		97%	Sigma-Aldrich
α-Phellandrene	APH	≥75%	Sigma-Aldrich
α-Pinene	AP	98%	Sigma-Aldrich
Terpinen-4-ol (sum of enantiomers)	T	≥95%	Sigma-Aldrich
Sesquiterpenes			
α-Copaene		≥90%	Merck
α-Humulene		≥96%	Sigma-Aldrich
Irregular terpene			
β-Ionone	I	≥96%	Sigma-Aldrich

[#]*Abbreviation also used in Ramírez et al. (2002). *Negative control. [a](1S,4R,6S)-1-Methyl-4-(prop-1-en-2-yl)-7-oxabicyclo[4.1.0]heptan-2-one, in the following text referred to as: (−)-(E)-Carvone epoxide. [b]Synthetized (after Garver et al., 1976; Yasuda et al., 1979; Wang et al., 2006; Takita et al., 2011); see* ***Supplementary Figure 1****.*

walk process, in which the probability of divergence in antennal responses increases uniformly over time. Second, we fitted a single-optimum Orenstein-Uhlenbeck (OU) model, in which the variance in antennal responses decreased over time as trait values converge around a global phenotypic optimum. The OU model has a global evolutionary rate parameter (σ2), a global phenotypic optimum parameter (θ), and a global strength of selection (α) parameter. Parameter estimates and the associated likelihood values for continuous character evolution in univariate datasets (i.e., responses to a specific compound) were calculated using the "fitContinuous" function, which we compared using the corrected Akaike information criterion (AICc). Lower AICc values (AICc $\leq$ 10) thereby indicate better evidence for a given model. We estimated models without (AICc) and with standard errors (AICc_SE).

Additionally, we calculated disparity through time (DTT) plots ("dtt" function) to investigate how antennal responses occupy trait space throughout the evolutionary history of the lineages included in our study. To do this, we compared the observed DTT trajectory across the phylogeny relative to antennal responses simulated via a pure Brownian motion model of trait evolution (random-walk model; see also Harmon et al., 2003). We assessed the difference between the observed disparities and the simulated disparities using the morphological diversity index (MDI) statistics after Harmon et al. (2003), a measure of the area between the mean observed and simulated DTT. Significance of MDI expectation was assessed according to the 95% confidence interval of 100 simulations with a level of significance defined at $\alpha \leq 0.05$.

For graphical representation of the combined data, we plotted a phylogenetic tree with a heatmap reflecting the standardized mean antennal responses (in percent, see above; "phylo.heatmap" function).

RESULTS

Electroantennographic Measurements (EAGs)

The statistical analyses comparing the antennal response profiles of tested bees revealed a significant difference among euglossine genera (PERMANOVA: Pseudo-$F_{3,233} = 8.31$, $P < 0.001$; **Figure 2**). Pair-wise comparisons showed that antennal responses of the two genera *Euglossa* and *Eulaema* differed significantly from each other and also from the other genera ($P < 0.05$ each). The only genera that did not significantly differ were *Eufriesea* and *Exaerete* ($P = 0.19$). Also, the dispersion of antennal response profiles differed among the genera (PERMDISP: $F_{3,258} = 9.33$, $P < 0.001$; **Figure 2**). The responses of *Euglossa* were most diverse, followed by *Eulaema*, *Eufriesea* and finally *Exaerete*. Thus, the dispersion is related with the number of species sampled per genus. The SIMPER analysis revealed that antennal responses to the chemical compounds, methyl *o*-anisate, α-copaene, eugenol separated *Eufriesea* and *Exaerete* bees from the other two genera, while the responses to β-ionone, ipsdienol, methyl salicylate and (−)-(*E*)-carvone epoxide seem to be mostly responsible for the dissimilarity between *Euglossa* and *Eulaema* bees (**Figure 2**).

There was also a significant difference between antennal responses when comparing species within the subgenera (nested in genus) (PERMANOVA: Pseudo-$F_{3,233}$: 8.87, $P < 0.001$). Pair-wise comparisons within *Euglossa* showed that antennal responses of *Euglossa s. str.* species, *Glossurella* species and *Glossura* species differed from each other ($P < 0.01$ each). Antennal responses to the chemical compounds α-humulene, α-copaene, terpinen-4-ol, α-pinene, (−)-(*E*)-carvone epoxide and ipsdienol were mainly responsible for the differences among all three subgenera (**Figure 3A**). Within the genus *Eulaema* antennal responses differed significantly among the two subgenera *Eulaema s. str.* and *Apeulaema* ($P < 0.001$). The responses to α-phellandrene, eugenol, ipsdienol, eucalyptol and (−)-(*E*)-carvone epoxide explained most of the response differences between these two subgenera (**Figure 3B**).

We also found a significant difference in antennal responses when comparing distinct species (nested in subgenera) among

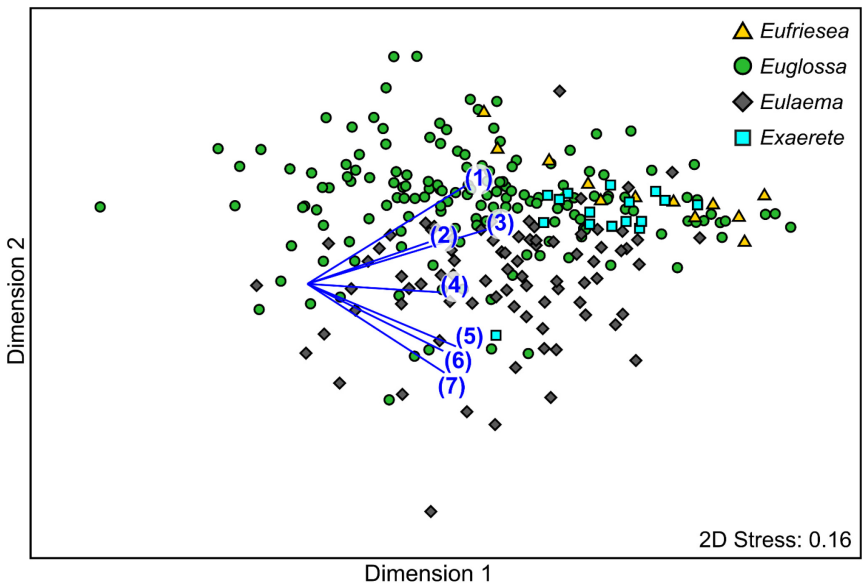

FIGURE 2 | Non-metric multidimensional scaling (nMDS) representation of antennal responses of different euglossine genera to 23 compounds, based on a Bray Curtis similarity matrix (standardized responses in percent). The single dots represent single bee individuals. Vectors represent the Pearson correlations for compounds most responsible for the dissimilarity in antennal response profiles between genera as indicated in a SIMPER analysis: (1) methyl *o*-anisate, (2) α-copaene, (3) eugenol, (4) β-ionone (5) ipsdienol, (6) methyl salicylate, (7) (−)-(*E*)-carvone epoxide.

each other (PERMANOVA: Pseudo-$F_{22,233}$: 3.03, $P < 0.001$; **Figure 4**).

The comparisons of absolute EAG responses to n-hexane and each chemical compound at the tested concentration of 10^{-2} revealed significant differences in all tested species ($P < 0.01$ each; **Supplementary Figure 2**). Generally, all tested species, irrespective of genus, showed strong antennal responses to benzyl acetate, 1,4-dimetoxy-benzene and veratrole. In addition, benzyl alcohol, eugenol, linalool, methyl benzoate and methyl salicylate elicited strong responses in most species. Weak antennal responses were found to the compounds α-copaene, α-humulene, methyl *o*-anisate, α-pinene, and 1,2,4-trimethoxy benzene and cannot be perceived by all tested bee species.

Phylogenetic Analyses

The Blomberg's *K* test revealed a significant level of phylogenetic signal in antennal response profiles of euglossine bees ($n = 624$, $K = 0.68$, $P < 0.01$), indicating that close relatives are more similar in their antennal response profiles than random pairs of species. These findings were supported by the calculated parameter estimates and the likelihood for continuous character evolution in a BM model (sigSq < 0.001, log-likelihood = 116.56, AICc < 1) as well as by the OU model (sigSq < 0.001, log-likelihood = 116.69, AICc < 1). Optimal antennal responses in all tested euglossine species were suggested for the chemical compounds benzyl alcohol (sigSq < 0.01, log-likelihood = −1.46, AICc ≤ 10; BM and OU model), 1,4-dimetoxybenzene (sigSq < 0.01, log-likelihood = −0.63, AICc ≤ 10; OU model), eugenol (sigSq < 0.01, log-likelihood = −2.22, AICc < 10; BM model), linalool (sigSq < 0.01, log-likelihood = −1.6, AICc ≤ 10; BM model), methyl benzoate (sigSq < 0.01, log-likelihood = 1.91, AICc < 5; BM and OU model), methyl salicylate (sigSq < 0.01, log-likelihood = −0.99, AICc < 10; OU model) and veratrole (sigSq < 0.01, log-likelihood = 1.85, AICc < 1; BM model). These compounds offer best evidence to the given models and are also reflected by the strong antennal responses of euglossine species shown in the heatmap of **Figure 5**.

Our analyses on the disparity of antennal response profiles through time show that the observed disparity in antennal responses was higher than expected under a neutral Brownian motion model of trait evolution (MDI: *Average square* = 0.24; **Figure 6**). In fact, we found that the relative disparity was most pronounced towards recent times (equivalent to the tips of the phylogeny in **Figure 5**).

DISCUSSION

In support to our hypothesis, the data revealed that antennal responses differ among euglossine genera, subgenera and species. Antennal responses to the chemical compounds methyl *o*-anisate, α-copaene, eugenol, β-ionone, ipsdienol, methyl salicylate and (−)-(*E*)-carvone epoxide were most responsible for these differences. Our phylogenetic analyses revealed that antennal response profiles to some compounds exhibit a phylogenetic signal and the variation in responses across the phylogeny are congruent with a Brownian motion model of trait evolution. This was the case with the antennal responses to benzyl alcohol, 1,4-dimetoxy-benzene, eugenol, linalool, methyl benzoate, methyl salicylate and veratrole. Our data also demonstrates that throughout the evolutionary history of the species we tested, the

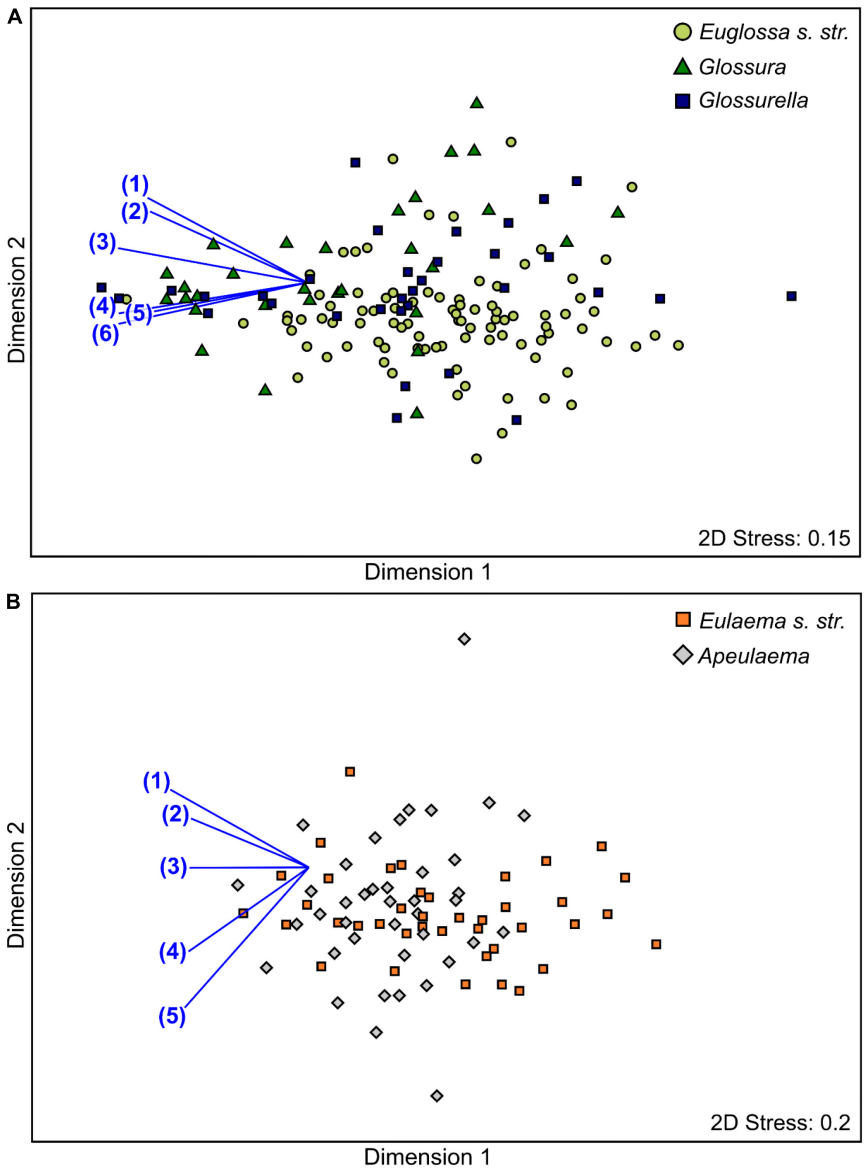

FIGURE 3 | Non-metric multidimensional scaling (nMDS) representation of antennal responses of different euglossine genera to 23 compounds, based on a Bray Curtis similarity matrix (standardized responses in percent). The single dots represent single bee individuals. Vectors represent the Pearson correlations for compounds most responsible for the dissimilarity in antennal response profiles between genera as indicated in a SIMPER analysis. **(A)** Subgenera within *Euglossa* (i.e., *Euglossa s. str.*, *Glossurella,* and *Glossura*); (1) α-humulene, (2) α-copaene, (3) terpinen-4-ol, (4) α-pinene, (5) (−)-(*E*)-carvone epoxide, (6) ipsdienol. **(B)** Subgenera within *Eulaema* (i.e., *Eulaema s. str.* and *Apeulaema*); (1) α-phellandrene, (2) eugenol, (3) ipsdienol, (4) eucalyptol, (5) (−)-(*E*)-carvone epoxide.

overall disparity in response between species was greater than expected under a null model of Brownian evolution.

The observed variation among antennal response profiles of tested euglossine bee species and taxonomic groups suggest that the antennae of the different bee species possess distinct types of ORs for different chemical compounds or different amounts of specific ORs. However, previous studies have shown that the sensitivity of ORs can also be influenced by further processes, such as tuning via metabotropic auto-regulation (Getahun et al., 2013) or variability in molecular receptive ranges (Hallem and Carlson, 2006), demonstrating the complexity of the olfactory periphery that could be responsible for the different antennal responses among tested species. Neural processing in the insect brain could also influence the olfactory perception in euglossine bees (e.g., Renou, 2014). To investigate the antennal responses of euglossine bees to specific compounds on the neuronal level, several approaches can be taken, including assaying individual olfactory receptors or measuring neural activity of brain regions *in vivo* (see e.g., Renou, 2014). For example, methods like single sensillum recording (SSR), the empty neuron system (Brand et al., 2020), or calcium imaging of glomerular responses in the antennal lobe (Galizia and Vetter, 2004) could further

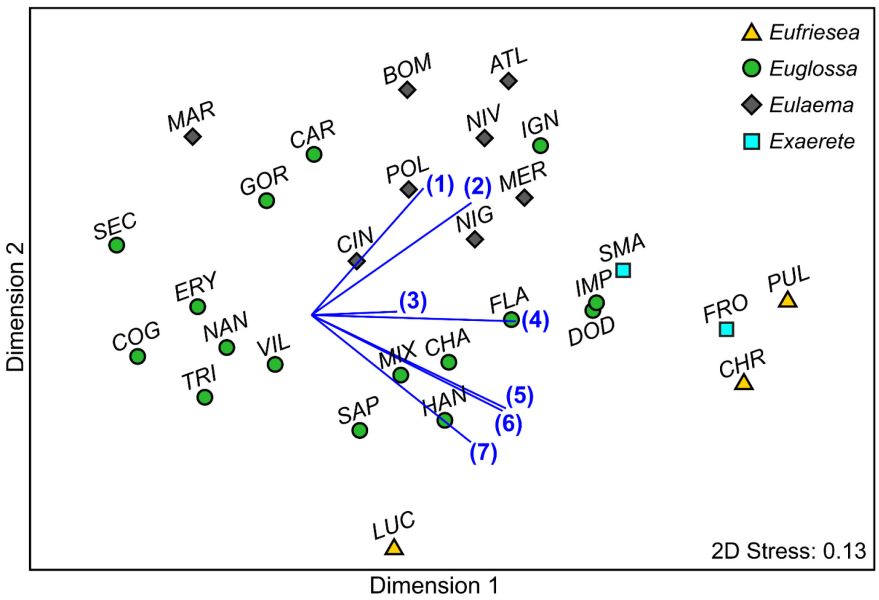

FIGURE 4 | Non-metric multidimensional scaling (nMDS) representation of mean antennal responses of different euglossine bees to 23 compounds, based on a Bray Curtis similarity matrix (standardized responses in percent). Vectors represent the Pearson correlations for compounds most responsible for the dissimilarity in antennal response profiles between species as indicated in a SIMPER analysis: (1) (−)-(*E*)-carvone epoxide, (2) ipsdienol, (3) β-ionone, (4) α-pinene, (5) α-humulene, (6) α-copaene, (7) methyl *o*-anisate. *Eg. carolina* (*CAR*), *Eg. championi* (*CHA*), *Eg. cognata* (*COG*), *Eg. dodsoni* (*DOD*), *Eg. erythrochlora* (*ERY*), *Eg. flammea* (*FLA*), *Eg. gorgonensis* (*GOR*), *Eg. hansoni* (*HAN*), *Eg. ignita* (*IGN*), *Eg. imperialis* (*IMP*), *Eg. mixta* (*MIX*), *Eg. nanomelanotricha* (*NAN*), *Eg. securigera* (*SEC*), *Eg. sapphirina* (*SAP*), *Eg. tridentata* (*TRI*), *Eg. villosiventris* (*VIL*), *Ef. chrysopyga* (*CRY*), *Ef. lucifera* (*LUC*), *Ef. pulchra* (*PUL*), *El. atleticana* (*ATL*), *El. bombiformis* (*BOM*), *El. cingulata* (*CIN*), *El. marcii* (*MAR*), *El. meriana* (*MER*), *El. nigrita* (*NIG*), *El. niveofasciata* (*NIV*), *El. polychroma* (*POL*), *Ex. frontalis* (*FRO*), and *Ex. smaragdina* (*SMA*).

contribute to the understanding of the complexity of olfactory tuning, processing and encoding in euglossine bees to chemical compounds that are used during courtship display and that several plants lineages, including orchids, have exploited for pollination services. In addition, sequences of the genome of all tested species could be used in further phylogenetic investigations to study the diversity of OR genes.

The results of our electroantennographic analyses revealed a clear difference among the antennal response profiles among euglossine bee genera (especially between *Euglossa* and *Eulaema*). Our study offers the first experimental evidence for the assumption that properties of the sensory equipment assort according to major taxonomic groups of euglossine bees. Bees of different genera respond differently to compounds, such as α-copaene, eugenol, ipsdienol, and (−)-(*E*)-carvone epoxide. In agreement to these patterns, the chemical composition of floral scents of perfume-rewarding orchids has been shown to differ among *Euglossa*- and *Eulaema*-pollinated species. Several chemical compounds which seem to be typically found in the floral scent bouquets of either *Euglossa*- (i.e., ipsdienol and myrcene; Milet-Pinheiro and Gerlach, 2017; Brandt et al., 2020) or *Eulaema*-pollinated species (e.g., α-pinene and (−)-(*E*)-carvone epoxide; Whitten et al., 1986; Milet-Pinheiro and Gerlach, 2017) coincide with the compounds most responsible for the separation of antennal response profiles among tested euglossine genera in our study. Altogether, these findings underline the finding of Ramírez et al. (2011) suggesting that distinct sensory biases between euglossine bee lineages have shaped the evolution of floral scents in perfume-rewarding plants. Under such scenario, floral scent bouquets evolve to target the compounds with strong sensory responses and behavioral attraction (Milet-Pinheiro and Gerlach, 2017) and lead to a genus specific attraction of pollinators. This is important because of the highly specialized pollination mechanisms exhibited by some perfume-producing orchids (see Dodson, 1962; Vogel, 1966; Romero and Carnevali, 2009) in which the morphological properties (i.e., the size) of euglossine bees, which typically differ among genera, ensure successful pollinarium transfer from male to female flowers (Dodson, 1962, 1978). For example, *Catasetum* species that are pollinated by *Euglossa* bees (8–18 mm in size; Dressler, 1982; Carvalho and Machado, 2002; Ramírez et al., 2002) are usually visited by two or more congeneric species, but rarely by species of *Eulaema* with a larger body size (20–35 mm in size; Dressler, 1982; Ramírez et al., 2002), and vice versa (Whitten et al., 1986, 1988; Ramírez et al., 2002). In fact, a similar pattern has also been reported in the orchid genus *Gongora*, which is also exclusively pollinated by euglossine bees (Hetherington-Rauth and Ramírez, 2015).

Within the tested euglossine bee genera, we found that the antennal response profiles are also specific at the subgenera and species levels. This observation provides further evidence for the idea of sensory niche partitioning provided by Zimmermann et al. (2009a). In areas where many different euglossine species of the same genus occur sympatrically, species-specific attraction of pollinators to perfume-rewarding flowers is not only essential to ensure pollinator fidelity and avoid pollinator competition

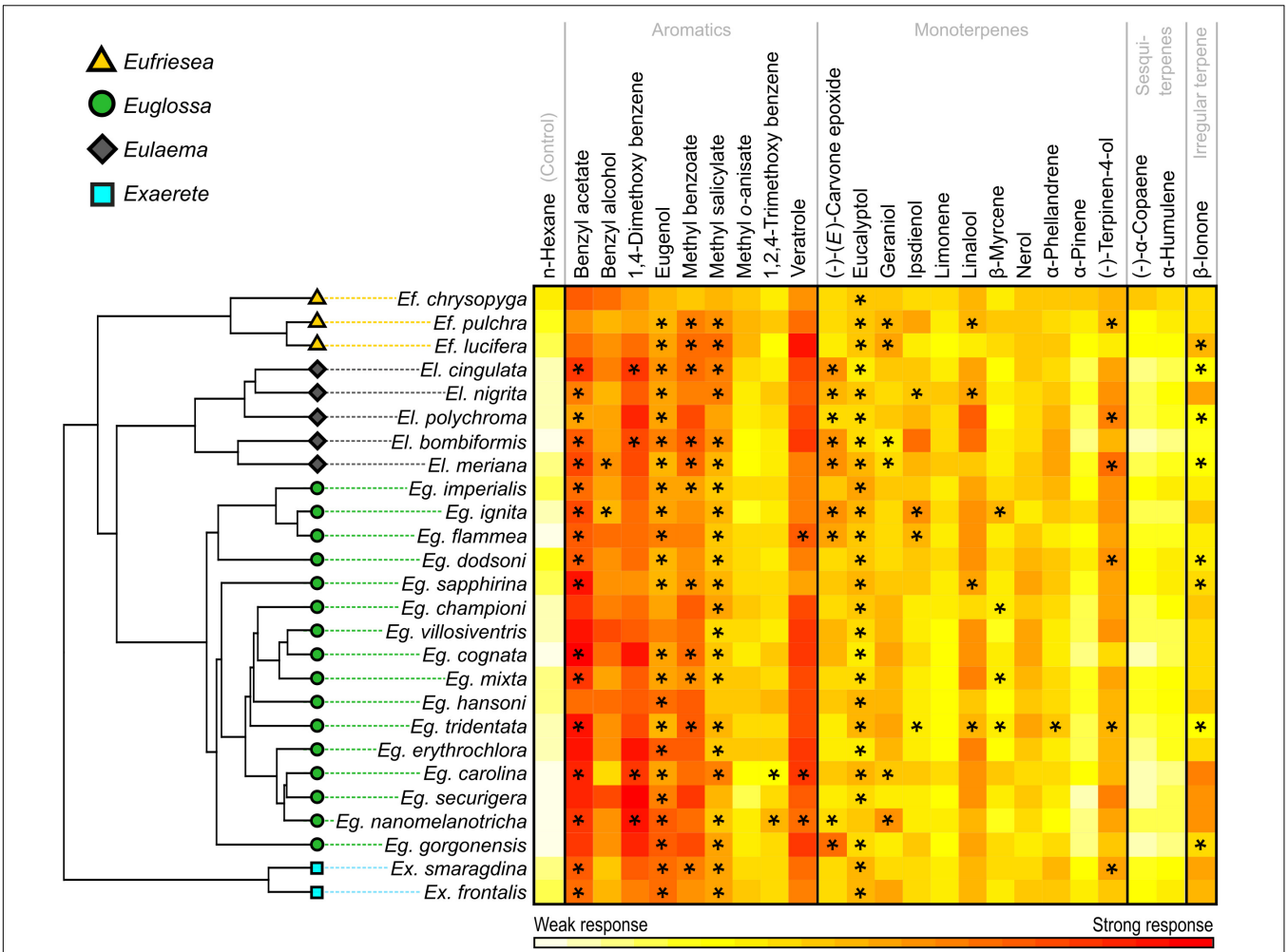

FIGURE 5 | Phylogenetic relationships of euglossine bee species based on data available for 26 of our 29 tested euglossine species (Ramírez et al., 2010b) included in this study along with a heatmap of the standardized antennal response profiles (standardized responses in percent). Colors indicate relative values of antennal strength to different chemical compounds, ranging from weak (bright yellow) to strong (deep red) responses. *Chemicals known to act as attractants for species; see also **Table 1**.

but also to ensure reproductive isolation among closely related orchid species. Appropriate mixtures of chemical compounds or the presence of specific major compounds in the floral scents enable the attraction of only few out of many different euglossine species (Dodson, 1970; Whitten et al., 1986). Together with further isolating mechanisms (see e.g., Hills et al., 1972; Williams and Whitten, 1983) the resulting highly specific attraction of pollinators in euglossinophilous plants can act as an effective reproductive barrier among otherwise interfertile plant species (Milet-Pinheiro and Gerlach, 2017). This is possible due to compound-specific differences in antennal perception even among closely related euglossine species, as we report here. For example, Eltz et al. (2008) showed how males of *Eg. dilemma* are strongly attracted to hydroxy-6-nona-1,3-dienyl-benzaldehyde (HNDB) and show strong antennal responses, while bees of the closely related and morphologically (Eltz et al., 2011) as well as ecologically (Villanueva-Gutierrez et al., 2013) similar species *Eg. viridissima* neither responds to this compound behaviorally nor electroantennographically. Brand et al. (2015, 2020) found that this divergence can be explained by a different selection among one single olfactory receptor gene (i.e., OR41), proving that (1) changes in the chemosensory gene family occur among closely related species and that (2) strong divergent selection acting on chemosensory receptor genes plays an important role in the evolution and diversification of the olfactory system in euglossine bees. The high species-specificity in antennal response profiles among species could be explained by the patterns of evolution of chemical sexual signaling. For example, the study of Cardé and Baker (1984) suggests that female preferences for a signal (receiver) impose strong stabilizing selection on male signal traits (sender), favoring the stability of the signal among populations and leading to a high species-specificity of chemical traits even across large geographic distances (Ord and Stamps, 2009). In this context, Zimmermann et al. (2006) and Ramírez et al. (2010a) revealed a qualitative consistency in perfume composition of tibial organs within euglossine bee species even when comparing

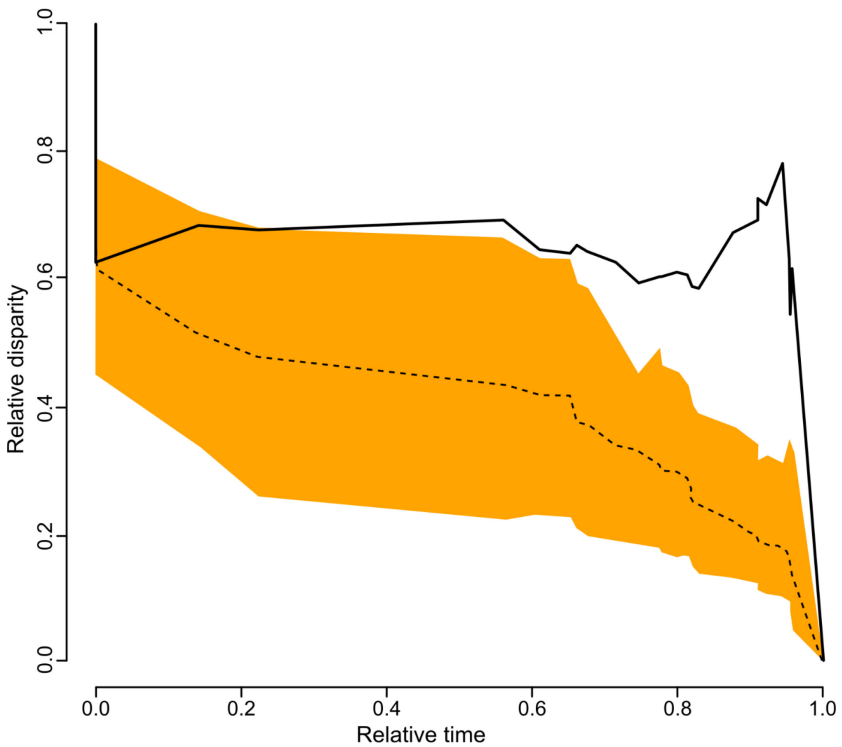

FIGURE 6 | Disparity through time (DDT) based on 100 simulations of phenotypic evolution of antennal response profiles (standardized responses in percent) based on data available for 26 of our 29 tested euglossine species. The relative time ranges from the beginning of the simulated evolution (0.0) to recent times (1.0; equivalent to the tips of the phylogeny in **Figure 5**). The dashed line represents the mean change in disparity across 100 replicates of simulated diversification and trait evolution as expected under a Brownian motion model with a 95% confidence interval of DDT range (orange area). The solid black line represents the actual mean change in disparity as calculated across the trees.

populations from distant geographic regions that harbor different perfume sources.

The results of the Blomberg's *K* test indicate the presence of a phylogenetic signal in the antennal response profiles of euglossine bee species. More specifically, there seems to be a tendency for species within a lineage to resemble each other more in their antennal responses than they resemble other lineages or random pairs of species, indicating that the diversification of the olfactory system of euglossine bee clades (genera) is phylogenetically conserved. Some chemical compounds (i.e., benzyl alcohol, 1,4-dimetoxy benzene, eugenol, linalool, methyl benzoate, methyl salicylate and veratrole) revealed an optimal level of antennal responses in the Brownian motion or Orenstein-Uhlenbeck model. A similar pattern was already described by Mitko et al. (2016) comparing the antennal responses of males belonging to 15 sympatric *Euglossa* species stimulated with compounds present in the hind tibiae. The results of this study suggest that sensory specialization has occurred within multiple lineages due to strong antennal responses for some chemicals that are present as major compounds in the perfume of the same species. Such a pattern is congruent with strong stabilizing selection acting to maintain antennal responses to specific compounds across the phylogeny (Hansen, 1997). The compounds affected by that pattern in our study have been frequently reported, not only within the floral scents of perfume-rewarding pollination systems (e.g., Montalvo and Ackerman, 1986; Gerlach and Schill, 1991; Hentrich et al., 2010), but in a variety of angiosperms worldwide (Knudsen et al., 2006). Therefore, we can assume that the selection on antennal response profiles of euglossine bee species could not only be driven by the association of perfume-rewarding plants but also by other aspects. For example, the compounds promoting an optimal level of antennal responses, as suggested by our phylogenetic analysis, could be important signal traits in the discrimination of sex partners by female orchid bees (see also Cardé and Baker, 1984) or in the search for nectar (see e.g., Borrell, 2005). Since perfume-rewarding flowers seem to contribute only little to the aromatic richness found in the tibial organs of male euglossine bees (Whitten et al., 1993; Ramírez et al., 2010a), we also cannot exclude, for example, the influence of non-floral perfume sources for male euglossine bees, such as rotting plant material, bark, leaves, and feces (Whitten et al., 1993).

At the same time, the results derived from the DDT plots indicate that closely related euglossine diverge more quickly on their antennal response profiles than expected under a Brownian motion model of neutral trait evolution, especially at the species level underlining the specificity of antennal responses on the species level of euglossine bees found in our electroantennographic analyses. Generally, these results resemble the patterns that have already been described on the

macroevolution of perfume signaling in euglossine bees (i.e., perfumes collected in the tibial organs of males). For example, the study of Weber et al. (2016) revealed both high species-specificity and elevated rates of evolution in perfume signals found in extracts of the tibial organs of distinct *Euglossa* species and stated that perfume evolution may be tied to the high number of orchid bee species coexisting together in neotropical communities. Furthermore, they described a rapid divergence at speciation and character displacement (see also Zimmermann et al., 2009b). Because of the high diversity of antennal response profiles on the species level, we cannot totally exclude the possibility that there might exist co-evolutionary adaptations between perfume flowers and euglossine bees, especially during the latest stage of evolution. Earlier studies have, indeed, suspected a (rather loose) coevolution for perfume rewarding orchids and their euglossine pollinators (e.g., Kiester et al., 1984). However, the already mentioned findings that (1) floral scent of perfume flowers developed much later than the collection behavior in euglossine bees (Ramírez et al., 2011) and that (2) a great part of chemical compounds collected by euglossine bees to build their unique tibial blends derive from non-floral rather than floral sources (Whitten et al., 1993; Ramírez et al., 2010a), suggest that sexual selection/changes in mating ecology might influence the evolution of the olfactory equipment of distinct euglossine bee species rather than the floral sources or their availability.

Altogether, the results of our study offer an overview of antennal responses for many different euglossine bee species belonging to distinct genera. The differences in antennal responses between distinct euglossine genera and subgenera, as well as species-specific patterns, reinforce the findings for the floral scent compositions in different species of perfume-rewarding flowers and offer first experimental evidence for the hypothesis of pollinator-mediated selection of floral scents driven by preexisting sensory biases in euglossine bees (Ramírez et al., 2011). The findings of our phylogenetic analyses indicate that a diversification of the olfactory system between euglossine bee genera could be (at least partly) phylogenetically conserved. Moreover, our results are congruent with a scenario of stabilizing selection acting on antennal responses to individual compounds, in particular to chemical compounds commonly found in perfume-rewarding flowers. At the same time, closely related species within taxonomic groups can differ considerably in their olfactory system due to a rapid evolution and a high level of disparity (Brand et al., 2020). Further phylogenetic investigations, for example on chemosensory genes of euglossine species (similar to the work of Brand et al., 2015) in combination with electroantennographic comparisons could shed more light into the evolution of the sensory periphery of euglossine bees and, consequently, in the evolution of floral scents in perfume-rewarding flowers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KB, SD, PM-P, and MA developed the experimental design and the idea. OR and DD synthetized the tested compound carvone epoxide. KB and PM-P conducted all experiments and collected the data. KB wrote the first draft of the manuscript and analyzed the results of the electroantennography. SR was mainly responsible for the phylogenetic analyses. All authors contributed to the revision of the manuscript.

FUNDING

This work was supported by the Deutsche Forschungsgemein schaft (AY 12/12-1), the Fundaçao de Apoio ao Desenvolvimento da Universidade Federal de Pernambuco (FADE-UFPE), the Deutscher Akademischer Austauschdienst (DAAD grant number 57210526 to KB), the "Ulrike Goldschmid-Grant" (grant to KB), the Coordenação de Aperfeiçoamento de Nível Superior (CAPES, Brazil – Finance Code 001 – grant to PM-P), the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (grant number FACEPE/BCT-0288-2.05/17 to PM-P), and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (grant number CNPq/PDJ-152077/2016-2 to PM-P).

ACKNOWLEDGMENTS

We thank the staff of the field station La Gamba in Costa Rica for providing research facilities, accommodation and personal care. We further thank Ulrike Goldschmid and all responsible people for awarding the private "Ulrike Goldschmid-Grant" in cooperation with the University of Vienna.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.727471/full#supplementary-material

REFERENCES

Ackerman, J. D. (1983). Diversity and seasonality of male euglossine bees (Hymenoptera: Apidae) in Central Panama. *Ecology* 64, 274–283. doi: 10.2307/1937075

Ackerman, J. D. (1989). Geographic and seasonal variation in fragrance choices and preferences of male euglossine bees. *Biotropica* 21, 340–347. doi: 10.2307/2388284

Anderson, M. J., Gorley, R. N., and Clarke, K. R. (2008). *PERMANOVA + for PRIMER: Guide to Software and Statistical Methods.* Plymouth: PRIMER-E.

Armbruster, W. S., and McCormick, K. D. (1990). Diel foraging patterns of male euglossine bees: ecological causes and evolutionary responses by plants. *Biotropica* 22, 160–171. doi: 10.2307/2388409

Armbruster, W. S., Keller, S., Matsuki, M., and Clausen, T. (1989). Pollination of *Dalechampia magnoliifolia* (Euphorbiaceae) by male euglossine bees. *Am. J. Bot.* 76, 1279–1285. doi: 10.1002/j.1537-2197.1989.tb15109.x

Ayasse, M., and Dötterl, S. (2014). The role of preadaptations or evolutionary novelties for the evolution of sexually deceptive orchids. *New Phytol.* 203, 710–712. doi: 10.1111/nph.12914

Ayasse, M., Paxton, R. J., and Tengö, J. (2001). Mating behavior and chemical communication in the order Hymenoptera. *Annu. Rev. Entomol.* 46, 31–78. doi: 10.1146/annurev.ento.46.1.31

Ayasse, M., Schiestl, F. P., Paulus, H. F., Ibarra, F., and Francke, W. (2003). Pollinator attraction in a sexually deceptive orchid by means of unconventional chemicals. *Proc. R. Soc. Lond. B Biol. Sci.* 270, 517–522. doi: 10.1098/rspb.2002.2271

Ayasse, M., Stökl, J., and Francke, W. (2011). Chemical ecology and pollinator-driven speciation in sexually deceptive orchids. *Phytochemistry* 72, 1667–1677. doi: 10.1016/j.phytochem.2011.03.023

Birch, M. C., and Haynes, K. F. (1982). *Insect Pheromones*. London: Edward Arnold.

Blomberg, S. P., Garland, T. Jr., and Ives, A. R. (2003). Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution* 57, 717–745. doi: 10.1111/j.0014-3820.2003.tb00285.x

Borrell, B. J. (2005). Long tongues and loose niches: evolution of euglossine bees and their nectar flowers 1. *Biotropica* 37, 664–669. doi: 10.1111/j.1744-7429.2005.00084.x

Brand, P., Hinojosa-Díaz, I. A., Ayala, R., Daigle, M., Obiols, C. L. Y., Eltz, T., et al. (2020). The evolution of sexual signaling is linked to odorant receptor tuning in perfume-collecting orchid bees. *Nat. Commun.* 11:244. doi: 10.1038/s41467-019-14162-6

Brand, P., Ramírez, S. R., Leese, F., Quezada-Euan, J. J. G., Tollrian, R., and Eltz, T. (2015). Rapid evolution of chemosensory receptor genes in a pair of sibling species of orchid bees (Apidae: Euglossini). *BMC Evol. Biol.* 15:176. doi: 10.1186/s12862-015-0451-9

Brandt, K., Dötterl, S., Fuchs, R., Navarro, D. M. D. A. F., Machado, I. C. S., Dobler, D., et al. (2019). Subtle chemical variations with strong ecological significance: stereoselective responses of male orchid bees to stereoisomers of carvone epoxide. *J. Chem. Ecol.* 45, 464–473. doi: 10.1007/s10886-019-01072-6

Brandt, K., Machado, I. C., Navarro, D. M. D. A. F., Dötterl, S., Ayasse, M., and Milet-Pinheiro, P. (2020). Sexual dimorphism in floral scents of the neotropical orchid *Catasetum arietinum* and its possible ecological and evolutionary significance. *AoB Plants* 12:laa030. doi: 10.1093/aobpla/plaa030

Cancino, A. D. M., and Damon, A. (2007). Fragrance analysis of euglossine bee pollinated orchids from Soconusco, south-east Mexico. *Plant Species Biol.* 22, 129–134. doi: 10.1111/j.1442-1984.2007.00185.x

Cardé, R. T., and Baker, T. C. (1984). "Sexual communication with pheromones," in *Chemical Ecology of Insects*, eds W. J. Bell and R. T. Cardé (Boston, MA: Springer), 355–383. doi: 10.1007/978-1-4899-3368-3_13

Carvalho, R., and Machado, I. (2002). Pollination of *Catasetum macrocarpum* (Orchidaceae) by *Eulaema bombiformis* (Euglossini). *Lindleyana* 17, 85–90.

Chow, Y., and Wang, S. (1981). Attraction responses of the American cockroach to synthetic periplanone-B. *J. Chem. Ecol.* 7, 265–272. doi: 10.1007/BF00995749

Clarke, K. R., and Gorley, R. N. (2006). *PRIMER V6: User Manual / Tutorial*. Plymouth: PRIMER-E, Ltd.

Couto, A., Alenius, M., and Dickson, B. J. (2005). Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Curr. Biol.* 15, 1535–1547. doi: 10.1016/j.cub.2005.07.034

Dodson, C. (1970). "The Role of Chemical Attractants in Orchid Pollination," in *Biochemical Co-Evolution,* ed. K. L. Chambers (Corvallis: Oregon State University Press), 83–107.

Dodson, C. (1978). The catasetums (Orchidaceae) of tapakuma, Guyana. *Selbyana* 2, 159–168.

Dodson, C. H. (1962). Pollination and variation in the subtribe Catasetinae (Orchidaceae). *Ann. Mo. Bot. Gard.* 49, 35–56. doi: 10.2307/2394740

Dodson, C. H., Dressler, R. L., Hills, H. G., Adams, R. M., and Williams, N. H. (1969). Biologically active compounds in orchid fragrances. *Science* 164, 1243–1249. doi: 10.1126/science.164.3885.1243

Dressler, R. L. (1968). Pollination by euglossine bees. *Evolution* 22, 202–210. doi: 10.1111/j.1558-5646.1968.tb03463.x

Dressler, R. L. (1982). Biology of the orchid bees (Euglossini). *Annu. Rev. Ecol. Syst.* 13, 373–394. doi: 10.1146/annurev.es.13.110182.002105

Eltz, T., Ayasse, M., and Lunau, K. (2006). Species-specific antennal responses to tibial fragrances by male orchid bees. *J. Chem. Ecol.* 32, 71–79. doi: 10.1007/s10886-006-9352-0

Eltz, T., Fritzsch, F., Pech, J. R., Zimmermann, Y., Ramirez, S. R., Quezada-Euan, J. J. G., et al. (2011). Characterization of the orchid bee *Euglossa viridissima* (Apidae: Euglossini) and a novel cryptic sibling species, by morphological, chemical, and genetic characters. *Zool. J. Linn. Soc.* 163, 1064–1076. doi: 10.1111/j.1096-3642.2011.00740.x

Eltz, T., Roubik, D. W., and Whitten, M. W. (2003). Fragrances, male display and mating behaviour of *Euglossa hemichlora*: a flight cage experiment. *Physiol. Entomol.* 28, 251–260. doi: 10.1111/j.1365-3032.2003.00340.x

Eltz, T., Roubik, D. W., and Lunau, K. (2005a). Experience-dependent choices ensure species-specific fragrance accumulation in male orchid bees. *Behav. Ecol. Sociobiol.* 59:149. doi: 10.1007/s00265-005-0021-z

Eltz, T., Sager, A., and Lunau, K. (2005b). Juggling with volatiles: exposure of perfumes by displaying male orchid bees. *J. Comp. Physiol. A* 191, 575–581. doi: 10.1007/s00359-005-0603-2

Eltz, T., Whitten, W., Roubik, D., and Linsenmair, K. (1999). Fragrance collection, storage, and accumulation by individual male orchid bees. *J. Chem. Ecol.* 25, 157–176. doi: 10.1023/A:1020897302355

Eltz, T., Zimmermann, Y., Pfeiffer, C., Pech, J. R., Twele, R., Francke, W., et al. (2008). An olfactory shift is associated with male perfume differentiation and species divergence in orchid bees. *Curr. Biol.* 18, 1844–1848. doi: 10.1016/j.cub.2008.10.049

Engel, M. S. (1999). *The First Fossil Euglossa and Phylogeny of Orchid Bees (Hymenoptera, Apidae, Euglossini). American Museum novitates; No. 3272*. New York, NY: American Museum of Natural History.

Frankie, G., Haber, W., Opler, P., and Bawa, K. (1983). "Characterstics and organization of the large bee pollination system in the Costa Rican dry forest," in *Handbook of Experimental Pollination Biology*, eds C. E. Jones and R. J. Little (Berkeley, CA: University of California), 411–447.

Galizia, C. G., and Vetter, R. S. (2004). "Optical methods for analyzing odor-evoked activity in the insect brain," in *Methods in Insect Sensory Neuroscience*, ed. T. A. Christensen (Boca Raton, FL: CRC-Press), 349–392.

Garver, L., Van Eikeren, P., and Byrd, J. E. (1976). A facile synthesis of (+)-pinol from (−)-carvone. *J. Org. Chem.* 41, 2773–2774. doi: 10.1021/jo00878a030

Gerlach, G., and Schill, R. (1991). Composition of orchid scents attracting euglossine bees. *Bot. Acta* 104, 379–384. doi: 10.1111/j.1438-8677.1991.tb00245.x

Getahun, M. N., Olsson, S. B., Lavista-Llanos, S., Hansson, B. S., and Wicher, D. (2013). Insect odorant response sensitivity is tuned by metabotropically autoregulated olfactory receptors. *PLoS One* 8:e58889. doi: 10.1371/journal.pone.0058889

González, J. (1996). Fauna of the Henri Pittier national park: Euglossini (Hymenoptera: Apidae: Bombinae). Keys and preliminary list. *Mem. Soc. Cien. Nat. Salle* 56, 45–54.

Gruber, M. H., Morawetz, L., and Wiemers, M. (2008). Diversidad de Euglossini (Hymenoptera, Apidae) en bosques lluviosos de tierras bajas primarios y secundarios en el sudoeste de Costa Rica. *Katal. Oberösterreichischen Landesmuseen Neue Ser.* 80, 257–266.

Hallem, E. A., and Carlson, J. R. (2006). Coding of odors by a receptor repertoire. *Cell* 125, 143–160. doi: 10.1016/j.cell.2006.01.050

Hansen, T. F. (1997). Stabilizing selection and the comparative analysis of adaptation. *Evolution* 51, 1341–1351. doi: 10.1111/j.1558-5646.1997.tb01457.x

Hansson, B. S., and Stensmyr, M. C. (2011). Evolution of insect olfaction. *Neuron* 72, 698–711. doi: 10.1016/j.neuron.2011.11.003

Harmon, L. J., Schulte, J. A., Larson, A., and Losos, J. B. (2003). Tempo and mode of evolutionary radiation in iguanian lizards. *Science* 301, 961–964. doi: 10.1126/science.1084786

Hentrich, H., Kaiser, R., and Gottsberger, G. (2010). Floral biology and reproductive isolation by floral scent in three sympatric Aroid species in French Guyana. *Plant Biol.* 12, 587–596. doi: 10.1111/j.1438-8677.2009.00256.x

Hetherington-Rauth, M. C., and Ramírez, S. R. (2015). Evolutionary trends and specialization in the euglossine bee-pollinated orchid genus Gongora. *Ann. Mo. Bot. Gard.* 100, 271–299. doi: 10.3417/2014035

Hildebrand, J. G., and Shepherd, G. M. (1997). Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. *Annu. Rev. Neurosci.* 20, 595–631. doi: 10.1146/annurev.neuro.20.1.595

Hills, G. (1968). *Fragrance Analysis in Chemotaxonomy of the Genus Catasetum (Orchidaceae)* Ph. D. Thesis. Coral Gables, FL: University of Miami.

Hills, H. G., Williams, N. H., and Dodson, C. H. (1972). Floral fragrances and isolating mechanisms in the genus *Catasetum* (Orchidaceae). *Biotropica* 4, 61–76. doi: 10.2307/2989728

Janzen, D. H., DeVries, P. J., Higgins, M. L., and Kimsey, L. S. (1982). Seasonal and site variation in Costa Rican euglossine bees at chemical baits in lowland deciduous and evergreen forests. *Ecology* 63, 66–74. doi: 10.2307/1937032

Kamilar, J. M., and Cooper, N. (2013). Phylogenetic signal in primate behaviour, ecology and life history. *Philos. Trans. R. Soc. B* 368:20120341. doi: 10.1098/rstb.2012.0341

Kiester, A. R., Lande, R., and Schemske, D. W. (1984). Models of coevolution and speciation in plants and their pollinators. *Am. Nat.* 124, 220–243.

Kimsey, L. S. (1982). *Systematics of Bees of the Genus Eufriesea (Hymenoptera, Apidae)*. Berkeley, CA: University of California Press.

Knudsen, J. T., Eriksson, R., Gershenzon, J., and Ståhl, B. (2006). Diversity and distribution of floral scent. *Bot. Rev.* 72:1. doi: 10.1663/0006-8101(2006)72[1:DADOFS]2.0.CO;2

López, D. F. (1963). Two attractants for *Eulaema tropica* L. *J. Econ. Entomol.* 56:540. doi: 10.1093/jee/56.4.540

Meeuse, B., and Morris, S. (1984). *The Sex Life of Flowers*. London: Faber and Faber.

Melo, G. A. (2014). Notes on the systematics of the orchid-bee genus Eulaema (Hymenoptera, Apidae). *Rev. Bras. Entomol.* 58, 235–240. doi: 10.1590/S0085-56262014000300003

Milet-Pinheiro, P., and Gerlach, G. (2017). Biology of the neotropical orchid genus *Catasetum*: a historical review on floral scent chemistry and pollinators. *Perspect. Plant Ecol. Evol. Syst.* 27, 23–34. doi: 10.1016/j.ppees.2017.05.004

Milet-Pinheiro, P., Silva, J. B. F., Navarro, D. M., Machado, I. C., and Gerlach, G. (2018). Notes on pollination ecology and floral scent chemistry of the rare neotropical orchid *Catasetum galeritum* R chb. f. *Plant Species Biol.* 33, 158–163. doi: 10.1111/1442-1984.12202

Mitko, L., Weber, M. G., Ramirez, S. R., Hedenström, E., Wcislo, W. T., and Eltz, T. (2016). Olfactory specialization for perfume collection in male orchid bees. *J. Exp. Biol.* 219, 1467–1475. doi: 10.1242/jeb.136754

Montalvo, A. M., and Ackerman, J. D. (1986). Relative pollinator effectiveness and evolution of floral traits in *Spathiphyllum friedrichsthalii* (Araceae). *Am. J. Bot.* 73, 1665–1676. doi: 10.1002/j.1537-2197.1986.tb09697.x

Morato, E., Campos, L. D. O., and Moure, J. (1992). Abelhas euglossini (Hymenoptera, Apidae) coletadas na Amazônia central. *Rev. Bras. Entomol.* 36, 767–771.

Nemesio, A. (2009). Orchid bees (Hymenoptera: Apidae) of the Brazilian Atlantic forest. *Zootaxa* 2041, 1–242. doi: 10.11646/zootaxa.2041.1.1

Olsson, S. B., and Hansson, B. S. (2013). "Electroantennogram and single sensillum recording in insect antennae," in *Pheromone Signaling*, ed. K. Touhara (Berlin: Springer), 157–177. doi: 10.1007/978-1-62703-619-1_11

Ord, T. J., and Stamps, J. A. (2009). Species identity cues in animal communication. *Am. Nat.* 174, 585–593. doi: 10.1086/605372

Pearson, D. L., and Dressler, R. L. (1985). Two-year study of male orchid bee (Hymenoptera: Apidae: Euglossini) attraction to chemical baits in lowland south-eastern Peru. *J. Trop. Ecol.* 1, 37–54. doi: 10.1017/S0266467400000067

Pennell, M. W., Eastman, J. M., Slater, G. J., Brown, J. W., Uyeda, J. C., FitzJohn, R. G., et al. (2014). geiger v2. 0: an expanded suite of methods for fitting macroevolutionary models to phylogenetic trees. *Bioinformatics* 30, 2216–2218. doi: 10.1093/bioinformatics/btu181

Peruquetti, R. C., Campos, L. D. O., Coelho, C. D. P., Abrantes, C. V. M., and Lisboa, L. D. O. (1999). Abelhas Euglossini (Apidae) de áreas de Mata Atlântica: abundância, riqueza e aspectos biológicos. *Rev. Bras. Zool.* 16(Suppl. 2), 101–118. doi: 10.1590/S0101-81751999000600012

Ramírez, S. R., Eltz, T., Fritzsch, F., Pemberton, R., Pringle, E. G., and Tsutsui, N. D. (2010a). Intraspecific geographic variation of fragrances acquired by orchid bees in native and introduced populations. *J. Chem. Ecol.* 36, 873–884. doi: 10.1007/s10886-010-9821-3

Ramírez, S. R., Roubik, D. W., Skov, C., and Pierce, N. E. (2010b). Phylogeny, diversification patterns and historical biogeography of euglossine orchid bees (Hymenoptera: Apidae). *Biol. J. Linn. Soc.* 100, 552–572. doi: 10.1111/j.1095-8312.2010.01440.x

Ramírez, S. R., Eltz, T., Fujiwara, M. K., Gerlach, G., Goldman-Huertas, B., Tsutsui, N. D., et al. (2011). Asynchronous diversification in a specialized plant-pollinator mutualism. *Science* 333, 1742–1746. doi: 10.1126/science.1209175

Ramírez, S., Dressler, R. L., and Ospina, M. (2002). Abejas euglosinas (Hymenoptera: Apidae) de la región Neotropical: listado de especies con notas sobre su biología. *Biota Colomb.* 3, 7–118.

Rebelo, J. M. M., and Moure, J. S. (1995). The species of Euglossa Latreille from the northeast of São Paulo, Brazil (Apidae, Euglossinae). *Rev. Bras. Zool.* 12, 445–466. doi: 10.1590/S0101-81751995000300001

Renou, M. (2014). "Pheromones and general odor perception in insects," in *Neurobiology of Chemical Communication*, 1 Edn, ed. C. Mucignat-Caretta (Boca Raton, MA: CRC Press). doi: 10.1201/b16511-3

Revell, L. J. (2012). phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* 3, 217–223. doi: 10.1111/j.2041-210X.2011.00169.x

Robertson, H. M., and Wanner, K. W. (2006). The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. *Genome Res.* 16, 1395–1403. doi: 10.1101/gr.5057506

Roelofs, W. L. (1984). "Electroantennogram assays: rapid and convenient screening procedures for pheromones," in *Techniques in Pheromone Research*, eds H. E. Hummel and T. A. Miller (New York, NY: Springer), 131–159. doi: 10.1007/978-1-4612-5220-7_5

Romero, G. A., and Carnevali, G. (2009). "Catasetum," in *Genera Orchidearum, Epidendroidea – Part II*, eds A. M. Pridgeon, P. J. Cribb, M. W. Chase, and F. N. Rasmussen (New York: Oxford University Press), 13–18.

Roubik, D. W., and Hanson, P. E. (2004). *Abejas de Orquídeas de la América Tropical: Biología y Guía de Campo. Orchid Bees of Tropical America*. Santo Domingo: Editorial Institutuo Nacional de Biodiversidad.

Schäffler, I., Steiner, K. E., Haid, M., van Berkel, S. S., Gerlach, G., Johnson, S. D., et al. (2015). Diacetin, a reliable cue and private communication channel in a specialized pollination system. *Sci. Rep.* 5:12779. doi: 10.1038/srep12779

Schneider, D., and Steinbrecht, R. A. (1968). Checklist of insect olfactory sensilla. *Symp. Zool. Soc. Lond.* 23, 279–297.

Shields, V., and Hildebrand, J. G. (2001). Responses of a population of antennal olfactory receptor cells in the female moth *Manduca sexta* to plant-associated volatile organic compounds. *J. Comp. Physiol. A* 186, 1135–1151. doi: 10.1007/s003590000165

Silva, F., and Rebêlo, J. M. M. (1999). Euglossine bees (Hymenoptera: Apidae) of buriticupu, amazonia of Maranhão, Brazil1. *Acta Amaz.* 29, 587–599. doi: 10.1590/1809-43921999294599

Steinbrecht, R. A. (1997). Pore structures in insect olfactory sensilla: a review of data and concepts. *Int. J. Insect Morphol. Embryol.* 26, 229–245. doi: 10.1016/S0020-7322(97)00024-X

Strausfeld, C. Z., and Kaissling, K.-E. (1986). Localized adaptation processes in olfactory sensilla of Saturniid moths. *Chem. Senses* 11, 499–512. doi: 10.1093/chemse/11.4.499

Takita, S., Yokoshima, S., and Fukuyama, T. (2011). A practical synthesis of (−)-kainic acid. *Org. Lett.* 13, 2068–2070. doi: 10.1021/ol200434a

Villanueva-Gutierrez, R., Quezada-Euan, J., and Eltz, T. (2013). Pollen diets of two sibling orchid bee species, Euglossa, in Yucatán, southern Mexico. *Apidologie* 44, 440–446. doi: 10.1007/s13592-013-0194-9

Vogel, S. (1966). Parfümsammelnde Bienen als Bestäuber von Orchidaceen und Gloxinia. *Österr. Bot. Z.* 113, 302–361. doi: 10.1007/BF01373435

Wang, Q., Huang, Q., Chen, B., Lu, J., Wang, H., She, X., et al. (2006). Total synthesis of (+)-machaeriol D with a key regio-and stereoselective

SN2' reaction. *Angew. Chem.* 118, 3733–3735. doi: 10.1002/ange.20060 0006

Weber, M. G., Mitko, L., Eltz, T., and Ramírez, S. R. (2016). Macroevolution of perfume signalling in orchid bees. *Ecol. Lett.* 19, 1314–1323. doi: 10.1111/ele. 12667

Whitten, W. M., Hills, H. G., and Williams, N. H. (1988). Occurrence of ipsdienol in floral fragrances. *Phytochemistry* 27, 2759–2760. doi: 10.1016/0031-9422(88) 80657-5

Whitten, W. M., Williams, N. H., Armbruster, W. S., Battiste, M. A., Strekowski, L., and Lindquist, N. (1986). Carvone oxide: an example of convergent evolution in euglossine pollinated plants. *Syst. Bot.* 11, 222–228. doi: 10.2307/24 18960

Whitten, W. M., Young, A. M., and Stern, D. L. (1993). Nonfloral sources of chemicals that attract male euglossine bees (Apidae: Euglossini). *J. Chem. Ecol.* 19, 3017–3027. doi: 10.1007/BF00980599

Williams, N. H., and Dodson, C. H. (1972). Selective attraction of male euglossine bees to orchid floral fragrances and its importance in long distance pollen flow. *Evolution* 26, 84–95. doi: 10.1111/j.1558-5646.1972.tb00 176.x

Williams, N. H., and Whitten, W. M. (1983). Orchid floral fragrances and male euglossine bees: methods and advances in the last sesquidecade. *Biol. Bull.* 164, 355–395. doi: 10.2307/1541248

Yasuda, A., Yamamoto, H., and Nozaki, H. (1979). A stereoselective 1, 3-transposition reaction of allylic alcohols. *Bull. Chem. Soc. Jpn.* 52, 1757–1759. doi: 10.1246/bcsj.52.1757

Zacharuk, R. Y. (1980). Ultrastructure and function of insect chemosensilla. *Annu. Rev. Entomol.* 25, 27–47. doi: 10.1146/annurev.en.25.010180.000331

Zimmermann, Y., Roubik, D. W., and Eltz, T. (2006). Species-specific attraction to pheromonal analogues in orchid bees. *Behav. Ecol. Sociobiol.* 60:833. doi: 10.1007/s00265-006-0227-8

Zimmermann, Y., Ramírez, S. R., and Eltz, T. (2009a). Chemical niche differentiation among sympatric species of orchid bees. *Ecology* 90, 2994–3008. doi: 10.1890/08-1858.1

Zimmermann, Y., Roubik, D., Quezada-Euan, J., Paxton, R., and Eltz, T. (2009b). Single mating in orchid bees (Euglossa, Apinae): implications for mate choice and social evolution. *Insectes Soc.* 56, 241–249.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 11 November 2021
doi: 10.3389/fevo.2021.662336

Naïve and Experienced Honeybee Foragers Learn Normally Configured Flowers More Easily Than Non-configured or Highly Contrasted Flowers

Scarlett R. Howard[1], Adrian G. Dyer[2,3]*, Jair E. Garcia[2], Martin Giurfa[4,5,6], David H. Reser[3,7], Marcello G. P. Rosa[3] and Aurore Avarguès-Weber[4]*

[1] Centre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University, Burwood, VIC, Australia, [2] Bio-Inspired Digital Sensing Lab, School of Media and Communication, RMIT University, Melbourne, VIC, Australia, [3] Department of Physiology and Neuroscience Program, Biomedicine Discovery Institute, Monash University, Clayton, VIC, Australia, [4] Centre de Recherches sur la Cognition Animale, Centre de Biologie Intégrative, Université de Toulouse, CNRS, UPS, Toulouse, France, [5] College of Animal Science (College of Bee Science), Fujian Agriculture and Forestry University, Fuzhou, China, [6] Institut Universitaire de France, Paris, France, [7] Monash Rural Health Churchill, Churchill, VIC, Australia

OPEN ACCESS

Edited by:
Christopher Irwin Smith, Willamette University, United States

Reviewed by:
Kathryn Theiss, California State University, Dominguez Hills, United States
Darren Incorvaia, McCann Health, United States
Daniel Papaj, University of Arizona, United States

***Correspondence:**
Scarlett R. Howard
s.howard@deakin.edu.au
Adrian G. Dyer
adrian.dyer@rmit.edu.au

Specialty section:
This article was submitted to Coevolution, a section of the journal Frontiers in Ecology and Evolution

Received: *01 February 2021*
Accepted: *04 October 2021*
Published: *11 November 2021*

Citation:
Howard SR, Dyer AG, Garcia JE, Giurfa M, Reser DH, Rosa MGP and Avarguès-Weber A (2021) Naïve and Experienced Honeybee Foragers Learn Normally Configured Flowers More Easily Than Non-configured or Highly Contrasted Flowers. Front. Ecol. Evol. 9:662336. doi: 10.3389/fevo.2021.662336

Angiosperms have evolved to attract and/or deter specific pollinators. Flowers provide signals and cues such as scent, colour, size, pattern, and shape, which allow certain pollinators to more easily find and visit the same type of flower. Over evolutionary time, bees and angiosperms have co-evolved resulting in flowers being more attractive to bee vision and preferences, and allowing bees to recognise specific flower traits to make decisions on where to forage. Here we tested whether bees are instinctively tuned to process flower shape by training both flower-experienced and flower-naïve honeybee foragers to discriminate between pictures of two different flower species when images were either normally configured flowers or flowers which were scrambled in terms of spatial configuration. We also tested whether increasing picture contrast, to make flower features more salient, would improve or impair performance. We used four flower conditions: (i) normally configured greyscale flower pictures, (ii) scrambled flower configurations, (iii) high contrast normally configured flowers, and (iv) asymmetrically scrambled flowers. While all flower pictures contained very similar spatial information, both experienced and naïve bees were better able to learn to discriminate between normally configured flowers than between any of the modified versions. Our results suggest that a specialisation in flower recognition in bees is due to a combination of hard-wired neural circuitry and experience-dependent factors.

Keywords: bottom-up processing, configural processing, pollinator, spatial configuration, top-down processing, visual learning

INTRODUCTION

Angiosperms display a combination of olfactory and visual cues to attract or deter pollinators. For example, in terms of visual spatial cues, bee-flies prefer large, and dissected flower models (Johnson and Dafni, 1998), while beetles have a preference for large and circular "bowl-shaped" flowers (Dafni and Kevan, 1997). Different species of small bees prefer to visit small flowers presenting broken outlines while larger bee species preferentially visit large circular flowers (Dafni and Kevan, 1997). The specific spatial preferences of pollinators may thus drive the evolution of angiosperms and the phenotypes of their flowers and in turn, the coevolution of plants and pollinators (Giurfa et al., 1999a; Fenster et al., 2004, 2006; Lázaro and Totland, 2014; Gómez et al., 2016).

Flowers pollinated by bees often share a similar, centrally symmetric, star-like configuration (Dafni et al., 1997). Howard et al. (2019e, 2021) suggested that shape may be a cue used by bees when attracted to images of unknown insect-pollinated flowers, as opposed to bird-pollinated flowers when bees are confronted with a binary choice between these options. A preference for star-like flower configurations has been demonstrated in both eusocial (Lehrer et al., 1995; Dafni et al., 1997; Howard et al., 2019e) and non-eusocial bees (Howard et al., 2021).

Honeybees can rapidly learn to discriminate between flower signals and/or cues including scent, colour, shape, size, and symmetry (scent: Deisig et al., 2001; Vergoz et al., 2007; Giurfa and Sandoz, 2012; colour: Dyer and Chittka, 2004; Giurfa, 2004; Dyer and Neumeyer, 2005; Dyer et al., 2008; Dyer and Murphy, 2009; Dyer, 2012b; Avarguès-Weber and Giurfa, 2014; Sommerlandt et al., 2016; shape: Lehrer et al., 1995; de Ibarra and Giurfa, 2003; Morawetz et al., 2013; size: Avarguès-Weber et al., 2014; Howard et al., 2017a,b; symmetry: Giurfa et al., 1996). This learning fits their foraging lifestyle when considering that foraging bees must discriminate between flowers to make a choice on where to collect nectar and/or pollen. Furthermore, efficient decision making promotes optimal nutrition collection to help enable colony survival (Burns and Dyer, 2008).

Previously, we have reported initial evidence that experienced honeybees show significantly improved learning of configured flowers over scrambled versions (Dyer et al., 2013). Bees are highly sensitive to spatial configuration, including prioritising global configurations over local features when memorising stimuli (Avarguès-Weber et al., 2015). Bees are also able to use spatial configurations to categorise stimuli (Avarguès-Weber et al., 2010), and can use holistic processing, an advanced form of configural processing where the predictive power of certain features is enhanced by the way such features are arranged (Maurer et al., 2002; Avarguès-Weber et al., 2018). For example, considering human vision and face processing, if features like noses, eyes, and mouths are scrambled to different positions within a stimulus, then we are less accurate at processing the face, even though the same basic featural information is available (Tanaka and Farah, 1993; Collishaw and Hole, 2000; Maurer et al., 2002). Importantly, relying on spatial configurations to identify visual objects is thought to be advantageous, as spatial arrangements of features are robust to changes in luminosity, viewpoint and partial occlusion, and holistic processing in humans facilitates subtle discrimination between highly similar objects such as human faces (Tanaka and Farah, 1993; Maurer et al., 2002).

Interestingly, bees learn normal contrast flower pictures more quickly compared to highly contrasted pictures (black and white) (Dyer et al., 2013). This high contrast picture modification was consistent with frequent forms of parameterised stimulus presentation in experiments involving honey bee vision (Lehrer et al., 1995; Dafni and Kevan, 1997; Dafni et al., 1997; Horridge, 1997; Avarguès-Weber et al., 2011). However, recordings of contrast sensitivity functions for orientation sensitive neurons in honeybees show that neural responses increase for a low-contrast range, but saturate beyond intermediate contrast values. This result suggests that lower and intermediate contrast images may already induce efficient discrimination due to maximal activation of feature detectors in the visual brain (Yang and Maddess, 1997). The question of how completely flower-naïve bees may learn and process flower configurations is crucial to start unravelling the mechanisms of flower learning and discrimination by experienced foragers.

High-level performance in bees when processing and discriminating between flower stimuli could be explained by top-down processing due to intensive experience. Top-down processing is a form of information processing where prior learnt knowledge or experience is used to inform behaviour (Sarter et al., 2001). Interestingly, honeybees have demonstrated evidence of using top-down processing (Zhang and Srinivasan, 1994; Chittka and Niven, 2009). In a classic experiment by Zhang and Srinivasan (1994), honeybees initially failed to learn to discriminate three-dimensional camouflaged shapes against a similar patterned background; but when bees were provided with prior experience with salient three-dimensional shapes they could subsequently solve the more complex camouflaged problem. Thus, bees demonstrated a capacity to use acquired information in the brain to improve subsequent discrimination performance. This is opposed to automatic and unidirectional reactions to stimuli inputs without using other knowledge acquired by the brain, which is described as a bottom-up way of processing. Bottom-up processing could potentially explain flower discrimination if bees possess specific cue detectors or filters in the early stage of neuronal visual processing that respond preferentially to key flower cues using a matched filtering type process (Wehner, 1987). In this case, bees should also be efficient in discriminating normally configured flowers within a short number of learning events. For example, honeybees perform well when learning salient colours within 3–7 visits to only a target colour (Menzel, 1967). Matched filter mechanisms can be easily exploited by deceptive species (Warrant, 2016), and thus animals may also require a capacity to learn to be flexible. In this scenario, a choice strategy driven by matched filters, which may underlie initial preferences by naïve animals, could easily been overridden by experience, which may lead to different choices. Considering colour processing in honeybees, fine colour differences require differential conditioning to both target and distractor stimuli for greater than 15 choices (Giurfa, 2004; Reser et al., 2012; Sommerlandt et al., 2016) to learn how to avoid deceptive options, and such learning promotes changes in the higher levels

of visual processing in a bee brain and the development of long-term memories (Dyer and Chittka, 2004; Dyer and Garcia, 2014; Sommerlandt et al., 2016). Thus, simple bottom-up or more advanced top-down type processes can be approached by testing stimuli that require differential conditioning for multiple trials (Dyer, 2012a). In such circumstances, animals are expected to assign a higher weight to the role of experience than to innate preferences (if any). The latter would manifest in the very first choices, yet they could be rapidly overshadowed by information acquired through individual experience.

Honeybees show evidence of learning spatial stimuli in a complex and dynamic way depending upon factors including stimulus salience; type of conditioning; and length of training. For example, considering simple stimuli composed of a low number of salient elements, honeybees and bumblebees have been observed to scan each element, which appears to be an innate initial learning behaviour (Lehrer et al., 1985; Dafni et al., 1997; MaBouDi et al., 2020) in a fashion somewhat analogous to how human vision moves eyes to initially scan salient elemental features in a scene when learning a new face, for instance (Henderson et al., 2005). This initial scanning behaviour can permit elemental learning based on spatial frequencies which could be explained by a matched filtering type process and subsequently allow a simple stimulus to be discriminated from alternative dissimilar stimuli (Srinivasan and Lehrer, 1988). One form of such learning can be described as absolute conditioning, where with a low number of learning events, sometimes within less than 10 trials, fast but relatively coarse discrimination is possible (Horridge and Zhang, 1995; Giurfa et al., 1999b). An alternative type of conditioning is termed differential conditioning, where rewarded target stimuli are learnt in the presence of non-rewarded and perceptually similar distractor stimuli. Differential conditioning enables finer levels of discrimination (Giurfa et al., 1999b; Stach et al., 2004). Learning with differential conditioning is dependent on the length of training. For instance, Stach and Giurfa (2005) reported that long training of 42 learning events (trials) led to significantly improved learning outcomes, and a fundamentally different type of visual processing compared to short training of 21 learning events (trials). This difference based upon experience explains why honeybees show evidence of moving from a simple scanning behaviour to a more complex, "cortical-like" way of processing spatial information (Srinivasan et al., 1993). Subsequent work has confirmed the dynamic nature of honeybee vision, and the dependence of training time required to enable different types of visual processing on task complexity (Avarguès-Weber et al., 2020). Interestingly, the human visual system also shows some similar patterns. For instance, in early stages of learning, eye movements are required to initiate learning (Henderson et al., 2005). Yet, later recognition of complex stimuli, such as faces, develops into robust holistic-type processing (Tanaka and Farah, 1993; Maurer et al., 2002) and can occur in the absence of scanning (Thorpe et al., 1996). Thus, visual learning and recognition appear to be enabled by dynamic processes in brains, although our understanding of these processes in insects is still emerging.

Here, we tested the performance of both flower-naïve and experienced bees learning to discriminate between pictures of either normally configured or scrambled flowers. In parallel, we tested the learning abilities of bees with high contrast pictures of normally configured flowers. We decided to employ a differential conditioning procedure with enough stimuli exposure to allow the bees to develop a complete representation of the stimuli.

We aimed to determine if the bee brain is inherently tuned to process flower cues, or if this type of specialisation emerges from foraging experience. To explore this question, we tested both flower-experienced and flower-naïve honeybee foragers with respect to their ability to learn rewarding vs. non-rewarding images depending on how the flower information was presented. We trained bees to discriminate between two flowers when flower spatial information was normally configured, symmetrically scrambled (experienced foragers only), asymmetrically scrambled, or high-contrasted. Experienced foragers were tested on all four stimulus comparisons, while naïve bees were tested on three of the comparisons. Our results suggest that flower shape recognition and memorisation is influenced by both hard-wired neural circuitry and experience-dependent factors.

MATERIALS AND METHODS

Study Species and Groups

Two groups of bees were trained to differentiate between two flower stimuli. One group consisted of experienced foragers (free-flying honeybee foragers; n = 137), while a second group consisted of flower-naïve foragers (bees living within a greenhouse which had never been exposed to flowers, hereafter referred to as naïve bees/naïve foragers; n = 30). Experienced foragers were allowed to freely forage and visit flowers from hives placed outside at Johannes Gutenberg University of Mainz, which has extensive biological gardens (see below). The experienced bees had previously visited flowers, and were collected while foraging at gravity feeders. These bees were typically 3–4-week-old individuals engaged in intensive foraging activities during the summer when the experiments were performed. Naïve foragers had not previously been exposed to either flowers nor any images of flowers. They were reared inside a greenhouse with only a clear glass von Frisch type gravity feeder to collect sucrose from and access to a water bowl (see below for more information). These bees were typically 3–4-week-old individuals trained and tested after exiting the hive in the greenhouse. While we had no control over the foraging experience of experienced bees prior to our experiments, naïve bees in the greenhouse never experienced appetitive rewards on flower-like visual stimuli. This approach has already been used to study innate colour preferences of bees raising within a greenhouse upon their first foraging flight (Giurfa et al., 1995).

Bees from both groups were divided into several subgroups to test for their ability to discriminate between the two stimuli, which consisted of (a) normal flower images (experienced foragers: n = 36; naïve foragers: n = 10), (b) scrambled flower images (experienced foragers: n = 36), (c) high contrast flower images (experienced foragers: n = 35; naïve foragers: n = 10),

and (d) asymmetric scrambled flower images (experienced foragers: n = 30; naïve foragers: n = 10; **Figure 1**). The difference between the number of groups was due to the challenges and limitations of training and testing honeybees in a greenhouse. Each bee only experienced one set of four images for the training and testing phases. Each set included two identical rewarded images and two identical non-rewarded images, different from the rewarded ones. Bees in all groups then underwent an unconditioned learning test to determine their ability to discriminate between the two training stimuli in the absence of reinforcement. Images of scrambled flowers follows the protocols of Collishaw and Hole (2000), who showed that image scrambling provides experimental access to understand featural or configural mechanisms of visual perception.

Group and sample sizes differed between the experienced and naïve groups, as naïve bees living within the greenhouse were harder to maintain and test than the free-flying experienced foragers. It is generally accepted that honeybees are difficult to keep, train, and test when constrained to small spaces, such as greenhouses, resulting in the experiments with naïve bees having lower group sizes than the groups of experienced bees.

All groups were counterbalanced for the image that was rewarding during training with the exception of the group consisting of n = 35 bees.

Experienced Bee Recruitment

Gravity feeders containing approximately 5–10% sucrose solution (by volume) were placed among hives at the Johannes Gutenberg University of Mainz. Honeybees landing on the feeders were recruited to the experiment on a plexiglass spoon containing 25% sucrose. When a bee volitionary returned to the testing site it was marked with a unique identifying colour code and or queen marking number following the standard procedure established by von Frisch (1965). Marked bees were collected onto a spoon containing 25% sucrose solution and taken to the rotating screen apparatus for training. They were placed on the platforms with no stimulus present and allowed to drink until satiated after which they returned to the hive. When the bee next returned to the rotating screen, stimuli were displayed and the experiment commenced.

Naïve Bee Maintenance

To ensure we could use naïve honeybee foragers, which had not experienced flowers throughout their lifespan, a greenhouse was constructed (Giurfa et al., 1995). The greenhouse was made of a transparent plastic sheet for the roof and walls, a grey plastic sheet for the floor, and plastic poles to hold it up. It was approximately 5 m × 2.5 m × 2.5 m (L × W × H; **Figure 2**). The area under and around the greenhouse was mowed and vegetation was removed to ensure there were no flowers close to the hive. A hive was placed in the wall of the greenhouse, with a mesh divider through the hive to ensure half of the bees could still fly out to collect nectar and pollen. We ensured the queen was in the half of the hive that only had access to the greenhouse. The greenhouse contained a grey gravity feeder, which provided 30% sucrose solution to foraging bees, and access to a water dish. After a few days of acclimatisation of the hive to the new position inside the greenhouse, we began to place colour marks on bees which were newly emerging from cells each morning on the greenhouse side of the hive. This was done for approximately 3 weeks before the marked bees emerged as foragers. Once the marked bees began exiting the hive to forage in the greenhouse, we could test the bees, which were marked and visiting the feeder, thereby ensuring that only naïve honeybee foragers which had lived their whole lives within the greenhouse were tested. Bees exiting the hive were placed on the feeder to allow them to find it within the greenhouse, or they found it themselves. Recruitment of bees to the experiment was performed as above with experienced bees. All marking, training, and testing of naïve bees was conducted within the greenhouse. We trained and tested 30 naïve foragers, with n = 10 per experiment.

Training

Both groups (naïve and experienced foragers) were presented with four flowers (two identically correct flower images and two identically incorrect flower images, pseudo-randomised per bee; see **Figure 3**) on a rotating screen for 30 choices. The rotating screen (**Figure 3**) was 50 cm in diameter and was made of a grey plexiglass material (Dyer et al., 2005; Avarguès-Weber et al., 2010). It contained hangers made of the same material used to present stimuli to bees. The hangers contained a landing platform on which sucrose solution could be placed, and bees could land to drink the solution directly below the stimuli. Bees were trained one at a time. Each time a bee landed on the correct flower type, it received a 10 μL drop of 50% sucrose solution as a reward for choosing the correct flower. If it landed on the incorrect flower option, it received a drop of water, a neutral outcome. Once a bee made a correct decision, it was removed from the apparatus using a transparent plexiglass spoon containing a 10 μL drop of 50% sucrose solution. The bee was placed behind an opaque screen while the apparatus was cleaned with ethanol, dried, stimulus positions were changed, and the screen was rotated to randomise the position of correct and incorrect stimuli. Once the bee finished imbibing the sucrose from the spoon, it could choose to either return to the hive or make another choice. Once a bee completed 30 choices, it was given sucrose on a spoon until it was satiated and returned to the hive. While the bee was in the hive, the apparatus was cleaned and set-up for the non-rewarded test. The training phase lasted approximately 2–3 h.

Testing

Both groups of bees were given a non-rewarded learning test of 20 choices to determine if they had learnt to choose the correct flower image during training. Each bee was only tested on the stimuli they had been trained on. For the test each bee was presented with the same four flowers from the training phase (two identically correct flower images and two identically incorrect flower images; see **Figure 3**) on a rotating screen. The number of test choices to be conducted was determined in a pilot study. As tests should occur in the absence of sucrose reward, we replaced it by water which was placed on the platforms to induce landings. Bees were tested using the same stimuli that they had been trained on: (i) normally configured flowers, (ii) scrambled flowers (experienced foragers only), (iii) high contrast

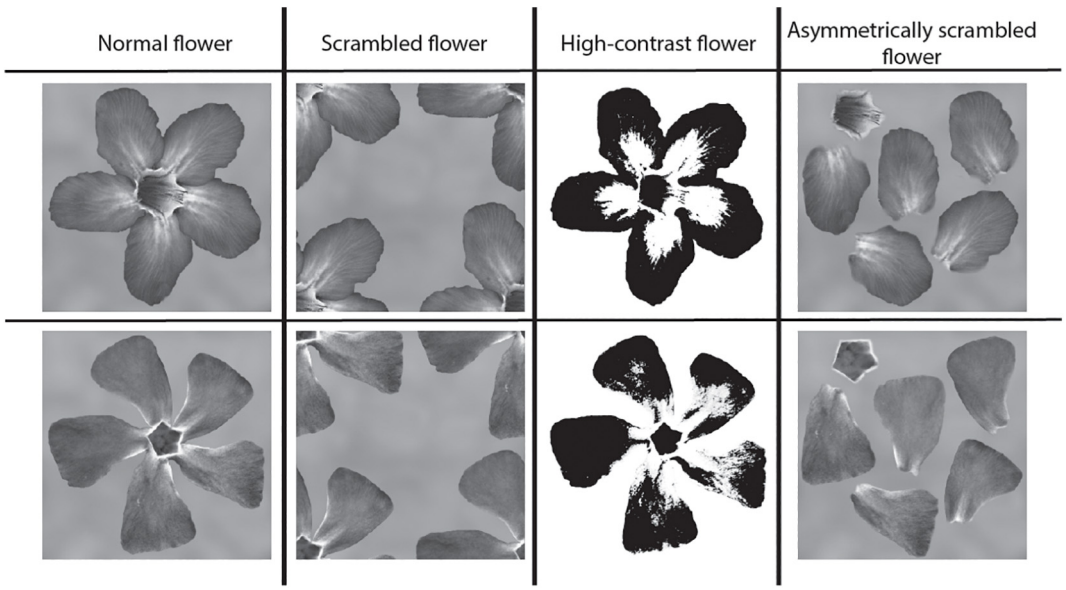

FIGURE 1 | The pairs of stimuli presented to bees learning to differentiate between two normal flower stimuli, two scrambled flower stimuli, two high contrast flower stimuli, and two asymmetrically scrambled flower stimuli. All stimuli contained the same image information compared to the normal flower images but differed in either contrast (black and white images compared to greyscale) or configuration of flower information (e.g., scrambled petals in image).

flowers, or (iv) asymmetrically scrambled flowers. Prior to the unrewarded test, all stimuli and platforms were cleaned with ethanol and dried to avoid scent marks. Generally, bees made all test choices within 5 min.

Statistical Analysis

Training Phase

Data from naïve and experienced bees were analysed separately as the two groups of bees were not exposed to the same number of flower types nor tested in parallel and originated from different colonies. We tested the null hypothesis that training would have no effect [slope (m) = 0], and that there would be no interaction between flower type and number of trials by fitting an ANCOVA generalised linear mixed model (ANCOVA glmm) to the choice data for each bee group. The linear model included, in addition to the intercept and error term (ε), two predictors representing the number of trial blocks as a continuous variable (five blocks of six choices each) and the flower type group as a categorical predictor with four levels for the experienced bees and three levels for the naïve bees, and an interaction term between the two predictor variables (Equation 1).

$$\text{logit}\,(pC)_{ij} = Intercept + Trial + Flower\ type_j + (Trial \times Flower\ type_j) + \alpha_i + \varepsilon_{ij}. \quad (1)$$

We used the proportion of correct choices per block as the response variable [$p(C)$] and assumed that the observations followed a binomial distribution. A logit function was used to link the proportion of correct choices to the linear predictor function. We included the ID number of each bee as a random term (α_i) in the model to account for the repeated measurements collected from each bee participating in the experiment.

Testing Phase

We initially fitted a generalised linear model (GLM) using the proportion of correct choices per bee as the response variable and the flower treatment as a predictor to test for a potential effect of flower treatment on the number of correct choices. We assumed that the proportion of correct choices is described by a binomial distribution and used a logit function to link the response variable with the linear predictor. As each bee contributed a single pair of correct and incorrect responses, we did not include a random term into this model. Flower treatment had the same four levels for the experienced bees or three levels for the naïve bees used during the learning phase. The model is analogous to a one-way ANOVA design described by Equation 2:

$$\text{logit}\left(pC\right)_j = \beta_1 + Flower\ type_j + \varepsilon_j. \quad (2)$$

In addition to the omnibus test, we tested whether the mean number of correct choices observed for each flower treatment differed significantly from chance. This was done by fitting individual GLM models to response data for each treatment including only the intercept term as a predictor.

All analyses were performed within the R environment for statistical analysis, Version 1.1.456 (R Core Team, 2017).

RESULTS

Training Phase

A graphical representation of the models fitting the choice data for the experienced and naïve bees is given in **Figure 4**.

FIGURE 2 | The greenhouse which was constructed to house the naïve honeybees **(A,B)** and the marked bees inside of the hive on a frame **(C,D)**.

Experienced Bees

The initial model fitted to the data corresponding to the training phase of the experienced bees showed no interaction between trial number and the type of image [*Deviance* (G) = 0.883, P = 0.830]. Therefore, we fitted a reduced model to the data excluding the interaction term. The reduced model yielded a significant effect of both trial (G = 13.2, $P < 0.001$) and image type (G = 21.2, $P < 0.001$) on the proportion of correct choices performed by the experienced bees.

The reduced model suggests that trial had a significant effect on the performance for all image types, as all four groups of bees improved choice performance at the same rate (**Figure 4A**). There was a significant effect of image type, indicating that the mean number of correct choices observed for each flower type during training was different. Contrast analyses performed on the image type variable revealed that the mean proportion of correct choices for the scrambled (z = 3.05, P = 0.002), high contrast (z = 3.43, P = 0.001), and asymmetrically scrambled flowers (z = 4.51, $P < 0.001$) were significantly lower than the mean proportion of choices observed for the normal flowers. This showed that experienced bees learnt the normally configured flowers better than the other images.

Interestingly, when considering the proportion of correct choices for the different image types between the first and second trial blocks, the normal flowers appeared to be learnt faster than the other alternatives (**Figure 5**) as no bees had learnt the task significantly better than chance level in the first trial block, but bees had learnt the normally configured flower by the end of the second block of trials. Even though the mean proportion of correct choices at the end of the first trial block (**Figure 5A**) was similar for the normal images and the three flower image variations ($z_{scrambled}$ = −0.290, P = 0.772; $z_{high-contrast}$ = −0.367, P = 0.714; $z_{asymmetric}$ = −1.47, P = 0.142), this trend changed by the end of the second block of trials, suggesting that bees learnt the normally configured flower images more quickly in the initial training trials. After 12 trials (**Figure 5B**), the number of correct choices differed significantly between the normal and high contrast flowers ($z_{high-contrast}$ = −2.11, P = 0.035); and between the normal and asymmetric flowers ($z_{asymmetric}$ = −2.73, P = 0.006). No significant difference was observed between the normal and scrambled flowers ($z_{scrambled}$ = −1.85, P = 0.065).

Pairwise comparisons

The initial omnibus test was followed by pairwise comparisons of the means for the different levels of the fixed treatment factor using estimated marginal means. Results indicate that the mean number of correct choices for the images representing normal flowers was always significantly higher than for each of the manipulated images (**Table 1**). Interestingly, we could not reject the hypothesis of equality of means when comparing the different pairs representing the three experimental conditions (**Table 1**). This means that although there is a significant difference between the normal flowers and each of the treatments, there is no difference between the different treatment pairs.

Naïve Bees

The initial, full model revealed a significant interaction effect (G = 12.0, P = 0.003) between trial and flower type for naïve bees (**Figure 4B**), indicating a difference in learning rate at least between two of the three flower types considered. A contrast analysis of the interaction term revealed that there were significant differences in the learning rates of naïve bees when learning normal vs. high contrast flowers (z = −2.67, P = 0.008), and when learning normal vs. symmetric, scrambled flowers (z = −3.20, P = 0.001).

Pairwise comparisons

To better understand the nature of the interaction term, we compared the slopes for each pair of levels in the fixed experimental treatment factor. Results indicate that the learning rate of normal flowers by naïve bees is significantly higher than the learning rate for the two different experimental treatments, whilst the different experimental treatments were learnt at the same rate (**Table 2**). These relationships explain the crossing-over of the learning curves for the different treatments observed for the naïve bees (**Figure 1**).

Testing Phase

Experienced Bees

Experienced foragers successfully chose the correct flower option in the unreinforced learning test, when trained and tested with the normally configured flowers (z = 6.85, $P < 0.001$, n = 36), the scrambled flowers (z = 3.27, P = 0.001, n = 35), and the high contrast flowers (z = 2.57, P = 0.010, n = 36). However, experienced foragers did not choose the correct flower option during the learning test in the asymmetrically scrambled flower condition (z = 0.000, P = 1.000, n = 30) (see **Figure 6**).

Pairwise comparisons

The initial omnibus analysis suggests a significant difference between the mean proportion of correct choices for at least one pair of flower treatments (G = 23.2, $P < 0.001$). Pairwise

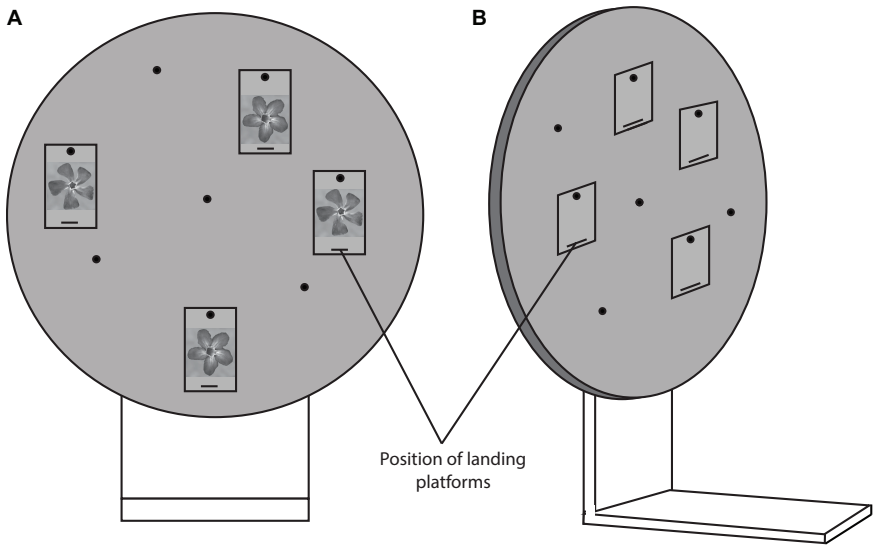

FIGURE 3 | The rotating screen apparatus (50 cm diameter) which was used in all experiments. In this diagram, the apparatus is shown with two identical normal flower stimuli which would be the correct option and two different but identical normal flower stimuli which would be the incorrect option **(A)**. The flower stimulus which was correct was pseudo-randomised between bees. The reward (drop of sucrose solution) was provided on the landing platforms. **(B)** Shows the apparatus from the side.

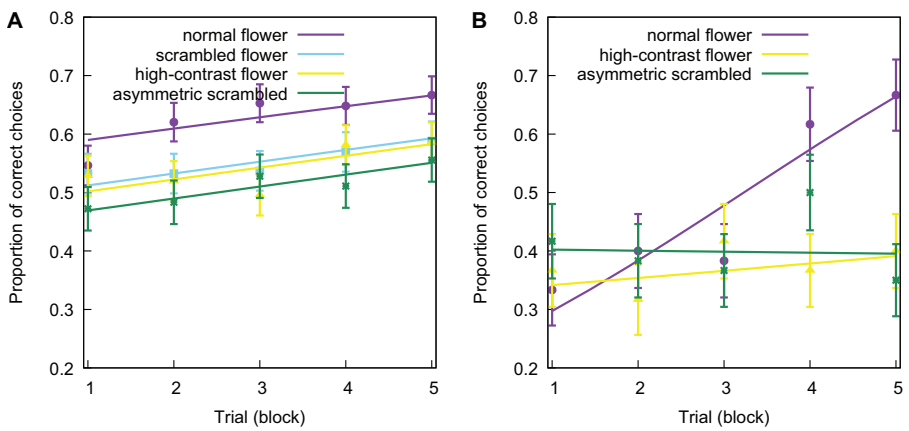

FIGURE 4 | Linear model (solid lines) and mean proportion of correct choices (markers) observed from a group of experienced **(A)** and naïve bees **(B)**. Different groups of bees were trained with differential conditioning to discriminate between two flower images in different configurations: normal flowers (purple line, circle markers), scrambled flowers (blue line, square markers), high contrast flowers (yellow line, triangle markers), and asymmetric scrambled flowers (green line, asterisk markers). Each training block represents the pooling of six choices following standard methods (Giurfa et al., 2001). Error lines represent the standard error of the mean proportion of correct choices for each trial block. The solid lines represent the fixed effects of the two models.

comparisons following the initial analysis revealed that the proportion of correct choices observed in bees trained on normal flowers were higher than in the other groups. There was no significant difference in the performance of bees trained and tested with any of the modified flower images (**Table 3**).

Naïve Bees

In contrast to the results of the learning test obtained for the experienced bees, naïve foragers chose all three flower treatments with a frequency significantly higher than chance expectation (normal flower stimuli: $z = 8.24$, $P < 0.001$, $n = 10$, high contrast flower: $z = 6.00$, $P < 0.001$, $n = 10$, and asymmetrically scrambled flower: $z = 2.25$, $P = 0.024$, $n = 10$; **Figure 6**).

Pairwise comparisons

As for the experienced bees, the initial analysis suggested a significant difference in the mean proportion of correct choices between the different flower treatments ($G = 2.83$, $P < 0.001$). Pairwise analyses following the omnibus test indicated a significant difference in the proportion of correct choices between the images representing normal flowers

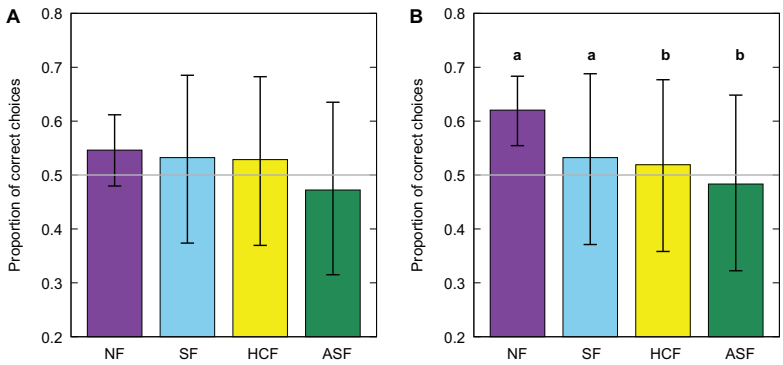

FIGURE 5 | Mean proportion of correct choices by experienced bees for normal (NF), scrambled (SF), high contrast (HCF), and asymmetric scrambled (ASF) flowers for trial blocks 1 **(A)** and 2 **(B)**. Error bars represent the 95% confidence intervals for the mean number of proportions and letters indicate statistically significant difference between normal flowers (NF) and the three experimental conditions. Refer to **Table 2** for the level of significance of each comparison. Grey line indicates chance level performance [p(C) = 50%]. Letters in panel **(B)** indicate significant differences between the manipulated flower groups and the normal flower group, which was used as a baseline for the analyses.

TABLE 1 | Pairwise comparisons of the marginal mean of proportions observed by a group of experienced bees for each treatment level during the training phase.

Treatment pair	Estimated marginal mean difference	95% CI	*z*	*P*-value
Normal/scrambled	0.323	0.120–0.532	3.05	0.012*
Normal/high contrast	0.364	0.160–0.575	3.43	0.003**
Normal/asymmetric	0.490	0.282–0.708	4.51	< 0.001***
Scrambled/high contrast	0.040	−0.160 to 0.241	0.398	0.979
Scrambled/asymmetric	0.160	−0.040 to 0.364	1.61	0.376
High contrast/asymmetric	0.120	−0.080 to 0.323	1.21	0.618

*P-values were adjusted for multiple comparisons using Tukey's honestly significant difference (HSD) method. * significant at $\alpha = 0.05$, ** significant at $\alpha = 0.01$, *** significant at $\alpha < 0.001$.*

TABLE 2 | Pairwise comparisons of the slopes of the learning curves observed by a group of naïve bees for each treatment level during the training phase.

Treatment pair	Estimated marginal mean difference	95% CI	*z*	*P*-value
Normal/high contrast	0.323	0.080–0.575	2.67	0.021*
Normal/asymmetric	0.405	0.160 to 0.619	3.20	< 0.004**
High contrast/asymmetric	0.040	−0.160 to 0.282	0.498	0.872

*P-values were adjusted for multiple comparisons using Tukey's honestly significant difference (HSD) method. * significant at $\alpha = 0.05$ and ** significant at $\alpha < 0.01$.*

and the two other treatments, and between the high-contrast and asymmetric treatments, with bees showing higher performance for learning normally configured flowers (**Table 4**).

DISCUSSION

Honeybees learnt to discriminate between normally configured pictures of flowers more efficiently than scrambled configurations or high-contrasted versions of the unscrambled pictures, even though these picture manipulations contained a similar level of featural information. Such superior performance for processing normal flower pictures was found both with experienced foragers and flower-naïve bees, providing evidence of an innate bias toward stimuli displaying flower-like configurations. Interestingly, bees which were completely flower naïve showed a higher rate of success for the modified pictures than experienced bees, which we discuss below.

Having an innate template and ability to process flowers would be beneficial to honeybees in a complex natural environment. Such a template would enable naïve foragers to efficiently detect and recognise rewarding flowers whilst avoiding deceptive alternatives like mimics that are known to exist in nature (Dyer and Chittka, 2004; Garcia et al., 2020). Our results (**Figures 4–6**) suggest that there is a component of innate preference for configured flowers – much like a flower template which is most likely "hard-wired" in the synaptic structure of the brain. Our findings also indicate that with experience, bees may improve their flower recognition and discrimination. When emerging from the hive, naïve honeybees may consequently employ a flower template, as previously shown by Lehrer et al. (1995) for star shaped stimuli, that permits efficient learning of similar star shaped flowers. The

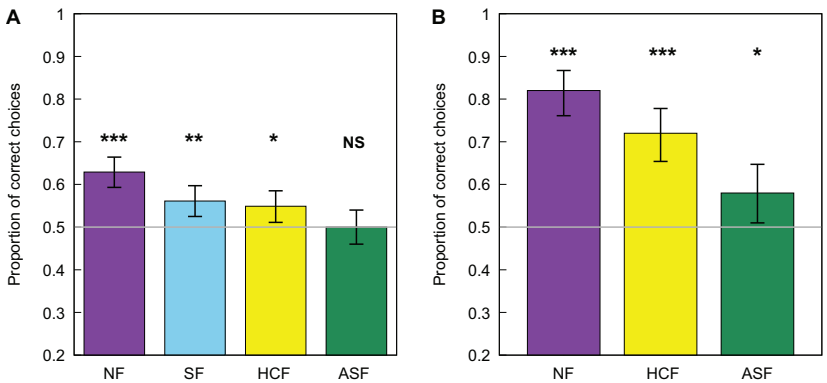

FIGURE 6 | Mean number of correct choices for images representing normal (NF), scrambled (SF), high contrast (HCF), and asymmetric scrambled (ASF) images of flowers by experienced **(A)** and naïve bees **(B)** observed during the learning tests. Error bars represent the 95% confidence intervals for the mean in all cases. Asterisks indicate a mean number of correct choices significantly different from chance level expectation of 50% (grey line) at $*P < 0.05$, $**P < 0.001$, and $***P < 0.001$. NS indicates a mean proportion of choices not significantly different from chance.

TABLE 3 | Pairwise comparisons of the marginal mean of proportions observed by a group of experienced bees for each treatment level during the learning tests.

Treatment pair	Estimated marginal mean difference	95% CI	*z*	*P*-value
Normal/scrambled	0.282	0.080–0.490	2.63	0.043*
Normal/high contrast	0.323	0.120–0.532	3.08	0.011***
Normal/asymmetric	0.532	0.323–0.754	4.71	< 0.001***
Scrambled/high contrast	0.040	−0.160 to 0.282	0.475	0.965
Scrambled/asymmetric	0.241	0.000–0.447	2.22	0.119
High contrast/asymmetric	0.201	−0.040 to 0.405	1.75	0.299

*P-values were adjusted for multiple comparisons using Tukey's honestly significant difference (HSD) method. * significant at $\alpha = 0.05$ and *** significant at $\alpha < 0.001$.*

TABLE 4 | Pairwise comparisons of the marginal mean of proportions observed by a group of naïve bees for each treatment level during the learning tests.

Treatment pair	Estimated marginal mean difference	95% CI	*z*	*P*-value
Normal/high contrast	0.575	0.080–1.046	2.36	0.048*
Normal/asymmetric	1.208	0.754–1.658	5.12	< 0.001***
High contrast/asymmetric	0.619	0.201–1.046	2.92	0.010*

*P-values were adjusted for multiple comparisons using Tukey's honestly significant difference (HSD) method. * significant at $\alpha = 0.05$ and *** significant at $\alpha < 0.001$.*

neural basis for such a template could be the presence of "matched filters" in the nervous system (Wehner, 1987), that is, visual detectors that specialise in extracting and responding to specific relevant features or configurations in the external world. Whilst initially thought to operate at an early stage of visual processing, such filters show some evidence of operating at the more central levels of sensory processing (Warrant, 2016). The presence of such filters predicts rapid responding to biologically relevant stimuli (Warrant, 2016), although this is not fully consistent with our accumulated evidence that both naïve and experienced foragers take about 30 learning trials with differential conditioning to reliably discriminate between the similar flower images. Detectors in early stages of visual processing could thus only contribute to enhancing discrimination learning abilities by increasing stimuli saliency and by mobilising attentional processes. Indeed, extended learning, which involves some form of top-down use of information, is essential to how bees acquire a capacity to identify target or deceptive flower species. In mammalian systems it is now well appreciated that learning of fine perceptual tasks may involve both higher level neural representations, and also feedback mechanisms that potentially tune responses of feature detectors in the earlier stages of visual processing (Ahissar and Hochstein, 2004; Ahissar et al., 2009). Whilst such phenomenon have proved difficult to access in bees because learning of more complex tasks requires free flying test conditions (Dyer, 2012a; Dyer and Griffiths, 2012), this possibility for information processing remains an interesting area for future work to understand how a miniature brain can enable fine discrimination tasks. Neural elements specialised and tuned *via* experience in responding to radially organised stimuli could thus promote efficiency to enable avoidance of deceptive stimuli, maximising nutrition collection and promoting colony survival.

There are two potential evolutionary processes by which bees may have acquired specialisation for configured, star-shaped flowers (Howard et al., 2019e, 2021). The first process suggests that the flowers of angiosperms would

have evolved to exploit the preferences of insects, such as honeybees, for certain flower morphologies (Lehrer et al., 1995; Gegear et al., 2017). This theory is supported by the knowledge that Hymenoptera, including bees, arose during the Permian time period, millions of years before angiosperms arose during the Late Triassic (van der Kooi and Ollerton, 2020). In the other process, honeybees have evolved a preference for the star-like shapes of flowers pollinated by insects due to the nectar and pollen being generally more accessible in this morphology than in other morphological types (Brown et al., 2011).

The flower naïve test group very efficiently learnt to discriminate between the rewarding and non-rewarding real flower images that we used as stimuli, and this learning was significantly better than for the asymmetrically scrambled flowers that would disrupt an innate flower shape template (**Figure 6**). Interestingly, whilst not a specifically designed research question of the current study, the naïve bees appeared to learn to discriminate between the holistic flower stimuli to a higher level than the experienced bee group (**Figure 6**), although both groups demonstrated significant learning after 30 trials. One possible explanation for the potentially better learning in flower naïve honeybees is that the experienced bees had very likely visited a range of different flowers in the university garden prior to participating in the experiment. Previous research on naïve honeybee innate preferences for colour stimuli shows that an initial preference (Menzel, 1967) is overwritten by experience with other rewarding colours (Giurfa et al., 1995). A similar phenomenon has also been observed in free-flying bumblebees (Gumbert, 2000) where bees revert to innate preferences if conditions require. Thus, experience with the shapes of real flowers may lead to overwriting any initial innate preferences to some extent. However, a confound to this conclusion is that the naïve bees also showed the capacity to learn to recognise features in the asymmetric flower stimuli, whilst the experienced bees did not (**Figures 5**, **6**). Therefore, the naïve bees could potentially just have been better learners by chance, and/or the experienced bees had developed a more holistic processing strategy. The latter has been observed to emerge in honeybees through experience (Avarguès-Weber et al., 2020). In humans, holistic processing is known to reduce some featural processing capacity (Maurer et al., 2002). Dissecting how experience may influence innate preferences for flowers in bees appears to be a fruitful avenue for future research.

Our current study complements previous research establishing honeybee's preference for specific flower-like geometric patterns (star-shaped stimuli and stimuli with elements which radiate outward) (Lehrer et al., 1995; Dafni et al., 1997). The importance of flower configuration for bees may explain their tendency to preferentially approach unfamiliar insect-pollinated flowers, originating from a different continent, since insect-pollinated flowers tend to share star-like shapes and radiating elements by opposition to other flowers. The flower configuration not only attracts bees but also induces improved discrimination and learning performance probably due to a combination of bottom-up (feature detectors) and top-down (attentional bias and refinement of the template with experience) processing.

Brains have evolved to be tuned to important stimuli which are species specific. For example, humans and other primates are tuned to detect, process, recognise, and discriminate between the faces of conspecifics (Kanwisher et al., 1997; Pascalis et al., 2002; Wilmer et al., 2010; Young and Burton, 2018). Non-human primates have a network of cortical face-selective "patches" distributed across the inferior temporal and frontal cortex (Hung et al., 2015; Schaeffer et al., 2020), in which neuronal tuning to faces develops from a scaffolding that is already present at birth (Livingstone et al., 2017). Moreover, human infants prefer human face-like stimuli to other non-face-like stimuli (Goren et al., 1975; Valenza et al., 1996; Mondloch et al., 1999; Pascalis et al., 2002), although experience also plays a decisive role in tuning our capacity to discriminate and memorise faces (Pascalis et al., 2002; Feng et al., 2011). Within insects, paper wasps, capable of individual recognition in a hierarchical context, also possess enhanced capacities to learn conspecific faces by opposition to scrambled faces, prey or high-contrast geometric shapes (Sheehan and Tibbetts, 2011). The paper wasp's specialisation for processing conspecific faces is composed of an innate and acquired component (Tibbetts et al., 2019a,b).

Previous research shows that paper wasps (Sheehan and Tibbetts, 2011) learn to differentiate natural stimuli more efficiently than simple high contrast patterns. Our observations with naïve and experienced honeybee foragers are consistent with the previous observations for wasps, suggesting insects have visual systems tuned to solve the most biologically relevant tasks in their environment. Such a result might not be expected, as the majority of research on honeybees has used simple high contrast stimuli (e.g., Horridge, 1997), however, measurement of neural responses from feature detectors present in the primary visual centres of honeybees are saturated beyond a contrast of 35–40% (Yang and Maddess, 1997). This result then is not surprising when considering that the brain should likely have evolved to process natural rather than parameterised stimuli (Field, 1987). While high-contrast elemental stimuli may help in certain categorisation tasks, natural stimuli providing more complex information may be favoured to allow fine discrimination between similar stimuli. The current study suggests that this is the case at least for processing flowers, which are ecologically relevant stimuli for bees. Neural recordings in other insects like flies and hoverflies suggest optimal processing may indeed be tuned for more natural type scenes (Maddess and Laughlin, 1985; Straw et al., 2008). Future work should explore whether such a bias in processing natural stimuli presenting realistic contrast levels and coherent spatial arrangement of features stands also for any kind of visual object, thus suggesting a general property of the insect visual system.

Honeybees are a model invertebrate species for studying visual learning and rule acquisition such as size rules (Avarguès-Weber et al., 2014; Howard et al., 2017a,b), oddity rules (Giurfa et al., 2001), above vs. below rules (Avarguès-Weber et al., 2011), numerical rules (Gross et al., 2009; Howard et al., 2018, 2019b,c,d), and maze navigation (Collett et al., 1993; Zhang et al., 1996, 2000), among other tasks. Furthermore, honeybees are

also excellent learners of patterns and complex images. In the current study, bees were able to learn the configuration of certain flower types within 30 trials, a timeframe which is longer than might be expected if bees were only using a simple matched filtering type process, but is within the timeframe that bees start to develop more complex configural type representations of spatial stimuli (Stach and Giurfa, 2005). Previous studies have demonstrated similar results, showing that bees can learn to recognise and discriminate complex stimuli which may or may not be biologically relevant. For example, honeybees can learn to recognise images of human faces and discriminate between these faces (Dyer et al., 2005; Avarguès-Weber et al., 2010, 2018; Chittka and Dyer, 2012), learn complex pattern discrimination (Srinivasan et al., 1993; Zhang and Srinivasan, 1994; Giger and Srinivasan, 1996; Horridge, 1997; Giurfa et al., 1999b; Efler and Ronacher, 2000; Deisig et al., 2001; Stach et al., 2004; Dyer and Griffiths, 2012), associate abstract characters or colours with concepts (Howard et al., 2019a,b,d), and categorise complex images (Giurfa et al., 1996; Zhang et al., 2004; Benard et al., 2006; Avarguès-Weber et al., 2010). These studies suggest that bees have adapted to processing patterns and images, like those of flowers, and that plasticity is important to survival for a generalist forager. Our study further demonstrates that bees are efficient visual learners and may be innately primed for specific pattern learning. Performance improvement beyond the initial bias toward preferred flower configurations as a result of experience is a potential mechanism for maximising resource acquisition in the specific environments visited by foraging honeybees.

DATA AVAILABILITY STATEMENT

The datasets supporting this article have been uploaded as part of the **Supplementary Material**.

AUTHOR CONTRIBUTIONS

SRH, AGD, MG, DR, MGPR, and AA-W developed the original conceptual framework of the study. SRH and AGD collected the behavioural data. SRH and JEG analysed and modelled the data. SRH, AGD, JEG, and AA-W wrote the initial draft. All authors contributed to reviewing the manuscript and checking domain specific facts.

ACKNOWLEDGMENTS

We thank Dr. Patrick Schultheiss for his help constructing the greenhouse and Lucie Hotier for the construction and management of the greenhouse beehive. We also thank Dr. Jürgen Schramme and the students from Johannes Gutenberg University of Mainz for assistance in conducting the experiments with experienced foragers. SRH acknowledges the Alfred Deakin Postdoctoral Research Fellowship, Australian Government Research Training Program (RTP) Scholarship, and the Fyssen Foundation. AGD and MGPR were supported by Australian Research Council DP0878968. AGD acknowledges the Alexander von Humboldt Foundation and the Australian Research Council 160100161. MG thanks the European Research Council (ERC) for funding support (Research Grant "Cognibrains"). AA-W acknowledges the CNRS, Toulouse 3 University and the Fyssen Foundation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.662336/full#supplementary-material

REFERENCES

Ahissar, M., and Hochstein, S. (2004). The reverse hierarchy theory of visual perceptual learning. *Trends Cogn. Sci.* 8, 457–464. doi: 10.1016/j.tics.2004.08.011

Ahissar, M., Nahum, M., Nelken, I., and Hochstein, S. (2009). Reverse hierarchies and sensory learning. *Philos. Trans. R. Soc. B Biol. Sci.* 364, 285–299.

Avarguès-Weber, A., and Giurfa, M. (2014). Cognitive components of color vision in honey bees: how conditioning variables modulate color learning and discrimination. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 200, 449–461. doi: 10.1007/s00359-014-0909-z

Avarguès-Weber, A., D'amaro, D., Metzler, M., and Dyer, A. G. (2014). Conceptualization of relative size by honeybees. *Front. Behav. Neurosci.* 8:80. doi: 10.3389/fnbeh.2014.00080

Avarguès-Weber, A., D'amaro, D., Metzler, M., Finke, V., Baracchi, D., and Dyer, A. G. (2018). Does holistic processing require a large brain? Insights from honeybees and wasps in fine visual recognition tasks. *Front. Psychol.* 9:1313. doi: 10.3389/fpsyg.2018.01313

Avarguès-Weber, A., Dyer, A. G., and Giurfa, M. (2011). Conceptualization of above and below relationships by an insect. *Proc. Biol. Sci.* 278, 898–905. doi: 10.1098/rspb.2010.1891

Avarguès-Weber, A., Dyer, A. G., Ferrah, N., and Giurfa, M. (2015). The forest or the trees: preference for global over local image processing is reversed by prior experience in honeybees. *Proc. Biol. Sci.* 282:20142384.

Avarguès-Weber, A., Finke, V., Nagy, M., Szabó, T., d'Amaro, D., Dyer, A. G., et al. (2020). Different mechanisms underlie implicit visual statistical learning in honey bees and humans. *Proc. Natl. Acad. Sci. U.S.A.* 117, 25923–25934. doi: 10.1073/pnas.1919387117

Avarguès-Weber, A., Portelli, G., Benard, J., Dyer, A., and Giurfa, M. (2010). Configural processing enables discrimination and categorization of face-like stimuli in honeybees. *J. Exp. Biol.* 213, 593–601. doi: 10.1242/jeb.039263

Benard, J., Stach, S., and Giurfa, M. (2006). Categorization of visual stimuli in the honeybee *Apis mellifera*. *Anim. Cogn.* 9, 257–270. doi: 10.1007/s10071-006-0032-9

Brown, M., Downs, C. T., and Johnson, S. D. (2011). Covariation of flower traits and bird pollinator assemblages among populations of *Kniphofia linearifolia* (*Asphodelaceae*). *Plant Syst. Evol.* 294, 199–206.

Burns, J. G., and Dyer, A. G. (2008). Diversity of speed-accuracy strategies benefits social insects. *Curr. Bol.* 18, R953–R954. doi: 10.1016/j.cub.2008.08.028

Chittka, L., and Dyer, A. G. (2012). Your face looks familiar. *Nature* 481, 154–155. doi: 10.1038/481154a

Chittka, L., and Niven, J. (2009). Are bigger brains better? *Curr. Biol.* 19, R995–R1008. doi: 10.1016/j.cub.2009.08.023

Collett, T., Fry, S., and Wehner, R. (1993). Sequence learning by honeybees. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 172, 693–706. doi: 10.1007/BF00195395

Collishaw, S. M., and Hole, G. J. (2000). Featural and configural processes in the recognition of faces of different familiarity. *Perception* 29, 893–909. doi: 10.1068/p2949

Dafni, A., and Kevan, P. G. (1997). Flower size and shape: implications in pollination. *Isr. J. Plant Sci.* 45, 201–211. doi: 10.1080/07929978.1997.10676684

Dafni, A., Lehrer, M., and Kevan, P. (1997). Spatial flower parameters and insect spatial vision. *Biol. Rev.* 72, 239–282. doi: 10.1017/S0006323196005002

de Ibarra, N. H., and Giurfa, M. (2003). Discrimination of closed coloured shapes by honeybees requires only contrast to the long wavelength receptor type. *Anim. Behav.* 66, 903–910. doi: 10.1006/anbe.2003.2269

Deisig, N., Lachnit, H., Giurfa, M., and Hellstern, F. (2001). Configural olfactory learning in honeybees: negative and positive patterning discrimination. *Learn. Mem.* 8, 70–78. doi: 10.1101/lm.8.2.70

Dyer, A. G. (2012b). "Psychophysics of honey bee color processing in complex environments," in *Honeybee Neurobiology and Behavior*, eds C. Galizia, D. Eisenhardt, and M. Giurfa (Berlin: Springer), 303–314. doi: 10.1007/978-94-007-2099-2_23

Dyer, A. G. (2012a). The mysterious cognitive abilities of bees: why models of visual processing need to consider experience and individual differences in animal performance. *J. Exp. Biol.* 215, 387–395. doi: 10.1242/jeb.038190

Dyer, A. G., and Chittka, L. (2004). Fine colour discrimination requires differential conditioning in bumblebees. *Naturwissenschaften* 91, 224–227. doi: 10.1007/s00114-004-0508-x

Dyer, A. G., and Garcia, J. E. (2014). Color difference and memory recall in free-flying honeybees: forget the hard problem. *Insects* 5, 629–638. doi: 10.3390/insects5030629

Dyer, A. G., and Griffiths, D. W. (2012). Seeing near and seeing far; behavioural evidence for dual mechanisms of pattern vision in the honeybee (*Apis mellifera*). *J. Exp. Biol.* 215, 397–404. doi: 10.1242/jeb.060954

Dyer, A. G., and Murphy, A. H. (2009). Honeybees choose "incorrect" colors that are similar to target flowers in preference to novel colors. *Isr. J. Plant Sci.* 57, 203–210. doi: 10.1560/IJPS.57.3.203

Dyer, A. G., and Neumeyer, C. (2005). Simultaneous and successive colour discrimination in the honeybee (*Apis mellifera*). *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 191, 547–557. doi: 10.1007/s00359-005-0622-z

Dyer, A. G., Neumeyer, C., and Chittka, L. (2005). Honeybee (*Apis mellifera*) vision can discriminate between and recognise images of human faces. *J. Exp. Biol.* 208, 4709–4714. doi: 10.1242/jeb.01929

Dyer, A. G., Spaethe, J., and Prack, S. (2008). Comparative psychophysics of bumblebee and honeybee colour discrimination and object detection. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 194, 617–627. doi: 10.1007/s00359-008-0335-1

Dyer, A., Avargues-Weber, A., Reser, D., Rosa, M., and Giurfa, M. (2013). Hierarchical visual representations enhance complex pattern learning in honeybees (*Apis mellifera*). *Front. Physiol.* 4. doi: 10.3389/conf.fphys.2013.25.00042

Efler, D., and Ronacher, B. (2000). Evidence against a retinotopic-template matching in honeybees' pattern recognition. *Vis. Res.* 40, 3391–3403. doi: 10.1016/S0042-6989(00)00189-9

Feng, L., Liu, J., Wang, Z., Li, J., Li, L., Ge, L., et al. (2011). The other face of the other-race effect: an fMRI investigation of the other-race face categorization advantage. *Neuropsychologia* 49, 3739–3749. doi: 10.1016/j.neuropsychologia.2011.09.031

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., and Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.* 35, 375–403. doi: 10.1146/annurev.ecolsys.34.011802.132347

Fenster, C. B., Cheely, G., Dudash, M. R., and Reynolds, R. J. (2006). Nectar reward and advertisement in hummingbird-pollinated *Silene virginica* (*Caryophyllaceae*). *Am. J. Bot.* 93, 1800–1807. doi: 10.3732/ajb.93.12.1800

Field, D. J. (1987). Relations between the statistics of natural images and the response properties of cortical cells. *J. Opt. Soc. Am. A* 4, 2379–2394. doi: 10.1364/JOSAA.4.002379

Garcia, J. E., Phillips, R. D., Peter, C. I., and Dyer, A. G. (2020). Changing how biologists view flowers—color as a perception not a trait. *Front. Plant Sci.* 11:601700. doi: 10.3389/fpls.2020.601700

Gegear, R. J., Burns, R., and Swoboda−Bhattarai, K. A. (2017). "Hummingbird" floral traits interact synergistically to discourage visitation by bumble bee foragers. *Ecology* 98, 489–499. doi: 10.1002/ecy.1661

Giger, A., and Srinivasan, M. (1996). Pattern recognition in honeybees: chromatic properties of orientation analysis. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 178, 763–769. doi: 10.1007/BF00225824

Giurfa, M. (2004). Conditioning procedure and color discrimination in the honeybee *Apis mellifera*. *Naturwissenschaften* 91, 228–231. doi: 10.1007/s00114-004-0530-z

Giurfa, M., and Sandoz, J.-C. (2012). Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn. Mem.* 19, 54–66. doi: 10.1101/lm.024711.111

Giurfa, M., Dafni, A., and Neal, P. R. (1999a). Floral symmetry and its role in plant-pollinator systems. *Int. J. Plant Sci.* 160, S41–S50. doi: 10.1086/314214

Giurfa, M., Hammer, M., Stach, S., Stollhoff, N., Müller-Deisig, N., and Mizyrycki, C. (1999b). Pattern learning by honeybees: conditioning procedure and recognition strategy. *Anim. Behav.* 57, 315–324. doi: 10.1006/anbe.1998.0957

Giurfa, M., Eichmann, B., and Menzel, R. (1996). Symmetry perception in an insect. *Nature* 382, 458–461. doi: 10.1038/382458a0

Giurfa, M., Nunez, J., Chittka, L., and Menzel, R. (1995). Colour preferences of flower-naive honeybees. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 177, 247–259. doi: 10.1007/BF00192415

Giurfa, M., Zhang, S., Jenett, A., Menzel, R., and Srinivasan, M. V. (2001). The concepts of 'sameness' and 'difference' in an insect. *Nature* 410, 930–933. doi: 10.1038/35073582

Gómez, J. M., Torices, R., Lorite, J., Klingenberg, C. P., and Perfectti, F. (2016). The role of pollinators in the evolution of corolla shape variation, disparity and integration in a highly diversified plant family with a conserved floral bauplan. *Ann. Bot.* 117, 889–904. doi: 10.1093/aob/mcv194

Goren, C. C., Sarty, M., and Wu, P. Y. (1975). Visual following and pattern discrimination of face-like stimuli by newborn infants. *Pediatrics* 56, 544–549.

Gross, H. J., Pahl, M., Si, A., Zhu, H., Tautz, J., and Zhang, S. (2009). Number-based visual generalisation in the honeybee. *PLoS One* 4:e4263. doi: 10.1371/journal.pone.0004263

Gumbert, A. (2000). Color choices by bumble bees (*Bombus terrestris*): innate preferences and generalization after learning. *Behav. Ecol. Sociobiol.* 48, 36–43. doi: 10.1007/s002650000213

Henderson, J. M., Williams, C. C., and Falk, R. J. (2005). Eye movements are functional during face learning. *Mem. Cogn.* 33, 98–106. doi: 10.3758/BF03195300

Horridge, G. A. (1997). Pattern discrimination by the honeybee: disruption as a cue. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 181, 267–277. doi: 10.1007/s003590050113

Horridge, G. A., and Zhang, S. W. (1995). Pattern vision in honeybees (*Apis mellifera*): flower-like patterns with no predominant orientation. *J. Insect Physiol.* 41, 681–688. doi: 10.1016/0022-1910(95)00021-L

Howard, S. R., Shrestha, M., Schramme, J., Garcia, J. E., Avarguès-Weber, A., Greentree, A. D., et al. (2019e). Honeybees prefer novel insect-pollinated flower shapes over bird-pollinated flower shapes. *Curr. Zool.* 65, 457–465.

Howard, S. R., Avarguès-Weber, A., Garcia, J. E., Greentree, A. D., and Dyer, A. G. (2019b). Numerical cognition in honeybees enables addition and subtraction. *Sci. Adv.* 5:easv0961. doi: 10.1126/sciadv.aav0961

Howard, S. R., Avarguès-Weber, A., Garcia, J. E., Greentree, A. D., and Dyer, A. G. (2019c). Surpassing the subitizing threshold: appetitive–aversive conditioning improves discrimination of numerosities in honeybees. *J. Exp. Biol.* 222:jeb205658. doi: 10.1242/jeb.205658

Howard, S. R., Avarguès-Weber, A., Garcia, J. E., Greentree, A. D., and Dyer, A. G. (2019d). Symbolic representation of numerosity by honeybees (*Apis mellifera*): matching characters to small quantities. *Proc. R. Soc. B Biol. Sci.* 286: 20190238.

Howard, S. R., Avarguès-Weber, A., Garcia, J. E., Greentree, A. D., and Dyer, A. G. (2019a). Achieving arithmetic learning in honeybees and examining how individuals learn. *Commun. Integr. Biol.* 12, 166–170. doi: 10.1080/19420889.2019.1678452

Howard, S. R., Avarguès-Weber, A., Garcia, J. E., Greentree, A. D., and Dyer, A. G. (2018). Numerical ordering of zero in honey bees. *Science* 360, 1124–1126. doi: 10.1126/science.aar4975

Howard, S. R., Avarguès-Weber, A., Garcia, J., and Dyer, A. G. (2017a). Free-flying honeybees extrapolate relational size rules to sort successively visited artificial flowers in a realistic foraging situation. *Anim. Cogn.* 20, 627–638. doi: 10.1007/s10071-017-1086-6

Howard, S. R., Avarguès-Weber, A., Garcia, J. E., Stuart-Fox, D., and Dyer, A. G. (2017b). Perception of contextual size illusions by honeybees in restricted and unrestricted viewing conditions. *Proc. Biol. Sci.* 284:20172278. doi: 10.1098/rspb.2017.2278

Howard, S. R., Prendergast, K., Symonds, M. R. E., Shrestha, M., and Dyer, A. G. (2021). Spontaneous choices for insect-pollinated flower shapes by wild non-eusocial halictid bees. *J. Exp. Biol.* 224:jeb242457. doi: 10.1242/jeb.242457

Hung, C.-C., Yen, C. C., Ciuchta, J. L., Papoti, D., Bock, N. A., Leopold, D. A., et al. (2015). Functional mapping of face-selective regions in the extrastriate visual cortex of the marmoset. *J. Neurosci.* 35, 1160–1172. doi: 10.1523/JNEUROSCI.2659-14.2015

Johnson, S., and Dafni, A. (1998). Response of bee—flies to the shape and pattern of model flowers: implications for floral evolution in a Mediterranean herb. *Funct. Ecol.* 12, 289–297. doi: 10.1046/j.1365-2435.1998.00175.x

Kanwisher, N., Mcdermott, J., and Chun, M. M. (1997). The fusiform face area: a module in human extrastriate cortex specialized for face perception. *J. Neurosci.* 17, 4302–4311. doi: 10.1523/JNEUROSCI.17-11-04302.1997

Lázaro, A., and Totland, Ø (2014). The influence of floral symmetry, dependence on pollinators and pollination generalization on flower size variation. *Ann. Bot.* 114, 157–165. doi: 10.1093/aob/mcu083

Lehrer, M., Horridge, G., Zhang, S., and Gadagkar, R. (1995). Shape vision in bees: innate preference for flower-like patterns. *Philos. Trans. R. Soc. London B Biol. Sci.* 347, 123–137. doi: 10.1098/rstb.1995.0017

Lehrer, M., Wehner, R., and Srinivasan, M. (1985). Visual scanning behaviour in honeybees. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 157, 405–415. doi: 10.1007/BF00615140

Livingstone, M. S., Vincent, J. L., Arcaro, M. J., Srihasam, K., Schade, P. F., and Savage, T. (2017). Development of the macaque face-patch system. *Nat. Commun.* 8:14897. doi: 10.1038/ncomms14897

MaBouDi, H., Dona, H., Gatto, E., Loukola, O. J., Buckley, E., Onoufriou, P. D., et al. (2020). Bumblebees use sequential scanning of countable items in visual patterns to solve numerosity tasks. *Integr. Comp. Biol.* 60, 929–942. doi: 10.1093/icb/icaa025

Maddess, T., and Laughlin, S. B. (1985). Adaptation of the motion-sensitive neuron H1 is generated locally and governed by contrast frequency. *Proc. R. Soc. London B Biol. Sci.* 225, 251–275. doi: 10.1098/rspb.1985.0061

Maurer, D., Le Grand, R., and Mondloch, C. J. (2002). The many faces of configural processing. *Trends Cogn. Sci.* 6, 255–260. doi: 10.1016/S1364-6613(02)01903-4

Menzel, R. (1967). Untersuchungen zum erlernen von spektralfarben durch die honigbiene (*Apis mellifica*). *Z. Vgl. Physiol.* 56, 22–62. doi: 10.1007/BF00333562

Mondloch, C. J., Lewis, T. L., Budreau, D. R., Maurer, D., Dannemiller, J. L., Stephens, B. R., et al. (1999). Face perception during early infancy. *Psychol. Sci.* 10, 419–422. doi: 10.1111/1467-9280.00179

Morawetz, L., Svoboda, A., Spaethe, J., and Dyer, A. G. (2013). Blue colour preference in honeybees distracts visual attention for learning closed shapes. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 199, 817–827. doi: 10.1007/s00359-013-0843-5

Pascalis, O., De Haan, M., and Nelson, C. A. (2002). Is face processing species-specific during the first year of life? *Science* 296, 1321–1323. doi: 10.1126/science.1070223

R Core Team (2017). *R: A Language and Environment for Statistical Computing (Version 3.4. 2) [Computer software]*. Vienna: R Foundation for Statistical Computing.

Reser, D. H., Wijesekara Witharanage, R., Rosa, M. G., and Dyer, A. G. (2012). Honeybees (*Apis mellifera*) learn color discriminations via differential conditioning independent of long wavelength (green) photoreceptor modulation. *PLoS One* 7:e48577. doi: 10.1371/journal.pone.0048577

Sarter, M., Givens, B., and Bruno, J. P. (2001). The cognitive neuroscience of sustained attention: where top-down meets bottom-up. *Brain Res. Rev.* 35, 146–160. doi: 10.1016/S0165-0173(01)00044-3

Schaeffer, D. J., Selvanayagam, J., Johnston, K. D., Menon, R. S., Freiwald, W. A., and Everling, S. (2020). Face selective patches in marmoset frontal cortex. *Nat. Commun.* 11:4856. doi: 10.1038/s41467-020-18692-2

Sheehan, M. J., and Tibbetts, E. A. (2011). Specialized face learning is associated with individual recognition in paper wasps. *Science* 334, 1272–1275. doi: 10.1126/science.1211334

Sommerlandt, F. M., Spaethe, J., Rössler, W., and Dyer, A. G. (2016). Does fine color discrimination learning in free-flying honeybees change mushroom-body calyx neuroarchitecture? *PLoS One* 11:e0164386. doi: 10.1371/journal.pone.0164386

Srinivasan, M. V., and Lehrer, M. (1988). Spatial acuity of honeybee vision and its spectral properties. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 162, 159–172. doi: 10.1007/BF00606081

Srinivasan, M. V., Zhang, S., and Rolfe, B. (1993). Is pattern vision in insects mediated by 'cortical' processing? *Nature* 362, 539–540. doi: 10.1038/362539a0

Stach, S., and Giurfa, M. (2005). The influence of training length on generalization of visual feature assemblies in honeybees. *Behav. Brain Res.* 161, 8–17. doi: 10.1016/j.bbr.2005.02.008

Stach, S., Benard, J., and Giurfa, M. (2004). Local-feature assembling in visual pattern recognition and generalization in honeybees. *Nature* 429, 758–761. doi: 10.1038/nature02594

Straw, A. D., Rainsford, T., and O'carroll, D. C. (2008). Contrast sensitivity of insect motion detectors to natural images. *J. Vis.* 8, 32–32. doi: 10.1167/8.3.32

Tanaka, J. W., and Farah, M. J. (1993). Parts and wholes in face recognition. *Q. J. Exp. Psychol.* 46, 225–245. doi: 10.1080/14640749308401045

Thorpe, S., Fize, D., and Marlot, C. (1996). Speed of processing in the human visual system. *Nature* 381, 520–522. doi: 10.1038/381520a0

Tibbetts, E. A., Den Uyl, J., Dwortz, M., and Mclean, C. (2019a). The development and evolution of specialized face learning in paper wasps. *Anim. Behav.* 147, 1–7. doi: 10.1016/j.anbehav.2018.10.016

Tibbetts, E. A., Desjardins, E., Kou, N., and Wellman, L. (2019b). Social isolation prevents the development of individual face recognition in paper wasps. *Anim. Behav.* 152, 71–77. doi: 10.1016/j.anbehav.2019.04.009

Valenza, E., Simion, F., Cassia, V. M., and Umiltà, C. (1996). Face preference at birth. *J. Exp. Psychol.* 22:892. doi: 10.1037/0096-1523.22.4.892

van der Kooi, C. J., and Ollerton, J. (2020). The origins of flowering plants and pollinators. *Science* 368, 1306–1308. doi: 10.1126/science.aay3662

Vergoz, V., Roussel, E., Sandoz, J.-C., and Giurfa, M. (2007). Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. *PloS One* 2:e288. doi: 10.1371/journal.pone.0000288

von Frisch, K. (1965). "Die tänze der bienen," in *Tanzsprache und Orientierung der Bienen*, (Berlin: Springer), 3–330. doi: 10.1007/978-3-642-94916-6_2

Warrant, E. J. (2016). Sensory matched filters. *Curr. Biol.* 26, R976–R980. doi: 10.1016/j.cub.2016.05.042

Wehner, R. (1987). 'Matched filters'—neural models of the external world. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 161, 511–531. doi: 10.1007/BF00603659

Wilmer, J. B., Germine, L., Chabris, C. F., Chatterjee, G., Williams, M., Loken, E., et al. (2010). Human face recognition ability is specific and highly heritable. *Proc. Natl. Acad. Sci. U.S.A.* 107, 5238–5241. doi: 10.1073/pnas.0913053107

Yang, E.-C., and Maddess, T. (1997). Orientation-sensitive neurons in the brain of the honey bee (*Apis mellifera*). *J. Insect Physiol.* 43, 329–336. doi: 10.1016/S0022-1910(96)00111-4

Young, A. W., and Burton, A. M. (2018). Are we face experts? *Trends Cogn. Sci.* 22, 100–110. doi: 10.1016/j.tics.2017.11.007

Zhang, S., and Srinivasan, M. (1994). Prior experience enhances pattern discrimination in insect vision. *Nature* 368, 330–332. doi: 10.1038/368330a0

Zhang, S., Bartsch, K., and Srinivasan, M. (1996). Maze learning by honeybees. *Neurobiol. Learn. Mem.* 66, 267–282. doi: 10.1006/nlme.1996.0069

Zhang, S., Mizutani, A., and Srinivasan, M. V. (2000). Maze navigation by honeybees: learning path regularity. *Learn. Mem.* 7, 363–374. doi: 10.1101/lm.32900

Zhang, S., Srinivasan, M. V., Zhu, H., and Wong, J. (2004). Grouping of visual objects by honeybees. *J. Exp. Biol.* 207, 3289–3298. doi: 10.1242/jeb.01155

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 09 December 2021
doi: 10.3389/fevo.2021.721241

Flower Color as Predictor for Nectar Reward Quantity in an Alpine Flower Community

Martin Streinzer[1]†, Johann Neumayer[2†] and Johannes Spaethe[3]*

[1] Department of Neurobiology, Faculty of Life Sciences, University of Vienna, Vienna, Austria, [2] Independent Researcher, Elixhausen, Austria, [3] Department of Behavioral Physiology and Sociobiology (Zoology II), Biocenter, University of Würzburg, Würzburg, Germany

Entomophilous plants have evolved colorful floral displays to attract flower visitors to achieve pollination. Although many insects possess innate preferences for certain colors, the underlying proximate and ultimate causes for this behavior are still not well understood. It has been hypothesized that the floral rewards, e.g., sugar content, of plants belonging to a particular color category correlate with the preference of the flower visitors. However, this hypothesis has been tested only for a subset of plant communities worldwide. Bumble bees are the most important pollinators in alpine environments and show a strong innate preference for (bee) "UV-blue" and "blue" colors. We surveyed plants visited by bumble bees in the subalpine and alpine zones (>1,400 m a.s.l.) of the Austrian Alps and measured nectar reward and spectral reflectance of the flowers. We found that the majority of the 105 plant samples visited by bumble bees fall into the color categories "blue" and "blue-green" of a bee-specific color space. Our study shows that color category is only a weak indicator for nectar reward quantity; and due to the high reward variance within and between categories, we do not consider floral color as a reliable signal for bumble bees in the surveyed habitat. Nevertheless, since mean floral reward quantity differs between categories, naïve bumble bees may benefit from visiting flowers that fall into the innately preferred color category during their first foraging flights.

Keywords: flower color, color preference, nectar reward, alpine, *Bombus*

Edited by:
Isabel Marques,
University of Lisbon, Portugal

Reviewed by:
Jair E. Garcia,
RMIT University, Australia
Mario Vallejo-Marin,
University of Stirling, United Kingdom

****Correspondence:***
Martin Streinzer
martin.streinzer@univie.ac.at

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *06 June 2021*
Accepted: *17 November 2021*
Published: *09 December 2021*

Citation:
Streinzer M, Neumayer J and Spaethe J (2021) Flower Color as Predictor for Nectar Reward Quantity in an Alpine Flower Community. Front. Ecol. Evol. 9:721241. doi: 10.3389/fevo.2021.721241

INTRODUCTION

Flower color is a major trait by which plants convey information about their identity and location to a potential visitor. Color is not an intrinsic property of the flower organ, but rather a psychophysical phenomenon that depends on the visual system of the observer that perceives the reflected light spectrum (Kelber and Osorio, 2010; Skorupski and Chittka, 2011). Bees, one of the major pollinator groups of angiosperms, possess three distinct photoreceptor types in their compound eyes, which are most sensitive in the ultraviolet (UV), blue and green part of the light spectrum and enable them to use trichromatic color vision. The number of different photoreceptors and their sensitivity maxima are phylogenetically conserved among most bees (Peitsch et al., 1992; Briscoe and Chittka, 2001) and their origin predates that of angiosperms (Chittka, 1996).

Color, as other floral traits like scent, shape or size, are used by flower visitors to detect and identify specific plants and thus to associate reward quantity and quality after a visit with a particular floral display. After several visits, pollinators are able to predict the reward probability of a

particular flower type in subsequent foraging flights and thus may develop floral (color) preferences based on previous experience. In addition to this learned preference, bees and many other pollinator groups, show an innate preference for certain color(s) or color categories (Giurfa et al., 1995; Lunau and Maier, 1995; Goyret et al., 2008; Streinzer et al., 2019). This preference already exists before any learning took place and is usually overwritten after experience, although it has been shown that bumble bees may revert to their innate preference when confronted with novel floral features (Gumbert, 2000). However, the underlying proximate and ultimate reasons of an innate color preference are currently not well understood. Innate preferences may be adaptive, if they for example allow naïve individuals to better find rewarding food sources as compared to a random search strategy. The preferred flower type may provide a higher reward, an optimal nutritional composition or show a floral morphology that is adapted to the pollinator's mouthparts and allows an efficient exploitation. Flower-naïve hoverflies (*Eristalis tenax*), for example, possess an innate preference for yellow and white flowers, and after landing on a flower they reflexively extend their proboscis when confronted with small UV-absorbing yellow spots. This behavior is assumed to help naïve flies to efficiently find and extract the pollen or nectar of a flower (Lunau and Wacht, 1997).

In bees, several species are shown to have strong preferences for "UV-blue", "blue" and "blue-green" colors in a bee-specific color space (Giurfa et al., 1995; Gumbert, 2000; Raine and Chittka, 2007b; Dyer et al., 2016). In one of the earliest studies, aiming to link the color of flowers with its predictive value for reward, Giurfa et al. (1995) studied flowering plants in a nature reserve in northern Germany. The authors found that flowers, which fall in the above-mentioned bee-color categories, have, on average, higher reward quantities compared to flowers with unattractive colors. However, similar patterns have not been observed in other world regions, e.g., Australia, where the highest rewards were found in color categories like "green" and "UV-green" (Shrestha et al., 2020). Nevertheless, studies on flower communities, which investigated color frequencies and distribution, generally found a non-uniform distribution of flower colors among the different categories. Distributions were found to be remarkably similar across continents, with a maximum of spectral reflection patterns falling in the "blue-green" and "blue" sectors of a bee-specific color space (Chittka et al., 1994; Dyer et al., 2012; Bischoff et al., 2013; Shrestha et al., 2014; Ortiz et al., 2021).

In this study, we measured nectar reward quantity (nectar volume and sugar concentration) and spectral reflectance of the majority of bumble bee visited flowering plants in the Eastern Alps in Europe. At high altitudes, bumble bees (Hymenoptera: Apidae: *Bombus* Latreille) are the most important pollinators of bee-visited plant species (Bingham et al., 1998). In contrast to the abundant bumble bees, other bee species found in the same habitat (e.g., *Apis mellifera, Andrena rogenhoferi, A. lapponica,* and *Osmia* spp.) are less important due to their lower densities at high elevations with harsh weather conditions (Ebmer, 2003). Based on the finding that bumble bees possess an innate preference for particular flower colors (and thus a higher propensity to visit such flowers), we tested whether the preference is reflected in the nectar reward quantity of flowers of these colors and can thus be considered as being adaptive.

MATERIALS AND METHODS

Study Region

All measurements were conducted in the Hohe Tauern National Park located in the main chain of the Eastern Alps in Austria. All sampling sites were located in the subalpine to alpine range between 1,400 and 2,600 m a.s.l. The sites were scattered roughly along the Großglockner panoramic road in the National Park. Experiments were carried out in the years 1994–2020 (nectar measurements: 1994–2020; spectral reflectance measurements: 2006–2020). Research and sampling permits for sites within the National Park core region were issued by the "Land Salzburg" (permit no. 21 301-RI/547/161-2010, to JN).

Plant Identification

Plants were either identified in the field or taken to the lab for species-level identification using adequate identification keys. For consistency, all identifications followed the nomenclature by Fischer et al. (2008). Some species could only be identified to the level of a species-group of closely related species, which is indicated by the suffix "agg." (for aggregate; e.g., *Saxifraga oppositifolia* agg., *Thymus praecox* agg.). All sampled species are listed in **Supplementary Table 1**.

Visitor Observations

To include only those plant species that are actually used by bumble bees as a nectar source, we used a database containing museum specimens, observations and literature records pertaining to the majority of field observations made on bumble bees in Austria involving over 42,000 flower visitation records collected between 1848 and 2021. A minimum criterion of five databased observations of bumble bee visits for a certain plant species was set, to include the species in the analysis. For high altitude alpine plants for which the observation density is generally lower (e.g., *Phyteuma globulariifolium, Saxifraga* spp., alpine *Salix* spp.), we accepted a threshold of three databased records (in $N = 5$ plant species). All plant species that failed to meet these criteria were removed from the analysis.

Nectar Measurements

Nectar measurements were performed as a series of five measurements during consecutive 2-h intervals, covering the entire day between 7 am and 5 pm CET (7–9 am, 9–11 am, 11 am–1 pm, 1–3 pm, and 3–5 pm). Measurements were only performed on days without precipitation, and with a mean relative humidity below 80% during the day (11 am–4 pm). Since weather conditions are fairly unpredictable in the alpine area, some measurement series were aborted and missing time intervals were completed on the next appropriate day.

For each series, the nectar volume and concentration were measured in at least 10 flowers (5 in rare species), with a

maximum of three different flowers measured *per* individual plant and time interval. Flowers were randomly selected from the plants/inflorescences. In each measurement series, individuals were investigated from the same population. We then calculated the mean reward quantity for each species and time interval. Two different values were used in the analysis. First, we selected the maximum value across the time series. This value relates to the maximum nectar standing crop of flowers that were shielded from visitors for up to 24 h (in the bagged condition), and thus represents a value that is comparable to previous studies (Shrestha et al., 2020). Secondly, we selected the mean value across the time series, which serves as a more realistic standing crop that would, on average, be available for visitors throughout the day.

Open flowers are usually depleted by nectar-seeking visitors. To estimate both the intrinsic nectar production of a flower and the nectar standing crop that is actually available for visitors, we recorded series of measurements from flowers, which were covered to exclude visitors, and from unprotected ("open") flowers. For the intrinsic nectar production, a white plastic mesh was placed around the flowers and inflorescences in the early morning, before the first flower visitors were active (referred to as "bagged" in the text). A mesh width of 0.8–1.5 mm allowed sufficient airflow to minimize the microclimatic influence of solar radiation, extreme temperatures and humidity, which are known to affect nectar amount and concentration, but excluded all potential visitors (Corbet et al., 1979; Kearns and Inouye, 1993). Nectar measurement series on bagged flowers were carried out in parallel to series on open flowers at the same site and day.

Nectar was extracted from flowers following the protocol of Corbet (2003), with microcapillaries of 5.0 µl (Assistant, Germany), 1.0 µl, 0.5 µl, or 0.2 µl (Drummond, Great Britain) volume. For flowers with several separated nectaries, the nectar from all nectaries was extracted. The length of the liquid column was measured to the nearest 0.5 mm and converted to µl volume. The contained liquid was then expelled onto a hand refractometer suitable for small volumes, and calibrated for concentrations between 0 and 50% sucrose equivalent (Bellingham and Stanley, Great Britain). When a higher nectar concentration was anticipated, the nectar was diluted by dipping the microcapillary into distilled water (Aqua dest.) before the measurement (Corbet, 2003). The nectar volume was then determined before and after dilution, and the ratio was used to calculate the original sugar concentration. For flowers containing very small nectar volumes (<0.05 µl), the nectar was generally diluted with Aqua dest. to allow measurement with the refractometer. Conversion of the volumes was performed as described above. In the first years of the study (1994–2000), measurements on flowers with very small volumes (<0.1 µl) were conducted by pooling the nectar from several flowers. The total volume was then divided by the number of flowers used to reach the threshold volume for measurement with the refractometer. For extremely small nectar volumes (<0.02 µl) concentration measurements failed in a few cases. We then used the average concentration from the other flowers in the same 2 h interval to determine the sugar content of the flower. In open flowers, nectar can concentrate during hot and dry weather conditions. When concentrations exceeded 80%, measurements were excluded from the analysis, since bees do not visit flowers with highly concentrated nectar (own unpublished observations; see also Harder, 1986).

Since both nectar volume and nectar concentration are strongly influenced by ambient conditions, we calculated the amount of sugar in the nectar (Bertsch, 1983). To calculate the total amount of reward per flower, our measurements were converted to mg sucrose equivalent/flower using standard procedures (Cruden and Hermann, 1983; Kearns and Inouye, 1993; Corbet, 2003). In brief, the measured nectar volume was multiplied by the measured sugar concentration. The sugar concentration measured with the refractometer (calibrated for weight:weight concentration) was corrected to the appropriate unit (weight:volume concentration) using a correction factor according to Cruden and Hermann (1983).

Since flowers often form units, e.g., inflorescences, flower baskets or synflorescences consisting of individual inflorescences/baskets (Fischer et al., 2008), we also aimed to quantify the nectar reward of the entire flowering units. We first defined a "flowering unit" from a visual perspective. Each entity that constitutes a separate visual cue during approach was treated as a single unit. Parts of inflorescences which stand far apart, form no continuous unit and which force visitors to fly between, were treated as separate units (e.g., *Aconitum degenii* and *Adenostyles alliariae*). For each plant species in our dataset, we counted the number of individual flowers per unit in at least 10 plant individuals and calculated the mean.

Color Measurements and Modeling

Flowers and inflorescences were collected and brought to the lab for spectral measurements. Spectral measurements were performed by measuring a small (c. 5 mm^2) area of a given plant organ that was mounted on black insulation tape. All measurements were performed with either a USB2000 spectrometer equipped with a DH2000 BAL light source, or a JAZ spectrometer unit equipped with a pulsed Xenon light source (Ocean Optics, Dunedin, FL, United States). The spectrometers were calibrated against a white standard (WS-1-SL, Ocean Optics). Measurements were performed with a bifurcated fiber optics probe, with the incident and measuring angle set at 45° with respect to the surface normal, following standard protocols (Chittka and Kevan, 2005). A single measurement was performed on each plant individual. For single flowers or unicolored compound inflorescences (e.g., in *Knautia, Scabiosa,* and *Valeriana*) we measured the region of the most prominent flower organ, facing the viewing direction of the visitor (usually the upper surface of a petal). For plants with multicolored flowers or inflorescences, e.g., many Asteraceae, we only measured the part of the inflorescence that occupied the majority (>50%) of the surface (usually the upper surface of the petal lips). Since the flowers of dioecious species (*Salix* sp.) differ in their reward and appearance and are found on different plant individuals, they were treated as independent data points in our analysis. In *Trifolium pratense*, two distinct color morphs with a general form appearing pink for a human observer, and an alpine form appearing white, were measured and treated as independent

data points. In both cases, nectar and color measurements were also performed separately. Several specimens (between 1 and 127, median 3) were measured per species, depending on availability of the flowers.

To estimate how bee visitors perceive the flower color, we used the color hexagon (Chittka, 1992), a bee-specific color space that is widely employed in pollinator studies and has been repeatedly tested in laboratory settings (Chittka et al., 1992; Giurfa et al., 1995; Raine and Chittka, 2005; Théry et al., 2005; Dyer et al., 2008, 2012; Leonard et al., 2011). Color loci were calculated according to standard procedures (Chittka and Kevan, 2005) using standard illumination (D65; Wyszecki and Stiles, 1982) and photoreceptor spectral sensitivity functions specific for *Bombus terrestris* (Skorupski et al., 2007). Hymenopteran photoreceptor sensitivities are phylogenetically conserved and similar among bee species (Briscoe and Chittka, 2001) and were confirmed to be similar across bumble bee species in particular (Peitsch et al., 1992; Briscoe and Chittka, 2001; Skorupski et al., 2007; Skorupski and Chittka, 2010). We used an average reflection spectrum of green foliage as adaptation background (Chittka and Kevan, 2005). For each measurement, we determined the (absolute) green receptor contrast, the position of the locus in the color space, and the color contrast as the Euclidean distance between the hexagon center and the color locus (Spaethe et al., 2001; Chittka and Kevan, 2005). Brightness, considered as the summed response of all three photoreceptors, is used by bees only during phototactic response (Menzel and Greggers, 1985) and is not regarded as an important spectral feature during foraging (Ng et al., 2018).

Color hue refers to the direction of a locus in the color space and was calculated as the angle between the lines connecting the hexagon center with the blue corner (set as 0°) and the color locus, respectively. Color locus angles are reported as positive values in the clockwise direction with respect to the reference line (see **Figure 1A**). However, it must be noted that although several studies used angles as a measure for hue, there are no universally accepted standards on how to report it. Different studies used different reference lines and rotation directions (Chittka et al., 1994; Dyer et al., 2012; Shrestha et al., 2014 *vs.* Tai et al., 2020). For further analysis, the hues (angles) were binned to six categories, which correspond to distinct classes of reflectance functions (Chittka et al., 1994). The categories are referred to as "blue" (B; 330°–30° in the hexagon space), "blue-green" (BG; 30°–90°), "green" (G; 90°–150°), "green-UV" (GU; 150°–210°), "ultraviolet" (UV; 210°–270°), and "UV-blue" (UB; 270°–330°). In addition, we also plotted the hues to a finer scale of 10° bins to make the data comparable to other studies, which used this bin size (e.g., Chittka et al., 1994; Dyer et al., 2012; Shrestha et al., 2014). Flower species with color contrasts < 0.1 hexagon units were assigned as "achromatic" and excluded from further analysis ($N = 3$ species, see below).

Phylogenetic Reconstruction and Signal

To test for phylogenetic signal in the color and nectar reward data, we constructed a species-level phylogenetic tree, including all of our studied taxa. We initially used the phylogenetic tree ("ALLOTB" tree) published by Smith and Brown (2018). Tree manipulation was performed in R (version 4.1.1; R Development Core Team, 2021) using the packages "phytools" for R (Version 0.7-80; Revell, 2012), "ape" for R (Version 5.5; Paradis and Schliep, 2019), and "picante" for R (Version 1.8.2; Kembel et al., 2010). The tree was pruned to include only those taxa contained in our dataset. For two taxa which include distinct color morphs (*Trifolium pratense*) or different sexes (*Salix waldsteiniana*) of a single species, we introduced a dichotomy with branch length zero. Multitomies in the tree were resolved using the function "multi2di" in the "ape" package. The final tree used in our analysis can be found in the **Supplementary Material**.

To test whether color traits, number of flowers/inflorescence or nectar reward of the study species show phylogenetic signal, we calculated Pagel"s λ (Pagel, 1999). For continuous traits, λ calculation and significance tests were performed in the "phytools" package. Since the distribution of nectar reward quantities (i.e., sucrose equivalents) was significantly different from a normal distribution ($p < 0.05$; Shapiro-Wilks test), all values were log10-transformed for the analysis. For color category, λ was calculated using the "fitDiscrete" function in the "geiger" package. Significant difference between the fitted model and the null model ($\lambda = 0.00$; no phylogenetic signal) was tested using a log-likelihood ratio test.

Data Analysis

To test whether plant species are uniformly distributed among the color categories, we used a Chi-square test, followed by an analysis of the standardized residuals (Sharpe, 2015). To test whether nectar reward quantity differed significantly between color categories, we performed phylogenetic ANOVA using the package "geiger" for R (Version 2.0.7; Pennell et al., 2014), using log10-transformed nectar values as dependent and hexagon color category as independent variable. Our dataset contained a single species in the "UV"-category (*Crepis aurea*), which was removed prior to the ANOVA. Independent analyses were performed for the maximum and mean nectar reward and for the "open" and "bagged" treatments. Significant results in the omnibus test were followed by a post-hoc test, comparing all possible combinations and adjusting the *p*-level using the Bonferroni method.

Whether nectar reward quantity differed significantly between "open" and "bagged" flowers was tested using a paired *t*-test. To test the relationship between nectar reward quantity and visual and other traits we used phylogenetic generalized linear mixed models (PGLMM) with a Gaussian distribution. The model included log10-transformed nectar reward data as response variable, species (both as phylogenetic and non-phylogenetic covariate) as random factor, and color contrast, green contrast, brightness and the number of flowers *per* inflorescence as continuous covariates. All continuous variables were scaled prior to model preparation to facilitate interpretation of the effect sizes (Schielzeth, 2010). Separate models were calculated for each combination of nectar data (maximum & mean) and treatment (open and bagged). Model calculation was performed using the "pglmm" function in the "phyr" package for R (Version 1.1.0.; Li et al., 2020). All analyses were performed using the base version of R (Version 4.1.1; R Development Core Team, 2021) and the cited packages.

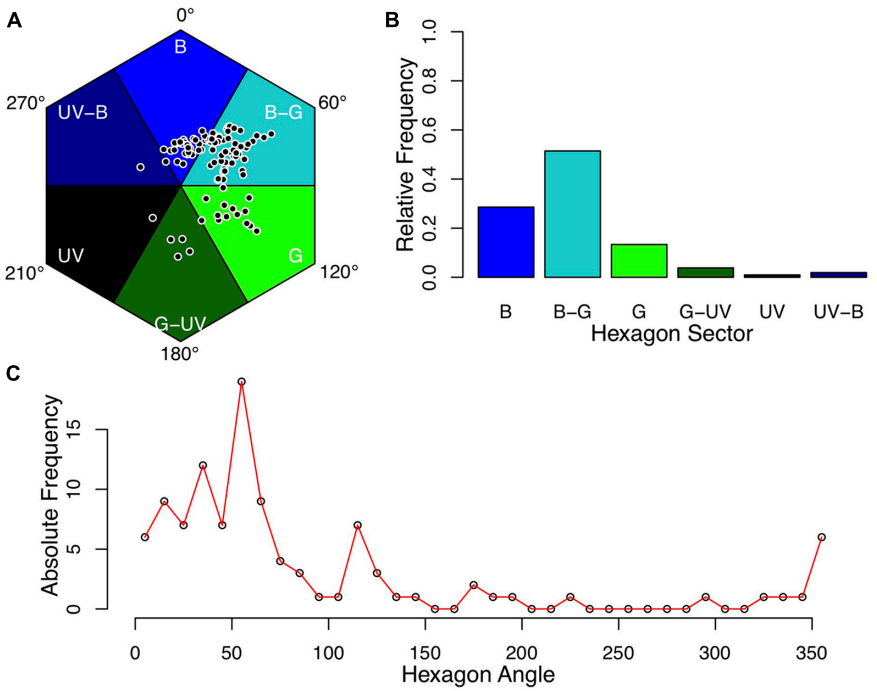

FIGURE 1 | Flower colors of alpine flowering plants. Mean color loci of bumble bee visited plant species for which full nectar data and spectral measurements were available ($N = 105$). **(A)** Color loci plotted in the hexagon color space. **(B,C)** Frequency of samples in color categories at a rough **(B)** and fine **(C)** scale (B, blue; B-G, blue-green; G, green; G–U, green-UV; UV, UV; UV-B, UV-blue). Colors assigned to the categories are for illustrative purposes and are not intended to reflect human or bee-specific perception. The convention for hexagon angle measurement is indicated in panel **(A)**.

RESULTS

Plant Sampling

We obtained full nectar (both open and bagged flowers) data and spectral measurements from 108 samples. Three species with color contrasts < 0.1 hexagon units were excluded from further analysis (*Pedicularis recutita, Salix hastata* female, and *Vaccinium myrtillus*). The remaining 105 samples constituted 103 unique species with two additional samples from a second color morph (*Trifolium pratense*) and a second sex of a dioecious species (*Salix waldsteiniana*).

Of the 112 flowering plant species for which bumble bee visits have been recorded in the study region (local communities of Rauris, Fusch and Heiligenblut above 1,400 m a.s.l.), the analyzed sample comprises 103 species, which accounted for the vast majority (98 %) of all recorded bumble bee visits ($N = 4{,}070$) in that region.

Color Distribution

The mean color loci of the samples were not distributed uniformly in the color space (**Figure 1A**), and the distribution of plant colors among the color categories differed significantly from a uniform distribution ($N = 105$, $\text{Chi}^2 = 125.46$, $p < 0.05$). The majority of samples were found in the "blue-green" category ($N = 54$), followed by the "blue" category ($N = 30$; **Figure 1B**). Analysis of the standardized residuals indicated that the observed frequencies in these two categories are significantly higher than expected by chance, while the categories "UV", "UV-blue," and "green-UV" had significantly less observations than expected. Color frequency distribution, when analyzed at a finer scale, showed a pronounced peak at 60°, which corresponds to the central part of the "blue-green" sector in the hexagon (**Figure 1**). Phylogenetic signal for flower color, calculated as Pagel's λ was estimated to be $\lambda = 0.87$, a value significantly different from $\lambda = 0.00$ ($p < 0.05$; **Table 1** and **Figure 2**).

Reward Quantity

Nectar reward quantity (expressed in mg sucrose per flower) varied among species and treatment (**Supplementary Table 1**).

TABLE 1 | Phylogenetic signal for measured traits and rewards in bumble bee visited plant species from the Eastern Alps.

Trait	λ	$p_\lambda = 0$
log10 (bagged flower maximum)	0.47	<0.05
log10 (open flower maximum)	0.57	<0.05
log10 (bagged flower mean)	0.43	<0.05
log10 (open flower mean)	0.56	<0.05
Color contrast	0.07	0.38
Green contrast	0.47	<0.05
Brightness	0.38	<0.05
Color category	0.87	<0.05
Flowers/inflorescence	0.14	<0.05

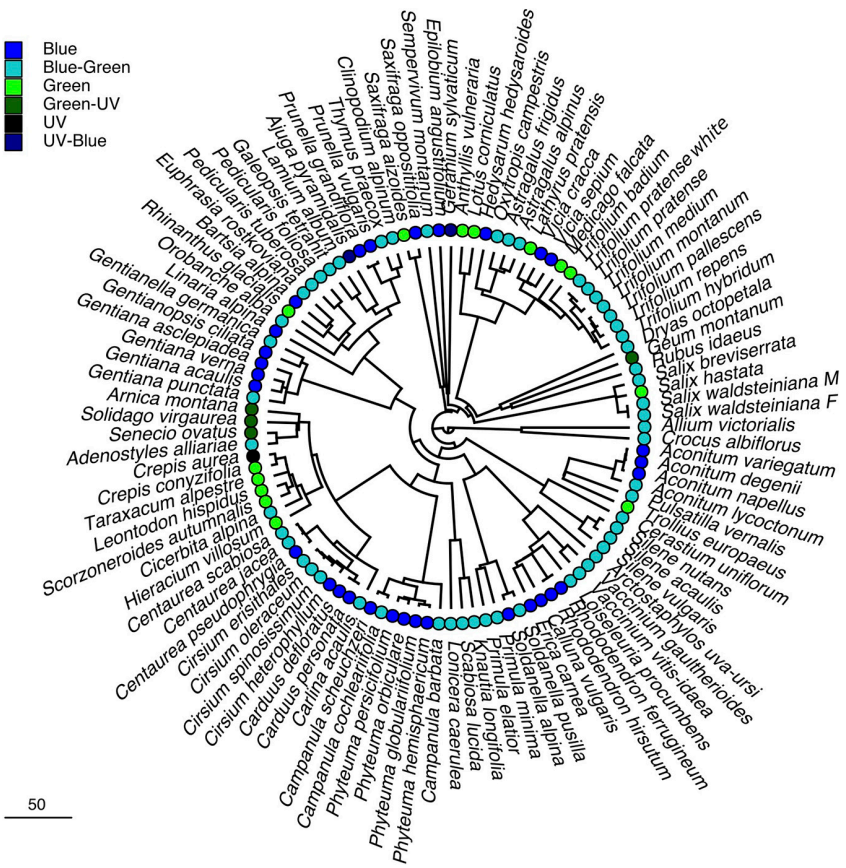

FIGURE 2 | Phylogenetic reconstruction of the studied plant species. Phylogenetic tree of the study species, based on the phylogenetic "ALLOTB" tree by Smith and Brown (2018). Colored circles refer to hexagon color category. Scale bar for branch length indicates divergence time in million years.

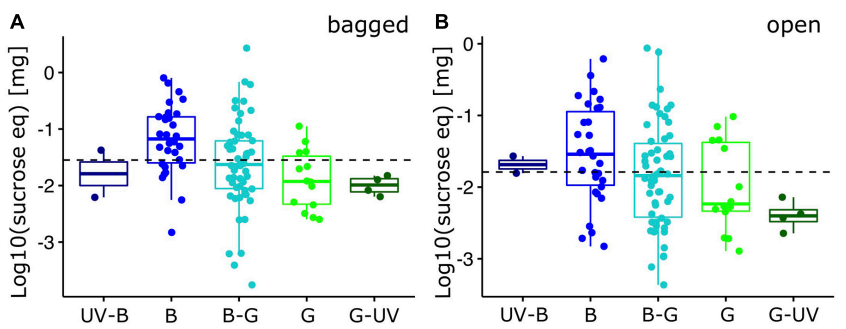

FIGURE 3 | Nectar standing crop of alpine flowering plants (individual flowers). Log10-transformed maximum reward quantity, expressed as mg sucrose equivalent per flower for bumble bee visited plant species ($N = 105$). Reward quantity was measured for **(A)** bagged flowers and **(B)** open flowers. Box-plots indicate the median (line) and interquartile range (IQR, i.e., Q25-Q75; box). Lower and upper whiskers indicate Q25-1.5*IQR and Q75+1.5*IQR, respectively. The horizontal dashed line indicates the overall mean. Individual data points have been added with random *X*-axis jitter. *X*-axis categories are the five hexagon categories used in the analysis (see **Figure 1**; UV-B, UV-blue; B, blue; B-G, blue-green; G, green; G-UV, green-UV). For statistics, see text.

Bagging had a significant effect on the measured reward quantity; flowers shielded from visitors had a significantly higher reward quantity than flowers which could be depleted by visitors (maximum nectar reward: $t(104) = 5.57$, $p < 0.05$; mean nectar reward: $t(104) = 6.88$, $p < 0.05$). Pagel's λ for the reward per flowers showed significant phylogenetic signal both in the mean and maximum value and in the "bagged" and "open" treatments (**Table 1**).

Reward and Color Category

Reward quantity of bagged flowers differed significantly between the color categories for the maximum nectar reward values

[Phylogenetic ANOVA: $F(4,99) = 3.51$, $p < 0.05$; **Figure 3A**], but just failed significance for the mean nectar reward values [$F(4,99) = 2.94$, $p = 0.07$; **Supplementary Figure 1A**]. While the individual flowers in the "blue" category had, on average, higher reward quantities than in the other categories, pair-wise post hoc comparison did not identify significant differences between any of the combinations after Bonferroni correction.

Reward quantities were generally smaller in open flowers. Their distribution across color categories did not differ significantly among categories for the maximum nectar reward values [$F(4,99) = 2.18$, $p = 0.20$; **Figure 3B**] and for the mean nectar reward values [$F(4,99) = 2.26$, $p = 0.18$; **Supplementary Figure 1B**]. When we extrapolated the nectar reward quantity to the entire functional unit (inflorescence), quantities did not differ significantly for both maximum [bagged: $F(4,99) = 3.37$, $p = 0.32$; open: $F(4,99) = 1.84$, $p = 0.56$; **Figure 4**] and mean [bagged: $F(4,99) = 2.81$, $p = 0.49$; open: $F(4,99) = 1.97$, $p = 0.59$; **Supplementary Figure 2**] quantity measures.

Phylogenetic generalized linear mixed models for maximum (**Tables 2**, **3**) and mean (**Supplementary Tables 2, 3**) nectar reward quantities identified a strong influence of species, followed by a smaller effect of the phylogeny-corrected species term. In the fixed effects, we identified equally strong negative effects of color contrast and the number of flowers *per* inflorescence, as well as small, non-significant effects of all other tested variables (**Tables 2**, **3** and **Supplementary Tables 2, 3**). In other words, nectar reward quantity correlated negatively with color contrast (i.e., flowers with higher color contrast contained less nectar) and flower number (i.e., inflorescences with fewer flowers had more nectar per flower).

DISCUSSION

In our study, we investigated the visual properties and the reward quantity of bumble bee visited flowering plants in an alpine environment. We found significant structure in the color signals, i.e., plant colors were not uniformly distributed across the color categories in a bee-specific color space. The reward quantity differed between the categories in bagged and open single flowers (although the latter was not significant) with higher average rewards in the "blue" and "blue-green" category. For entire flowering units this difference vanished. We hypothesize that naïve bumble bees, when visiting flowers of innately preferred colors, will find on average more reward per flower, although this effect was weak and almost disappeared when flowers were unbagged.

Color

Our analysis showed that the color loci of bumble bee visited flowers were scattered throughout the color hexagon, resulting in a variety of hues (angles) and chromatic contrasts to the background (distance to hexagon center). Interestingly, only three species appeared achromatic to bees. Achromatic cues are difficult to detect under natural conditions, and bees may not utilize them for flower detection and identification (Ng et al., 2018). The number of flowers found in each of the major bee-color categories (*sensu* Chittka et al., 1994) differed significantly from a uniform distribution, with the majority falling into the "blue-green" sector. This pattern, as well as that obtained when analyzed at a finer resolution (**Figure 1C**), showed remarkable similarity with data from other habitats and locations, e.g., Germany (Giurfa et al., 1995), Australia (Dyer et al., 2012), Nepal (Shrestha et al., 2014), New Zealand (Bischoff et al., 2013), and Taiwan (Tai et al., 2020). Interpretation and comparison of the distribution is problematic as it may depend on sampling strategy, habitat type, pollinator species and the choice of visual system selected for the color modeling (for a discussion, see Shrestha et al., 2019). For instance, most of the above-mentioned studies either combined samples from large regions rather than local communities and/or did not consider the pollinator composition.

Both abiotic factors and biotic factors are assumed to influence the flower color distribution. Previous studies demonstrated influences of e.g., day length and precipitation (Arista et al., 2013), soil composition (Horovitz, 1976), and vacuole pH (Grotewold, 2006). For the alpine environment, selection pressures for adaptations to cope with temperature extremes

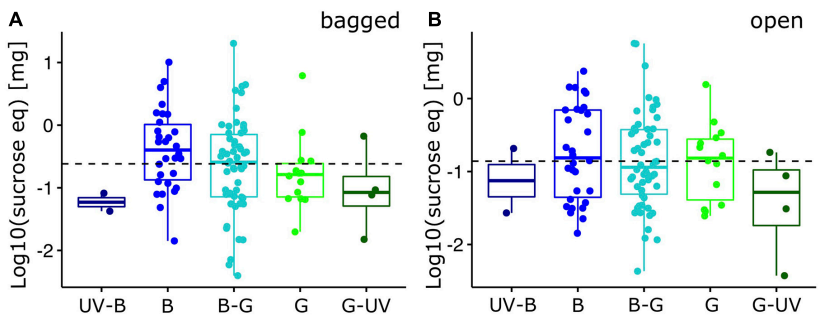

FIGURE 4 | Nectar standing crop of alpine flowering plants (inflorescences). Log10-transformed maximum reward quantity, expressed as mg sucrose equivalent per inflorescence for bumble bee visited plant species ($N = 105$). Reward quantity was measured for **(A)** bagged inflorescences and **(B)** and open inflorescences. Box-plots indicate the median (line) and interquartile range (IQR, i.e., Q25-Q75; box). Lower and upper whiskers indicate Q25-1.5*IQR and Q75+1.5*IQR, respectively. The horizontal dashed line indicates the overall mean. Individual data points have been added with random *X*-axis jitter. *X*-axis categories are the five hexagon categories used in the analysis (see **Figure 1**; UV-B, UV-blue; B, blue; B-G, blue-green; G, green; G-UV, green-UV). For statistics, see text.

TABLE 2 | PGLMM for maximum nectar reward of single bagged flowers.

Parameter	Variance	SD	Estimate	SE	Z	P
Maximum nectar reward (n = 105)						
*Species	0.334	0.578				
*Species_	0.068	0.261				
Color contrast			−0.248	0.091	−2.74	<0.05
Fl/inflorescence			−0.214	0.088	−2.42	<0.05
Green contrast			0.015	0.102	0.15	0.88
Brightness			0.059	0.099	0.60	0.55

**Denotes terms that were entered as random factors; _indicates that a phylogenetic covariance matrix was used in the random term. Continuous parameters were scaled before model generation.*

TABLE 3 | PGLMM for maximum nectar reward data of single open flowers.

Parameter	Variance	SD	Estimate	SE	Z	P
Maximum nectar reward (n = 105)						
*Species	0.238	0.488				
*Species_	0.075	0.274				
Color contrast			−0.164	0.080	−2.04	<0.05
Fl/Inflorescence			−0.184	0.078	−2.37	<0.05
Green contrast			0.031	0.091	0.34	0.73
Brightness			−0.070	0.088	−0.80	0.42

**Denotes terms that were entered as random factors; _indicates that a phylogenetic covariance matrix was used in the random term. Continuous parameters were scaled before model generation.*

and high irradiance are likely to influence the observed color frequencies (van der Kooi et al., 2019; Dalrymple et al., 2020).

For biotic selection pressures, color frequency differences have been hypothesized as resulting from selection by different pollinator assemblages, but (experimental) proof for this hypothesis is rare. Recently, data from regions that lack bees (Maquarie Island; Shrestha et al., 2016) or social bees (New Zealand; Ishii et al., 2019), show different plant color distributions and thus provide support for this hypothesis. For regions with highly overlapping visitor spectra, like the Alps, comparing color frequencies as function of pollinator group is more complex. Plant-visitor networks showed considerable overlap of visitor groups for most investigated plant species in this ecosystem (Lefebvre et al., 2018). While it can be assumed that many of the flower visitors also serve as pollinators, experimental proof of the actual pollinator identity and its share in the overall pollination of most generalist plant species is largely lacking. These bits of information are, however, crucial in understanding pollinator-mediated selection on traits like e.g., color, since the strength of selection can be assumed to critically depend on the pollination efficiency of the different pollinators of a plant species (Trunschke et al., 2021). To better understand the origin of the color frequency distribution that we observed in our study, we need further detailed information about the base line in the entire community (i.e., spectral reflectance data from all of the c. 400 flowering plant species that occur in the region) and quality information about the predominant pollinator(s) for each of them, which will be a challenge for future generations of pollination ecologists.

Nectar

Previous studies, which attempted to link flower color with reward quantity, either used literature data only (Giurfa et al., 1995), extrapolated nectar production rates from short measurement sequences to the entire day (Chittka et al., 2004; Raine and Chittka, 2007a), or measured sugar content of the nectar standing crop of bagged flowers only (Shrestha et al., 2020). In our study, we measured the nectar production capacity of the species (bagged flowers) as well as the nectar standing crop of open flowers, as a more direct measure of what is actually available during a typical day. As expected, the nectar standing crop was lower than the production capacity, due to depletion by visitors. Nectar reward quantities were more similar between color categories in open flowers, suggesting that those flowers that produce more nectar are preferentially depleted in the field under normal conditions. It is unclear whether these higher visitation rates originate initially from random or targeted visits of flower visitors, which have learned that certain flowers are more rewarding than others (Goulson et al., 2007).

Visitor Color Preferences and the Correlation With Nectar

Visual signals are used by flowering plants to convey information about their species identity and allow for easier detection in the usually cluttered visual environment. They can hold information about the reward or promote learning of the association between floral traits and the reward. Flower spectral reflectance is a complex mixture of different qualities that can be employed by the bee visual system separately or in combination. These qualities involve color contrast (contrast between the background and the flower color), achromatic contrast (modulation of the green receptor channel), brightness (the sum of the three photoreceptor excitations) and color hue. Aside from brightness, which is sometimes used in bee vision studies but has not been shown to be of importance for bees (Spaethe et al., 2001; Ng et al., 2018) all other signals and cues have been found to be relevant in bee foraging. Color contrast correlates with detection speed (Spaethe et al., 2001; Streinzer et al., 2009) and bees are known to prefer flowers of higher contrast when given a choice (Rohde et al., 2013). Achromatic contrast is used in object detection (Giurfa et al., 1996; Dyer et al., 2008), but is probably not or only rarely used as a sole cue in flower detection (Martínez-Harms et al., 2010; Lunau et al., 2011; Ng et al., 2018). Finally, hue is employed by bees to identify different reflectance spectra independent of lighting conditions and other visual traits (Reser et al., 2012). Bumble bees can learn to discriminate very small differences in hue when trained appropriately (Dyer and Chittka, 2004), but in the real world, such fine discrimination ability is probably of little value, given the existing variation of flower color within species, which sometimes overlaps with color of other species (Jersáková et al., 2016; Garcia et al., 2020).

Flower visiting insect have been shown to have (species-specific) innate preferences for certain colors (Lunau and Maier, 1995) which have been interpreted to help them find rewarding flowers more quickly during their first foraging flights. In a field study with *Bombus terrestris*, Raine and Chittka (2007b)

first determined the strength of the innate preference for "UV-blue" and then let them forage in the surrounding, where UV-blue flowers were also the most rewarding ones. They found a significantly higher colony-level success of colonies that showed a strong innate color preference, indicating that these preferences may be adaptive if color correlates with reward quantity. Due to the large variation of rewards in our study, color cannot be considered as a reliable signal for nectar reward quantity. After sampling the vast majority of plant species visited by bumble bees in our study region, we, however, found that flowers with colors from the preferred color categories do have, on average, higher reward production at the single flower level (**Figure 3A**). Smaller differences have also been found for open flowers (**Figure 3B**) and when comparing entire inflorescences (**Figure 4**), though the differences were statistically not significant. We thus conclude that color may be a weak (but honest) indicator for reward quantity, and that this overall (small) advantage may indeed allow the bees to increase foraging success, compared with an entirely random search. Similar correlations between the innately preferred color categories of bees and the reward quantity were also found in Central Europe (Giurfa et al., 1995; Raine and Chittka, 2005), but not e.g., in Australia (Shrestha et al., 2020). While in the European region, social bees are assumed to have a large share in the overall pollination of entomophilous plants, in the Australian communities, (social) bees are not the major pollinator guild. Furthermore, pollinator/visitor identity was not investigated in that study, which limits the comparison with our results.

For a complete understanding of how strongly innate color preferences affect flower color in the Alps, we must know the relative contribution of all flower visitors of a plant species to its pollination success, and to analyze in detail whether different nectar traits (like volume, concentration, sugar content, and sugar composition) differ between major visitor groups and color categories. Our study surprisingly showed a negative correlation between the color contrast and nectar reward, which seems to stand in contrast to the observation that bumble bees prefer flowers of high color contrast (Rohde et al., 2013). However, some previous studies found contrasting results regarding the relationship between color contrast and reward quantity (Kantsa et al., 2017; Shrestha et al., 2020). Color contrast is a highly variable trait in flower communities (Garcia et al., 2021) and it is currently not known how bees use this visual feature while foraging in natural environments.

Interestingly, we found no statistical difference of nectar rewards among color categories when we calculated the nectar reward of the entire inflorescence (**Figure 4** and **Supplementary Figure 2**). Flower and floral display size have been shown to correlate with nectar reward quantity and may constitute an honest signal of reward quantity for potential visitors (Ortiz et al., 2021). In our study, we found a significant negative relationship between nectar reward quantity of individual flowers and flower number of an inflorescence. From a plant's perspective, grouping several flowers with smaller reward quantity to larger units would constitute a strategy to attract more potential pollinators due to a larger display size (Spaethe et al., 2001; Wertlen et al., 2008) and thus promote learning through higher reward quantities that can be gathered during a single visit. While larger inflorescences may provide a larger total amount of reward, the energy and time needed to collect these rewards must also be considered in foraging economics (Harder et al., 2001). In future studies, one will need to investigate in more detail how the interplay between nectar reward of individual flowers, variation of inflorescence size (e.g., number of flowers) and spatial distribution of plant individuals within a population affect the foraging economics and color preferences of bumble bees (Geslin et al., 2014).

CONCLUSION

In an alpine community, investigating the majority of flowering plants that are confirmed to be visited by bumble bees, we found evidence that flower color may serve as a weak predictor for reward quantity. Since flowers of innately preferred colors produce either higher, or at least not smaller, reward quantities compared to less favored colors, naïve bumble bees may increase their foraging success by visiting flowers of such categories. Also, experienced foragers may also profit by visiting these flowers, e.g., when previously rewarding flowers become depleted or flowering season has ended. Although our study contributes to a better understanding of the origin and adaptiveness of color preferences of flower visitors, future studies are necessary to gather more quality data on pollination efficiency of the different flower visitors and thus their respective selection force on flower color.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JS, JN, and MS designed the study and collected the data. MS analyzed the data and drafted the manuscript. All authors contributed to manuscript writing and editing.

FUNDING

Nectar data were collected in part during projects supported by Austrian National Park fund (1994) and Glockner Öko-Fonds (2012).

ACKNOWLEDGMENTS

We would like to thank S. Dötterl for spectral measurements of some flowers, S. Streinzer for help with data preparation, and J. Plant for linguistic improvements. We further wish to express our thanks to the Großglockner-Hochalpenstraßen AG for the gratuitous use of the Großglocker high alpine road and

the museum "Haus der Natur" (Salzburg) for the opportunity to use the Eberhard-Stüber alpine research station. Parts of the data were collected during field courses of the University of Vienna. We would also like to thank all participating students. P. Pilsl provided estimates of flowering plant number in the region.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.721241/full#supplementary-material

REFERENCES

Arista, M., Talavera, M., Berjano, R., and Ortiz, P. L. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *J. Ecol.* 101, 1613–1622. doi: 10.1111/1365-2745.12151

Bertsch, A. (1983). Nectar production of *Epilobium angustifolium* L. at different air humidities; nectar sugar in individual flowers and the optimal foraging theory. *Oecologia* 59, 40–48. doi: 10.1007/BF00388069

Bingham, R. A., Orthner, A. R., and Takita, T. (1998). Efficient pollination of alpine plants. *Nature* 391, 238–239. doi: 10.1111/j.1365-2990.2004.00503.x

Bischoff, M., Lord, J. M., Robertson, A. W., and Dyer, A. G. (2013). Hymenopteran pollinators as agents of selection on flower colour in the New Zealand mountains: salient chromatic signals enhance flower discrimination. *N. Z. J. Bot.* 51, 181–193. doi: 10.1080/0028825X.2013.806933

Briscoe, A. D., and Chittka, L. (2001). The evolution of color vision in insects. *Annu. Rev. Entomol.* 46, 471–510. doi: 10.1146/annurev.ento.46.1.471

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543. doi: 10.1007/BF00199331

Chittka, L. (1996). Does bee color vision predate the evolution of flower color? *Naturwissenschaften* 83, 136–138. doi: 10.1007/s001140050263

Chittka, L., and Kevan, P. G. (2005). "Flower colour as advertisement," in *Practical Pollination Biology*, eds A. Dafni, P. G. Kevan, and B. C. Husband (Cambridge, ONT: Enviroquest Ltd), 157–230.

Chittka, L., Beier, W., Hertel, H., Steinmann, E., and Menzel, R. (1992). Opponent color coding is a universal strategy to evaluate the photoreceptor inputs in Hymenoptera. *J. Comp. Physiol. A* 170, 545–563.

Chittka, L., Ings, T. C., and Raine, N. E. (2004). Chance and adaptation in the evolution of island bumblebee behaviour. *Popul. Ecol.* 46, 243–251. doi: 10.1007/s10144-004-0180-1

Chittka, L., Shmida, A., Troje, N., and Menzel, R. (1994). Ultraviolet as a component of flower reflections, and the colour perception of Hymenoptera. *Vis. Res.* 34, 1489–1508. doi: 10.1016/0042-6989(94)90151-1

Corbet, S. A. (2003). Nectar sugar content: estimating standing crop and secretion rate in the field. *Apidologie* 34, 1–10. doi: 10.1051/apido:2002049

Corbet, S. A., Unwin, D. M., and Prŷs-Jones, O. E. (1979). Humidity, nectar and insect visits to flowers, with special reference to *Crataegus*, *Tilia* and *Echium*. *Ecol. Entomol* 4, 9–22. doi: 10.1111/j.1365-2311.1979.tb00557.x

Cruden, R. W., and Hermann, S. M. (1983). "Studying nectar? Some observations on the art," in *The Biology Of Nectaries*, eds B. L. Bentley and T. S. Elias (New York, NY: Columbia University Press), 223–242.

Dalrymple, R. L., Kemp, D. J., Flores-Moreno, H., Laffan, S. W., White, T. E., Hemmings, F. A., et al. (2020). Macroecological patterns in flower colour are shaped by both biotic and abiotic factors. *New Phytol.* 228, 1972–1985. doi: 10.1111/nph.16737

Dyer, A. G., and Chittka, L. (2004). Fine colour discrimination requires differential conditioning in bumblebees. *Naturwissenschaften* 91, 224–227. doi: 10.1007/s00114-004-0508-x

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G. P., Simonov, V., and Wong, B. B. M. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. B Biol. Sci.* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Dyer, A. G., Boyd-Gerny, S., Shrestha, M., Lunau, K., Garcia, J. E., Koethe, S., et al. (2016). Innate colour preferences of the Australian native stingless bee *Tetragonula carbonaria* Sm. *J. Comp. Physiol. A* 202, 603–613. doi: 10.1007/s00359-016-1101-4

Dyer, A. G., Spaethe, J., and Prack, S. (2008). Comparative psychophysics of bumblebee and honeybee colour discrimination and object detection. *J. Comp. Physiol. A* 194, 617–627. doi: 10.1007/s00359-008-0335-1

Ebmer, P. A. W. (2003). Die Höhenverbreitung der Bienen, ausgenommen Hummeln, im Nationalpark Hohe Tauern, Kärnten. *Beitr. Entomofaunistik* 4, 160–164.

Fischer, M. A., Oswald, K., and Adler, W. (2008). *Exkursionsflora für Österreich, Liechtenstein, Südtirol*, 3rd Edn. Linz: Biologiezentrum der Oberösterreichischen Landesmuseen.

Garcia, J. E., Dyer, A. G., Burd, M., and Shrestha, M. (2021). Flower colour and size signals differ depending on geographical location and altitude region. *Plant Biol.* 23, 905–914. doi: 10.1111/plb.13326

Garcia, J. E., Phillips, R. D., Peter, C. I., and Dyer, A. G. (2020). Changing how biologists view flowers—color as a perception not a trait. *Front. Plant Sci.* 11:601700. doi: 10.3389/fpls.2020.601700

Geslin, B., Baude, M., and Dajoz, I. (2014). Effect of local spatial plant distribution and conspecific density on bumble bee foraging behaviour. *Ecol. Entomol.* 39, 334–342. doi: 10.1111/een.12106

Giurfa, M., Núñez, J., Chittka, L., and Menzel, R. (1995). Color preferences of flower-naive honeybees. *J. Comp. Physiol. A* 177, 247–259.

Giurfa, M., Vorobyev, M., Kevan, P., and Menzel, R. (1996). Detection of coloured stimuli by honeybees: minimum visual angles and receptor specific contrasts. *J. Comp. Physiol. A* 178, 699–709. doi: 10.1007/BF00227381

Goulson, D., Cruise, J. L., Sparrow, K. R., Harris, A. J., Park, K. J., Tinsley, M. C., et al. (2007). Choosing rewarding flowers; perceptual limitations and innate preferences influence decision making in bumblebees and honeybees. *Behav. Ecol. Sociobiol.* 61, 1523–1529. doi: 10.1007/s00265-007-0384-4

Goyret, J., Pfaff, M., Raguso, R. A., and Kelber, A. (2008). Why do *Manduca sexta* feed from white flowers? Innate and learnt colour preferences in a hawkmoth. *Naturwissenschaften* 95, 569–576. doi: 10.1007/s00114-008-0350-7

Grotewold, E. (2006). The genetics and biochemistry of floral pigments. *Annu. Rev. Plant Biol.* 57, 761–780. doi: 10.1146/annurev.arplant.57.032905.105248

Gumbert, A. (2000). Color choices by bumble bees (*Bombus terrestris*): innate preferences and generalization after learning. *Behav. Ecol. Sociobiol.* 48, 36–43. doi: 10.1007/s002650000213

Harder, L. D. (1986). Effects of nectar concentration and flower depth on flower handling efficiency of bumble bees. *Oecologia* 69, 309–315. doi: 10.1007/BF00377639

Harder, L. D., Williams, N. M., Jordan, C. Y., and Nelson, W. A. (2001). "The effects of floral design and display on pollinator economics and pollen dispersal," in *Cognitive Ecology of Pollination: Animal Behaviour and Floral Evolution*, eds J. D. Thomson and L. Chittka (Cambridge: Cambridge University Press), 297–317. doi: 10.1017/CBO9780511542268.016

Horovitz, A. (1976). Edaphic factors and flower colour distribution in the *Anemoneae* (*Ranunculaceae*). *Plant Syst. Evol.* 126, 239–242. doi: 10.1007/BF00983363

Ishii, H. S., Kubota, M. X., Tsujimoto, S. G., and Kudo, G. (2019). Association between community assemblage of flower colours and pollinator fauna: a comparison between Japanese and New Zealand alpine plant communities. *Ann. Bot.* 123, 533–541. doi: 10.1093/aob/mcy188

Jersáková, J., Spaethe, J., Streinzer, M., Neumayer, J., Paulus, H., Dötterl, S., et al. (2016). Does *Traunsteinera globosa* (the globe orchid) dupe its pollinators through generalized food deception or mimicry? *Bot. J. Linn. Soc.* 180, 269–294. doi: 10.1111/boj.12364

Kantsa, A., Raguso, R. A., Dyer, A. G., Sgardelis, S. P., Olesen, J. M., and Petanidou, T. (2017). Community-wide integration of floral colour and scent in a Mediterranean scrubland. *Nat. Ecol. Evol.* 1, 1502–1510. doi: 10.1038/s41559-017-0298-0

Kearns, C. A., and Inouye, D. W. (1993). *Techniques For Pollination Biologists*. Boulder, CO: University Press of Colorado.

Kelber, A., and Osorio, D. (2010). From spectral information to animal colour vision: experiments and concepts. *Proc. R. Soc. B Biol. Sci.* 277, 1617–1625. doi: 10.1098/rspb.2009.2118

Kembel, S. W., Cowan, P. D., Helmus, M. R., Cornwell, W. K., Morlon, H., Ackerly, D. D., et al. (2010). Picante: R tools for integrating phylogenies and ecology. *Bioinformatics* 26, 1463–1464. doi: 10.1093/bioinformatics/btq166

Lefebvre, V., Villemant, C., Fontaine, C., and Daugeron, C. (2018). Altitudinal, temporal and trophic partitioning of flower-visitors in Alpine communities. *Sci. Rep.* 8:4706. doi: 10.1038/s41598-018-23210-y

Leonard, A. S., Dornhaus, A., and Papaj, D. R. (2011). Flowers help bees cope with uncertainty: signal detection and the function of floral complexity. *J. Exp. Biol.* 214, 113–121. doi: 10.1242/jeb.047407

Li, D., Dinnage, R., Nell, L. A., Helmus, M. R., and Ives, A. R. (2020). phyr: an R package for phylogenetic species-distribution modelling in ecological communities. *Methods Ecol. Evol.* 11, 1455–1463. doi: 10.1111/2041-210X.13471

Lunau, K., and Maier, E. J. (1995). Innate colour preferences of flower visitors. *J. Comp. Physiol. A* 177, 1–19. doi: 10.1007/BF00243394

Lunau, K., and Wacht, S. (1997). Innate flower recognition in the hoverfly *Eristalis tenax* L. *Mitt. Dtsch. Ges. Allg. Angew. Entomol.* 11, 481–484.

Lunau, K., Papiorek, S., Eltz, T., and Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *J. Exp. Biol.* 214, 1607–1612. doi: 10.1242/jeb.052688

Martínez-Harms, J., Palacios, A. G., Márquez, N., Estay, P., Arroyo, M. T. K. K., Mpodozis, J., et al. (2010). Can red flowers be conspicuous to bees? *Bombus dahlbomii* and South American temperate forest flowers as a case in point. *J. Exp. Biol.* 213, 564–571. doi: 10.1242/jeb.037622

Menzel, R., and Greggers, U. (1985). Natural phototaxis and its relationship to colour vision in honeybees. *J. Comp. Physiol. A* 157, 311–321. doi: 10.1007/BF00618121

Ng, L., Garcia, J. E., and Dyer, A. G. (2018). Why colour is complex: evidence that bees perceive neither brightness nor green contrast in colour signal processing. *Facets* 3, 800–817. doi: 10.1139/facets-2017-0116

Ortiz, P. L., Fernández-Díaz, P., Pareja, D., Escudero, M., and Arista, M. (2021). Do visual traits honestly signal floral rewards at community level? *Funct. Ecol* 35, 369–383. doi: 10.1111/1365-2435.13709

Pagel, M. (1999). Inferring the historical patterns of biological evolution. *Nature* 401, 877–884.

Paradis, E., and Schliep, K. (2019). ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* 35, 526–528.

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* 170, 23–40. doi: 10.1007/BF00190398

Pennell, M., Eastman, J., Slater, G., Brown, J., Uyeda, J., Fitzjohn, R., et al. (2014). geiger v2.0: an expanded suite of methods for fitting macroevolutionary models to phylogenetic trees. *Bioinformatics* 30, 2216–2218.

R Development Core Team (2021). *R: A Language And Environment For Statistical Computing*. Vienna: R Foundation statistical Computing.

Raine, N. E., and Chittka, L. (2005). Colour preferences in relation to the foraging performance and fitness of the bumblebee *Bombus terrestris*. *Uludag Bee J.* 5, 145–150.

Raine, N. E., and Chittka, L. (2007b). The adaptive significance of sensory bias in a foraging context: floral colour preferences in the bumblebee *Bombus terrestris*. *PLoS One* 2:e556. doi: 10.1371/journal.pone.0000556

Raine, N. E., and Chittka, L. (2007a). Mengen der Nektarerzeugung bei 75 von Hummeln besuchten Blumenarten in einem deutschen Pflanzenbestand (Hymenoptera: Apidae: *Bombus terrestris*). *Entomol. Gen.* 30, 191–192. doi: 10.1127/entom.gen/30/2007/191

Reser, D. H., Wijesekara Witharanage, R., Rosa, M. G., and Dyer, A. G. (2012). Honeybees (*Apis mellifera*) learn color discriminations via differential conditioning independent of long wavelength (green) photoreceptor modulation. *PLoS One* 7:e48577. doi: 10.1371/journal.pone.0048577

Revell, L. J. (2012). phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* 3, 217–223.

Rohde, K., Papiorek, S., and Lunau, K. (2013). Bumblebees (*Bombus terrestris*) and honeybees (*Apis mellifera*) prefer similar colours of higher spectral purity over trained colours. *J. Comp. Physiol. A* 199, 197–210. doi: 10.1007/s00359-012-0783-5

Schielzeth, H. (2010). Simple means to improve the interpretability of regression coefficients. *Methods Ecol. Evol.* 1, 103–113. doi: 10.1111/j.2041-210X.2010.00012.x

Sharpe, D. (2015). Your chi-square test is statistically significant: now what? *Pract. Assess. Res. Eval.* 20, 1–10. doi: 10.7275/tbfa-x148

Shrestha, M., Dyer, A. G., Bhattarai, P., and Burd, M. (2014). Flower colour and phylogeny along an altitudinal gradient in the Himalayas of Nepal. *J. Ecol.* 102, 126–135. doi: 10.1111/1365-2745.12185

Shrestha, M., Dyer, A. G., Garcia, J. E., and Burd, M. (2019). Floral colour structure in two Australian herbaceous communities: it depends on who is looking. *Ann. Bot.* 124, 221–232. doi: 10.1093/aob/mcz043

Shrestha, M., Garcia, J. E., Burd, M., and Dyer, A. G. (2020). Australian native flower colours: does nectar reward drive bee pollinator flower preferences? *PLoS One* 15:e0226469. doi: 10.1371/journal.pone.0226469

Shrestha, M., Lunau, K., Dorin, A., Schulze, B., Bischoff, M., Burd, M., et al. (2016). Floral colours in a world without birds and bees: the plants of Macquarie Island. *Plant Biol.* 18, 842–850. doi: 10.1111/plb.12456

Skorupski, P., and Chittka, L. (2010). Photoreceptor spectral sensitivity in the bumblebee, *Bombus impatiens* (Hymenoptera: Apidae). *PLoS One* 5:e12049. doi: 10.1371/journal.pone.0012049

Skorupski, P., and Chittka, L. (2011). Is colour cognitive? *Opt. Laser Technol.* 43, 251–260. doi: 10.1016/j.optlastec.2008.12.015

Skorupski, P., Döring, T. F., and Chittka, L. (2007). Photoreceptor spectral sensitivity in island and mainland populations of the bumblebee, *Bombus terrestris*. *J. Comp. Physiol. A* 193, 485–494. doi: 10.1007/s00359-006-0206-6

Smith, S. A., and Brown, J. W. (2018). Constructing a broadly inclusive seed plant phylogeny. *Am. J. Bot.* 105, 302–314. doi: 10.1002/ajb2.1019

Spaethe, J., Tautz, J., and Chittka, L. (2001). Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3898–3903. doi: 10.1073/pnas.071053098

Streinzer, M., Paulus, H. F., and Spaethe, J. (2009). Floral colour signal increases short-range detectability of a sexually deceptive orchid to its bee pollinator. *J. Exp. Biol.* 212, 1365–1370. doi: 10.1242/jeb.027482

Streinzer, M., Roth, N., Paulus, H. F., and Spaethe, J. (2019). Color preference and spatial distribution of glaphyrid beetles suggest a key role in the maintenance of the color polymorphism in the peacock anemone (*Anemone pavonina*, Ranunculaceae) in Northern Greece. *J. Comp. Physiol. A* 205, 735–743. doi: 10.1007/s00359-019-01360-2

Tai, K.-C., Shrestha, M., Dyer, A. G., Yang, E.-C., and Wang, C.-N. (2020). Floral color diversity: how are signals shaped by elevational gradient on the tropical-subtropical mountainous island of Taiwan? *Front. Plant Sci.* 11:582784. doi: 10.3389/fpls.2020.582784

Théry, M., Debut, M., Gomez, D., and Casas, J. (2005). Specific color sensitivities of prey and predator explain camouflage in different visual systems. *Behav. Ecol.* 16, 25–29. doi: 10.1093/beheco/arh130

Trunschke, J., Lunau, K., Pyke, G. H., Ren, Z.-X., and Wang, H. (2021). Flower color evolution and the evidence of pollinator-mediated selection. *Front. Plant Sci.* 12:617851. doi: 10.3389/fpls.2021.617851

van der Kooi, C. J., Kevan, P. G., and Koski, M. H. (2019). The thermal ecology of flowers. *Ann. Bot.* 124, 343–353. doi: 10.1093/aob/mcz073

Wertlen, A. M., Niggebrugge, C., Vorobyev, M., and Hempel de Ibarra, N. (2008). Detection of patches of coloured discs by bees. *J. Exp. Biol.* 211, 2101–2104. doi: 10.1242/jeb.014571

Wyszecki, G., and Stiles, W. S. (1982). *Color Science: Concepts And Methods, Quantitative Data And Formulae*, 2nd Edn. New York, NY: John Wiley & Sons.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

HYPOTHESIS AND THEORY
published: 17 February 2022
doi: 10.3389/fevo.2022.819436

Achromatic Cues Are Important for Flower Visibility to Hawkmoths and Other Insects

Casper J. van der Kooi[1*†] *and Almut Kelber*[2†]

[1] Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, Netherlands, [2] Lund Vision Group, University of Lund, Lund, Sweden

Studies on animal colour vision typically focus on the chromatic aspect of colour, which is related to the spectral distribution, and disregard the achromatic aspect, which is related to the intensity ("brightness") of a stimulus. Although the chromatic component of vision is often most reliable for object recognition because it is fairly context independent, the achromatic component may provide a reliable signal under specific conditions, for example at night when light intensity is low. Here we make a case for the importance of achromatic cues in plant-pollinator signalling, based on experimental data on naïve *Deilephila elpenor* and *Macroglossum stellatarum* hawkmoths, optical modelling and synthesising published experiments on bees, flies, butterflies and moths. Our experiments show that in ecologically relevant light levels hawkmoths express a strong preference for brighter stimuli. Published experiments suggest that for flower-visiting bees, butterflies, moths and flies, achromatic cues may be more important for object detection than often considered. Our optical modelling enabled disentangling the contribution of pigments and scattering structures to the flower's achromatic contrast, and illustrates how flower anatomy and background are important mediating factors. We discuss our findings in the context of the often-assumed dichotomy between detection and discrimination, chromatic versus achromatic vision, and the evolution of floral visual signals.

Keywords: brightness, hawkmoth, insect colour vision, pollination, achromatic contrast, achromatic vision, flower colour, pigment

OPEN ACCESS

Edited by:
Isabel Marques,
University of Lisbon, Portugal

Reviewed by:
Daniela Scaccabarozzi,
Curtin University, Australia
Michael John Munro Harrap,
University of Bristol, United Kingdom

****Correspondence:***
Casper J. van der Kooi
C.J.van.der.Kooi@rug.nl

†ORCID:
Casper J. van der Kooi
orcid.org/0000-0003-0613-7633
Almut Kelber
orcid.org/0000-0003-3937-2808

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *21 November 2021*
Accepted: *20 January 2022*
Published: *17 February 2022*

Citation:
van der Kooi CJ and Kelber A (2022) Achromatic Cues Are Important for Flower Visibility to Hawkmoths and Other Insects. Front. Ecol. Evol. 10:819436. doi: 10.3389/fevo.2022.819436

INTRODUCTION

Many animals rely on colour information for numerous tasks, such as finding mates and food or avoiding predators (reviewed by Kelber et al., 2003; Cronin et al., 2014; van der Kooi et al., 2021). Colour has a chromatic aspect, which is related to the spectral distribution of the reflected or emitted light, and an achromatic aspect, which is related to the intensity or total reflectance of a stimulus (Kelber et al., 2003; Osorio and Vorobyev, 2005; Kemp et al., 2015). Hue and saturation are terms used to characterise human perception of the chromatic aspect, and brightness is used to describe the achromatic aspect of colour, though these terms are not defined for animals. In this text, we use the word "bright" to describe a colour of higher intensity and "dark" for a colour of low intensity. Likewise, we will use human colour terms to describe how colours appear to the human eye.

Ever since the experiments on honeybee colour vision by von Frisch (1914), the use of chromatic information by animals has gained far more attention than their use of the achromatic aspect of colour. The use of the achromatic aspect of colour is seen as more "primitive" than the use of the chromatic aspect, because achromatic cues can be detected by a single spectral type of photoreceptor or a summed receptor signal (Osorio and Vorobyev, 2005), whereas chromatic vision depends on comparing signals from two or more spectral types of receptors (Kelber et al., 2003). Moreover, to demonstrate the use of colour vision in an animal, it is required to show that it uses chromatic information and to exclude the possibility that it used achromatic information (Kelber et al., 2003). This is usually done by making achromatic information unreliable in the experiment, which has led to the present situation that less is known about the use of achromatic than chromatic information in many animals.

In this essay, we call for an increased focus on the achromatic aspect of flower colour and make the case that achromatic information may be more important for flower detection and discrimination by insect pollinators than often acknowledged. The proposition is relevant for a better understanding of pollinator vision as well as flower colours. We will discuss this using five questions:

(1) Do flower-visiting insects use chromatic and achromatic signals for separate purposes as often suggested?
(2) Is there a difference between diurnal and nocturnal insects in the use of achromatic versus chromatic cues?
(3) Is there a difference in how chromatic and achromatic cues guide the innate preferences of naïve flower visitors and learned preferences for flower colours?
(4) Do chromatic and achromatic cues have different roles for the detection of flowers against the background, and for the discrimination between flowers?
(5) How do floral scattering and pigmentation properties determine the achromatic contrast?

In the following, after giving a short historical background, we will summarise basics on chromatic and achromatic vision in insects. We test the importance of achromatic contrast in an experiment with diurnal and nocturnal hawkmoths, and present evidence from literature on the importance of achromatic cues in plant-pollinator signalling. We use an optical model that enables disentangling the contribution of pigments, scattering structures and the type of background to discuss what determines the achromatic contrast between flowers and their background.

HISTORICAL BACKGROUND

Why is the use of achromatic cues often neglected? First, for many years, the goal was to understand colour vision. While already in the 17-hundreds, Sprengel (1793) noted that flowers were not colourful to appeal to humans but to attract pollinators, it took until the seminal work of Turner (1910) and von Frisch (1914) that it became broadly accepted that insects use colour information. At that time, colour vision, which is based on multiple photoreceptor types and neural comparison of their signals, was considered superior to or more derived than achromatic vision, which is possible with any spatially resolving eye.

The important notion made by von Frisch was that in order to show that an animal used colour, it needed to be clear that the animal used the chromatic aspect of colour. The achromatic aspect thus had to be negated by the experimenter (von Frisch, 1914; Kelber et al., 2003). Over the last century, colour vision has been documented in many insects, leading to the conclusion that most species use it. For example, in insects alone, the spectral sensitivities have been described in more than 200 species of 13 orders (van der Kooi et al., 2021). Behavioural studies aiming to test whether an insect species possesses colour vision need to exclude the use of achromatic information (Kelber et al., 2003; Spaethe et al., 2014; Arikawa et al., 2021), but the opposite, that is, rigorous exclusion of chromatic cues to investigate the use of achromatic cues, has very rarely been undertaken.

Second, starting with Turner's and von Frisch's work, there has long been a bias toward research on a single – albeit important – species of insect pollinators, European honeybees (*Apis mellifera*), because these are easy to obtain and maintain, and convenient in behavioural studies. Although research on honeybees has provided a leap in comprehension on colour vision, literature is taxonomically biased toward this species. A caveat pertaining honeybees as model system in vision research is that in honeybees there may be a comparatively strong difference between the use of chromatic and achromatic cues. Behavioural experiments suggested that honeybees (*A. mellifera* and *A. cerana*) use achromatic or chromatic information at different stages of their approach to flowers, depending on the subtended visual angle of a stimulus (e.g., Lehrer and Bischof, 1995; Giurfa et al., 1996, 1997; Menzel et al., 1997; Meena et al., 2021). Thus, in behavioural terms, honeybees use achromatic or chromatic contrast depending on the size of and distance to a flower. In other insects, this separation may not strictly be the case (e.g., in butterflies, Stewart et al., 2015).

Third, it was long assumed that insect chromatic and achromatic vision are processed by two different channels, with separate neural pathways (**Table 1**; for discussions, see Kelber and Henze, 2013). In the honeybee, behavioural experiments have shown that only the green photoreceptor (often called long-wavelength-sensitive receptor) is used for achromatic tasks such as motion and shape detection (Srinivasan, 1985; Srinivasan and Lehrer, 1988; Hempel de Ibarra and Giurfa, 2003). Achromatic vision is also used to control landing, a behaviour studied extensively in flies (e.g., Tinbergen and Abeln, 1983; Van Breugel and Dickinson, 2012) and bees (e.g., Lehrer et al., 1990). Patterns are very poorly detected by the bee's achromatic channel if they only present contrast in the UV (short-wavelength-sensitive) or blue (medium-wavelength-sensitive) photoreceptor. In flies, a similar case was posited. Six morphologically similar photoreceptors (named R1-6) with the same broadband sensitivity were assumed to feed into the motion and form vision channels, whereas the remaining two receptor types (R7/8) were assumed to feed into the colour vision channel. The fly eye is a mosaic of two ommatidial types, all with the same R1-6, but with two sub-types and different spectral

TABLE 1 | Examples of insect pollinators and their chromatic and achromatic visual channels.

	N spectral types of receptors	Chromatic vision based on	Achromatic vision based on	Selected references
Apidae (Hymenoptera)	UV, Blue, and Green	UV, Blue Green	Green	Peitsch et al., 1992; Giurfa et al., 1997
Diptera	R1-6 broadband R7/8 UV1, UV2, Blue, and Green	R7/8, plus some influence of R1-6	R1-6, plus some influence from R7/8	Wardill et al., 2012; Schnaitmann et al., 2013
Sphingidae (Lepidoptera)	UV, Blue, and Green	UV, Blue Green	Assumed the green receptor, but not confirmed	Telles et al., 2014
Papilio aegeus (Lepidoptera)	Five peak sensitivities plus broadband	UV, Blue, Green, and Red	Unknown	Arikawa, 2003
Pygopleurus israelitus (Coleoptera)	UV, Green, and Red	UV, Green, and Red	Unknown	Martínez-Harms et al., 2012

R indicates numbered receptor types, see references for clarification.

sensitivities in R7 (UV1 and UV2) and R8 (blue and green). Fly eyes thus have four spectrally different types of photoreceptors that are used for colour vision (Hardie, 1986). This general scheme seems to be universal among Diptera, at least for common flower visitors such as Syrphids (reviewed by van der Kooi et al., 2021).

Early work on bee and fly chromatic and achromatic colour processing led to the general assumption that achromatic and chromatic vision might be physically and behaviourally separated in insect vision in general, even though not much was known about other groups. For most insect groups, it is not clear which photoreceptor(s) is/are important for processing of achromatic information (see **Table 1**), but the green receptor is a suitable and likely candidate for the achromatic channel, because it is the most abundant photoreceptor type across insect eyes and thus, the green channel has the highest sensitivity (Kelber et al., 2003).

Over the last decade, however, work has revealed that the dichotomy in neural chromatic versus achromatic pathways is not universal, and that the pathways intersect in both flies and butterflies, meaning that chromatic processes feed into the achromatic channel and vice versa (Wardill et al., 2012; Kelber and Henze, 2013; Schnaitmann et al., 2013; Rusanen et al., 2018; Pagni et al., 2021). The same may apply to other insects such as moths and bees.

These three historical facets of studies on insect colour vision have contributed to our lack of understanding of the use of achromatic cues by flower visitors.

WHAT MAY ACHROMATIC CUES BE USED FOR?

The reliability of achromatic versus chromatic cues depends on biotic and abiotic conditions. Chromatic vision is often considered more reliable than achromatic vision, because it is less context dependent (Osorio and Vorobyev, 2005; Johnsen et al., 2006). Achromatic cues vary with illumination, for example caused by shadows or clouds, but also the spectral composition of the illumination, which differs between an open field and the forest, and between twilight, starlight and moonlight (Johnsen et al., 2006). By contrast, colour vision systems universally correct, at least partly, for illumination differences by means of colour constancy (reviewed by Chittka et al., 2014), so enabling the animal to recognise the same colour under varying illumination.

As compared to chromatic cues, achromatic cues represent the highest signal power and information content in any image (Osorio and Vorobyev, 2005; Vasas et al., 2017). The higher signal to noise ratio of achromatic cues has been taken as main reason why motion vision, polarisation vision as well as other visual domains that require high spatial and temporal resolution of insect vision mainly rely on achromatic input (Osorio and Vorobyev, 2005). Therefore, achromatic cues could be more important for flower detection and discrimination than often assumed.

Insect responses to achromatic cues vary markedly between species. Honeybees can detect flowers from the threefold distance using achromatic contrast, compared to chromatic contrast (**Table 2**). Spaethe et al. (2001) showed that in bumblebees, the relative importance of achromatic versus chromatic cues also varies with size of, or distance to, the stimulus. Smaller flowers grouped in inflorescences can be detected from further away, if they provide green receptor contrast (Wertlen et al., 2008). However, the difference between achromatic and chromatic spatial resolution is smaller in, for example, stingless bees (Dyer et al., 2016; Jezeera et al., 2021). *Eristalis tenax* hoverflies exhibit preferences for bright (yellow) stimuli (An et al., 2018) and *Papilio xuthus* butterflies prefer high achromatic contrast between flowers and their background (Kinoshita et al., 2012), but *Catopsilia pomona*, a diurnal pierid butterfly, did not prefer yellow flower models of higher intensity (Balamurali et al., 2020).

Even animals of the same species can give different weight to chromatic and achromatic cues, depending on context. Crepuscular flower-visitors such as the hawkmoth *Manduca sexta*, albeit showing a clear chromatic preference for blue flowers in twilight (Goyret et al., 2008), switch to an achromatic preference for bright flowers in dim starlight and, if flowers are seen in front of a dark background, also in moonlight levels (Kuenzinger et al., 2019). The nocturnal hawkmoth *Deilephila elpenor* can learn to discriminate flowers using chromatic information even in starlight, but when given the choice between different intensity versions, it exhibits preferences for darker or brighter shades of the training colour (Kelber et al., 2002). Differences in the achromatic

TABLE 2 | Studies that enable assessing the relative importance of chromatic versus achromatic contrast in the context of flower detection and discrimination.

Species	Stimulus	Activity	Innate/learned	Detection - discrimination	Additional findings/comments	References
Lepidoptera						
Macroglossum stellatarum	Paper stimuli	D	Naïve animals show a preference for the stimulus with higher intensity	Discrimination task		Present study
Macroglossum stellatarum	Mono-chromatic blue lights	D	Naïve animals show a preference for the stimulus with higher intensity. Achromatic cues are learned, but more slowly than chromatic cues	Discrimination task	In a conflicting situation, the chromatic aspect is given higher weight	Kelber, 2005
Macroglossum stellatarum	Paper stimuli	D	Naïve animals can use both chromatic and achromatic contrast to guide the proboscis to the nectary	Detection task: nectar guide versus surrounding petal colour		Goyret and Kelber, 2012
Deilephila elpenor	Paper stimuli	N	Naïve animals show a preference for the stimulus with higher intensity	Discrimination task		Present study
Deilephila elpenor	Paper stimuli	N	After training to a colour, animals use the chromatic aspect to choose. However, when provided the training colour in several intensities, they prefer brighter shades of yellow and darker shades of blue, suggesting a contribution of achromatic vision	Discrimination task		Kelber et al., 2002
Manduca sexta	Feeder	C	Naïve moths prefer bright white flowers in dim light and with dark background	Detection: Importance of achromatic contrast of the flower to the background increases with decreasing illumination intensity	Both used stimulus colours differed in both achromatic and chromatic aspect of colour; the preference for blue disappears in dim light	Kuenzinger et al., 2019
Manduca sexta	Paper stimuli	C	Naïve animals probed on white lines on a black or dark blue flower background, but avoid black or dark blue lines on a white background, indicating that proboscis control is mediated by achromatic cues independent of the chromatic aspect	Discrimination of nectar guides, as contrast was high enough for detection in all cases		Goyret, 2010
Helicoverpa armigera	Paper	N	Animals appeared to select the most reflective stimuli	Discrimination task	The setup was not ideally designed for testing achromatic vision, as the aim was different	Satoh et al., 2016

(Continued)

TABLE 2 | (Continued)

Species	Stimulus	Activity	Innate/learned	Detection - discrimination	Additional findings/comments	References
Catopsilia pomona	Paper	D	Naïve butterflies choose equally between three shades of yellow differing in intensity, no effect of achromatic differences. Learning of achromatic cues was not tested	Discrimination task	The butterflies have a preference for blue over all other colours, and blue has low intensity	Balamurali et al., 2020
Papilio xuthus	Paper	D	Achromatic contrast to background is required for the animals to land on a flower	Detection and landing on flower	The contrast seems to be for the broad-band receptor, alternatively for a summed receptor signal of UV, blue, green and red receptors	Koshitaka et al., 2011
Papilio xuthus	Paper	D	After training to a single colour stimulus, animals prefers high reflectance over low reflectance stimuli	Discrimination task		Kinoshita et al., 2011
Papilio xuthus	Paper, neutral density filters	D	Naïve animals prefer the brighter of two stimuli. They can learn to choose the brighter or darker of two stimuli, but learning is slower than for chromatic cues	Discrimination task	Tested with backgrounds of different intensities, the animals can use simultaneous brightness contrast, something rarely tested in insects	Kinoshita et al., 2012
Diptera						
Eristalis tenax	Paper	D	Trained animals preferred stimuli of higher intensity, particularly with yellow hues, independent of the brightness of the learned colour	Discrimination task	The main focus of the study was on chromatic vision	An et al., 2018
Hymenoptera						
Apis mellifera	Red ("bee-black"), blue and green paper	D	Learned behaviour	Discrimination task	Bees discriminated red from other colours (even though it appears as achromatic, black to them) so they must have used achromatic information	Turner, 1910
Apis mellifera	Mono-chromatic and white light	D	Learned behaviour	Discrimination	In dim light, bees choose the stimulus of higher intensity, even after training to the other stimulus	Menzel, 1981
Apis mellifera	Paper stimuli	D	Trained behaviour	Edge detection and landing	Edge detection and landing is colour-blind and requires contrast in the green receptor	Lehrer et al., 1990
Apis mellifera	Paper stimuli	D	Learned behaviour	Detection: For flower cues without chromatic contrast to background, detection threshold (3° angular size) depends on achromatic (green receptor) contrast		Lehrer and Bischof, 1995

(Continued)

TABLE 2 | (Continued)

Species	Stimulus	Activity	Innate/learned	Detection - discrimination	Additional findings/comments	References
Apis mellifera	Paper stimuli	D	Learned behaviour	Detection	Bees learn achromatic (green receptor) cues at small visual angles (5°), though this is harder for them than learning chromatic cues	Giurfa et al., 1996, 1997; Giurfa and Vorobyev, 1998
Apis mellifera	Paper stimuli	D	Learned behaviour	Both chromatic and achromatic (green receptor) contrast mediate detection of coloured patterns	For large targets (≥15°), chromatic contrast is most important	Hempel de Ibarra et al., 2000, 2001; Niggebrügge and Hempel de Ibarra, 2003
Apis mellifera	Paper stimuli	D	Learned behaviour	Discrimination	Only achromatic (green receptor) contrast is used for shape discrimination	Hempel de Ibarra and Giurfa, 2003
Apis mellifera	Paper stimuli	D	Learned behaviour	Detection	With achromatic (green receptor) contrast present, grouping of small stimuli improves detection	Wertlen et al., 2008
Apis mellifera	Paper stimuli	D	Learned behaviour	Detection (Experiment 1) and discrimination (Experiment 2) both use achromatic information	Only green receptor contrast explained the detection of orange from dark grey. Both green receptor contrast and chromatic contrast together explained discrimination from other flower colours	Reisenman and Giurfa, 2008
Apis mellifera	Paper stimuli	D	Learned behaviour	Detection: the animals could not detect flowers of 28° extension, if they presented no chromatic contrast to the grey background	Green receptor contrast and summed receptor contrast are not learned in this situation	Ng et al., 2018
Apis cerana	Paper stimuli	D	Learned behaviour	Detection threshold for flowers with achromatic and chromatic contrast is 7.7°, for flowers with only chromatic contrast 13.2°		Meena et al., 2021
Bombus terrestris	Artificial flowers of different sizes	D	Learned behaviour	Detection	When searching for large targets chromatic contrasts are used. For small targets, achromatic (green receptor) contrast is used	Spaethe et al., 2001
Bombus terrestris	Paper stimuli	D	Learned behaviour	Detection	With green receptor contrast present, grouping of small stimuli improves detection	Wertlen et al., 2008
Melipona mondury	Powder mixes	D	Preference in several colour combinations in series, visits to all stimuli were rewarded	Discrimination of two colours, no consistent effect of achromatic cues	Background colour (green or grey) had an effect	Koethe et al., 2016

(Continued)

TABLE 2 | (Continued)

Species	Stimulus	Activity	Innate/learned	Detection - discrimination	Additional findings/comments	References
Melipona quadrifasciata	Powder mixes	D	Preference in several colour combinations in series, visits to all stimuli were rewarded	Discrimination of two colours, no consistent effect of achromatic cues		Koethe et al., 2016
Tetragonula carbonaria	Paper stimuli	D	Learned behaviour	Detection threshold is similar (visual angle) for stimuli with and without achromatic (green receptor) contrast		Dyer et al., 2016
Tetragonula iridipennis	Paper stimuli	D	Learned behaviour	Detection threshold for target with both chromatic and achromatic contrast is 9°, with only chromatic contrast 11.5°		Jezeera et al., 2021
Tetralonia lerlandi	Paper perianth, real flower		At distances < 30 cm, the male search time for *Ophrys* dummy flowers with different perianth colours was correlated with achromatic, but not chromatic contrast to the green background	Detection task	At distances > 30 cm, search time was affected by wind speed (olfaction), but not by the visual properties of the perianth	Streinzer et al., 2009

D, diurnal; N, nocturnal; C, crepuscular.

cues of flowers may result in changes in plant reproductive success; for example, nutrient deficiency can lead to lower amounts of floral pigment, which reduces flower chromatic and achromatic contrast (Ausma et al., 2021), and shaded flowers may suffer from reduced visitation by insect pollinators (Ushimaru et al., 2021). Generally, the relative importance of chromatic versus achromatic signals in detection and discrimination of flowers by insect pollinators remains largely unknown, partly because studies on insect vision are greatly biassed toward the chromatic aspect (Kelber and Osorio, 2010; Hempel de Ibarra et al., 2014).

In the following, we take a three-way approach to evaluate the importance of achromatic information for flower detection by flower-visiting insects. We present results from a behavioural experiment on responses of flower-naïve diurnal and nocturnal hawkmoths to stimuli that differ solely in intensity. Thereafter we discuss what is known from literature on the use of chromatic cues by flower-visiting insects. Finally, we use an optical model to better understand the contribution of pigment, scattering structures and type of background for the achromatic contrast between flowers and their background.

BEHAVIOURAL EXPERIMENTS WITH DIURNAL AND NOCTURNAL HAWKMOTHS

Pupae of the nocturnal *D. elpenor* and the diurnal *Macroglossum stellatarum*, bred on the natural larval food plant (*Galium* sp.), were placed in a flight cage at room temperature in a 12:12 h light:dark cycle for eclosure. Tests were performed in a separate flight cage (65 cm × 65 cm × 80 cm height × depth × width) with three walls from light grey cloth and one long wall from transparent plastic for observation. The cage, placed in an otherwise dark room, was diffusely illuminated from above with an array of LEDs (Goobay LED strip flex 33 SMD white, max 132 lumen) pointing upward toward an aluminium reflector (**Figure 1A**). The intensity was adjusted to 10 lux for *D. elpenor* (≈nautical twilight) and to 100 lux (≈civil twilight) for *M. stellatarum*, as measured in the centre of the cage (using Hagner Screen Master, Hagner AB Solna Sweden). The two illumination intensities were chosen as similar as possible but also to account for the fact that *D. elpenor* is nocturnal, while *M. stellatarum* is diurnal, but extends its flight into the evening twilight (Herrera, 1992).

The flat visual display, printed on paper, 20 cm high and 28 cm wide, with a green background (spectrum in **Figure 2B**) and two circular blue flower-like stimuli of 2 cm diameter, in the centre, but 7 cm apart, was presented vertically on a stand about 5 cm in front of the cage wall opposite the wall covered with transparent plastic (for a figure of a similar set-up please see Telles et al., 2014). As the preference of naïve moths was to be tested, we did not provide any reward to the moths. The stimuli, hereafter called Light Blue and Dark Blue, had the same chromatic properties but different intensities (achromatic properties) (**Figure 1B**). The illumination spectrum (**Figure 1A**) was measured using a calibrated spectroradiometer (RSP900-R; International Light, Peabody, MA, United States). Reflectance spectra of stimuli and background (**Figure 1B**) were measured using an integrating sphere and the same setup, following standard routines

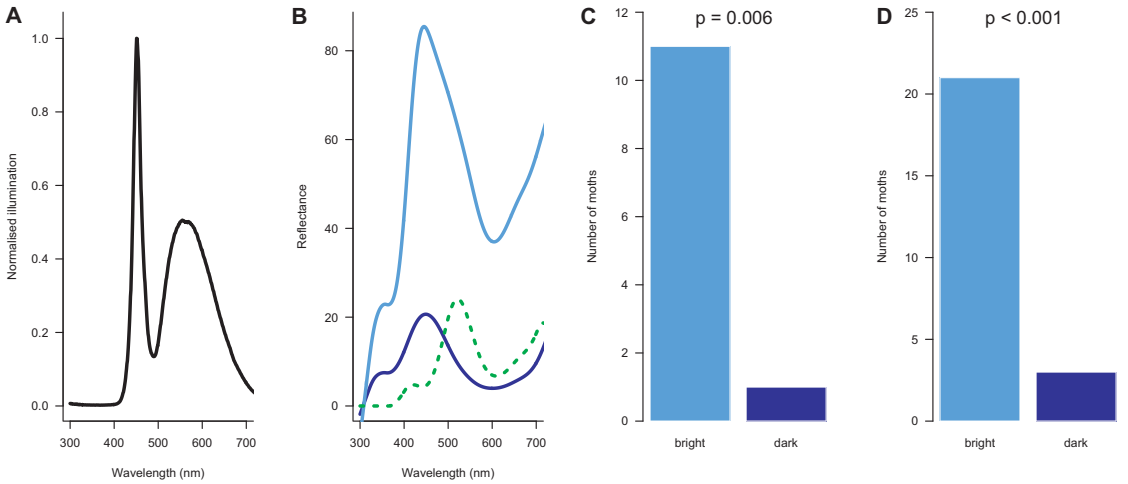

FIGURE 1 | Preference of hawkmoths for brighter stimuli. **(A)** Spectrum of the cage illumination. **(B)** Reflectance spectra of Dark Blue (dark blue curve) and Light Blue (light blue curve) stimuli and background (dashed green curve). **(C)** *Deilephila elpenor* first choices. **(D)** *Macroglossum stellatarum* first choices.

(van der Kooi et al., 2016, 2017). The used reference was a diffuse, white tile (Avantes WS-2).

Each newly eclosed, flower-naïve moth was tested once and individually, on the day of eclosion, in their respective activity period (first 2 h of night, for *D. elpenor*, daytime for *M. stellatarum*). A new stimulus array was used for each moth, and the position of Light Blue and Dark Blue (right or left side) was randomised. Hawkmoths do not land on flowers, but approach and probe them with the extended proboscis. Therefore, a single moth was released into the cage and given time to warm up, and the first time it approached and probed one of the stimuli with the extended proboscis was counted as choice. Only the first choice was registered to avoid pseudoreplication. Moths that did not approach and probe the stimulus display within 5 min after taking flight (three *D. elpenor* and four *M. stellatarum*), were considered not motivated to feed and removed. None of the moths probed the green background.

Twelve moths of *D. elpenor* approached the stimulus display with the extended proboscis and probed at least one stimulus. Of these moths, eleven approached Light Blue first, whereas one chose Dark Blue ($p = 0.006$, exact binomial test; 99% confidence interval: 0.52–1.00). Of 24 *M. stellatarum*, 21 probed Light Blue ($p < 0.01$, exact binomial test, confidence interval: 0.61–0.99). In both species, this indicates a significant preference for the stimulus with higher reflectance and intensity (**Figures 1C,D**). A similar preference for the brighter of the two shades of the same colour has earlier been shown for *M. stellatarum* (Kelber, 2005); that study had been performed under higher illumination intensity (≈4,000 lux) and used (monochromatic) light sources with fairly high intensity differences as stimuli, whereas the stimuli used here are more similar to natural flowers. Although the peak reflectance of the Light Blue stimulus used here is still somewhat higher (80%) than the highest reflectance found in flowers (∼60%; van der Kooi et al., 2016, 2017), the strong preference (**Figures 1C,D**) can likely be generalised to natural flowers.

WHAT DOES PUBLISHED LITERATURE SUGGEST ABOUT THE USE OF ACHROMATIC CUES BY INSECTS?

We synthesised the findings of 32 experimental studies that investigated the importance of achromatic versus chromatic aspects of colour in object detection/recognition for in total 15 insect species of bees, butterflies, moths and flies (**Table 2**). We did not list studies that solely investigated chromatic vision, without allowing any insight into the use or lack of use of achromatic cues, and also exclude literature dealing with the use of achromatic or single receptor cues for motion vision or polarisation vision. We also excluded most literature on landing responses, the use of polarised light stimuli and other behaviours that are not directly involved in flower detection or discrimination. We focussed on the use of achromatic cues in the direct context of flower detection and flower discrimination, separating between preferences of flower-naïve (or generally untrained) individuals and individuals that had learned to retrieve a reward from a specific type of flower. As many publications focus on chromatic aspects and mention reactions to achromatic contrast in passing, our list is likely not complete, but it clearly indicates the many ways in which achromatic information is used by different species of pollinators. We posed five questions, and summarise below what we have learned with respect to each of them, also giving some details in **Table 2**.

Do Flower-Visiting Insects Use Chromatic and Achromatic Signals for Separate Purposes as Often Suggested?

It is commonly assumed that insects use chromatic and achromatic cues for different purposes (see "Historical Background"). Specifically for bees, it has been suggested that chromatic cues are important for flower detection and recognition, whereas achromatic channels guide motion vision

and other behaviours. However, this separation may be less complete than previously assumed. Although chromatic cues are learned faster and used more frequently than achromatic cues, achromatic cues can be learned in the context of object – and flower – detection and recognition (**Table 2**). Several species of moths, flies and bees exhibited an innate preference for high intensity stimuli and/or chose stimuli more (rapidly) in the presence of achromatic cues (**Table 2**). Only in three species of stingless bees and one tested butterfly species, no effect of achromaticity was found (**Table 2**).

On the other hand, *P. xuthus* butterflies use both chromatic and achromatic cues for the control of landing (see **Table 2**), a task which is controlled exclusively by achromatic cues in bees and flies. Whereas landing is an important behaviour for pollination, the literature on landing responses was not included in **Table 2**, because there is general agreement that insects use achromatic vision for this behaviour (see "Historical Background"). *Macroglossum stellatarum* moths use both achromatic and chromatic cues for finding the entrance to the nectar reservoir (Goyret and Kelber, 2012). Thus, as to be expected from the anatomical and physiological findings that achromatic and chromatic channels are not entirely separated (see "Historical Background"), they both can, at least to some degree, influence the same behaviours to a larger degree than was earlier appreciated.

Is There a Difference Between Diurnal and Nocturnal Insects in the Use of Achromatic Versus Chromatic Cues?

Colour vision and the use of chromatic versus achromatic cues have been studied in fewer nocturnal than diurnal insects. A direct comparison can only be made between the diurnal hawkmoth *M. stellatarum* and the nocturnal species *D. elpenor* (see **Figure 1**). Although many nocturnal insects, including *D. elpenor* and the nocturnal carpenter bee *Xylocopa tranquebarica* (Somanathan et al., 2008), have (exquisite) colour vision, given the adaptations to increase light capture found in the eyes of nocturnal insects (reviewed by Nilsson, 2021), it is tempting to hypothesise that stimulus intensity plays a role in visibility to many nocturnal insects.

Our behavioural experiments showed that when given the choice between two flowers, naïve individuals express a clear preference for a more reflective, brighter stimulus (**Figure 1**). The crepuscular hawkmoth *Manduca sexta* was tested with conflicting cues and revealed that under bright light conditions, and with a bright background, they preferred dark blue to white (without UV) flowers, but this changed both with light and background, and in dimmest light levels and with dark backgrounds, the preference clearly shifted to white (**Table 2**; Kuenzinger et al., 2019).

It has been reported that in honeybees "colour vision disappears" in dim light (Menzel, 1981), but this happens in light intensities when they do not normally fly and forage. Only one nocturnal bee species, the carpenter bee *X. tranquebarica*, has ever been tested for colour vision at night, and this was not done in the context of flower visits, but at the nest entrance. If we assume that their use of cues is similar in both situations, we could conclude that chromatic information remains more important for that species than achromatic information. Bees trained to associate their nest entrance with a yellow marker, consistently searched at yellow markers, independent of intensity cues, and disregarded markers of other colours even if they matched the intensity of the learned marker (Somanathan et al., 2008).

Based on the limited number of species tested it seems that the use of achromatic information indeed differs between situations and light conditions, but whether there are overall differences between diurnal and nocturnal species remains unclear.

Is There a Difference in How Chromatic and Achromatic Cues Guide the Innate and Learned Preferences of Flower Visitors?

Innate colour preferences are considered to shape foraging behaviour in various insect groups. Both bees and moths have been shown to be able to learn colours other than what they innately prefer (van der Kooi et al., 2019), whereas syrphid flies seem to more strongly adhere to their innate preferences (Lunau and Maier, 1995; An et al., 2018). For the spontaneous preferences of flower-naïve animals, we see clear evidence for the use of achromatic cues in moths (Satoh et al., 2016; Kuenzinger et al., 2019; this study), but not in bees, flies or butterflies (**Table 2**). Bees, butterflies and moths can learn achromatic cues, but when given a conflicting situation, they will rely more heavily on the chromatic aspect. A tendency to rely on chromatic aspects in conflicting situations is likely directly related to the fact that chromatic information is more stable under changing light conditions. Colour constancy, demonstrated in these groups (Werner et al., 1988; Balkenius and Kelber, 2004) will allow an animal to commonly distinguish a yellow flower from a green background, independent of changes in illumination colour, which can be quite dramatic between shade and sun, and between late twilight an a moonlit night (Johnsen et al., 2006).

Do Chromatic and Achromatic Cues Have Different Roles for the Detection of Flowers Against the Background, and for the Discrimination Between Flowers?

Pollinators have two discrimination tasks to solve: first, they need to discriminate a flower from its background, and also from other flowers, which may not be rewarding. That first task is often referred to as detection. In addition to colour cues, insects can rely on depth cues to discriminate between objects and their background, often using motion parallax, which relies on achromatic contrast between flower and background (see "Historical Background," e.g., Lehrer et al., 1990). We have not included other references to this in **Table 2**, as little has been done on flower-visiting insects. However, butterflies require achromatic contrast to land on flowers (Koshitaka et al., 2011), and *M. stellatarum* needs achromatic contours to stabilise

hovering flight in front of a flowers (reviewed by Stöckl and Kelber, 2019).

The detection from the background is a more complex problem, as flowers can be seen in front of many different backgrounds, such as a clear blue featureless sky, a sky with moving clouds, dark green vegetation which can also be moving, and soil or human-made structures of various colours. Most of these backgrounds are different in colour from flowers. Although natural background structures (i.e., leaves, soil, and rocks) are roughly similarly achromatic (Menzel and Shmida, 1993; Ellis et al., 2021), recent studies showed that variation in background colour can determine the salience of flowers to pollinators. Bukovac et al. (2017) modelled flower salience against more than 500 natural backgrounds and found that background colour has the potential to significantly change a flower's colour contrast. A recent study by Finnell and Koski (2021) showed that bee and fly colour preferences for ultraviolet flower markings depends on the type of background, though this preference difference depended on chromatic and not achromatic properties. The findings from Menzel et al. (1997) that desert plants exhibit lower achromatic contrast than plants in the Mediterranean due to differences in background and not flower colour also suggests that achromatic aspects of backgrounds are important. However, the discrimination between neighbouring flowers presented against one background may be more challenging when these flowers have similar colours. The smaller the chromatic or achromatic difference is, the higher sensitivity is needed.

Studies on European honeybees have found that these flower-visitors can both detect (and discriminate) flowers that exhibit achromatic contrast from a three times larger distance (with a subtended visual angle of 5°) than flowers presenting chromatic contrast (15°), and a similar result is found for the Asian honeybee (see **Table 2** for references). A smaller difference has been observed in bumblebees (Dyer et al., 2008; Wertlen et al., 2008) and stingless bees (Dyer et al., 2016; Jezeera et al., 2021). Honeybees use achromatic and chromatic contrast in similar ways for detection of flowers from the background, for discrimination between differently coloured flowers and for discrimination of flower patterns (Hempel de Ibarra et al., 2000, 2001; Niggebrügge and Hempel de Ibarra, 2003). We therefore conclude that achromatic and chromatic information is used for both flower detection and discrimination. Nevertheless, because the achromatic channel often has a higher spatial resolution (see above) it might be more important in the detection task.

How Do Floral Scattering and Pigmentation Properties Determine the Achromatic Contrast?

Flowers differ from their background in both the chromatic and achromatic aspect of colour (Chittka et al., 1994; Kevan et al., 1996; Menzel et al., 1997; van der Kooi et al., 2019). It is generally assumed that achromatic contrast is determined by the type of pigment (Kevan et al., 1996; Narbona et al., 2021). For example, white and yellow flowers generally exhibit higher achromatic contrast than blue, purple and red flowers. However, flower colour is not only determined by pigments, but also by structures that scatter incident light, such as cell walls, starch granules and the flower's surface (van der Kooi et al., 2016; Stavenga et al., 2020). Flowers generally consist of different layers, such as the epidermis, mesophyll and starch layer, which all scatter part of the incident light. The reflectance of a flower increases when individual layers become more inhomogeneous or when the number of layers increases. How the scattering properties of flowers determine the achromatic contrast is largely unstudied.

To gain a more quantitative understanding of how achromatic contrast varies for different types of pigmentation and the amount of scattering, we deployed our previously devised optical model (Stavenga and van der Kooi, 2016). The optical model is based on the Kubelka-Munk theory for scattering and absorbing media and relies on spectral measurements as input. The model enables to systematically investigate the contribution of different optical properties, such as the amount of scattering or pigmentation (van der Kooi et al., 2016, 2017; van der Kooi and Stavenga, 2019). We used a white, blue, yellow and ultraviolet-reflecting red flower (*Silene latifolia-alba, Nolana paradoxa, Oenothera macrocarpa,* and *Papaver rhoeas, respectively*), systematically varied their scattering coefficient independent from pigmentation properties and calculated the achromatic contrast against a green leaf or blue-sky background (D65, midday). Achromatic contrast was calculated as the von Kries-corrected difference in excitation of the honeybee's long-wavelength (green) photoreceptor between the stimulus and background (**Supplementary Figure 1**), after Spaethe et al. (2001). For details on the modelling and parameter setting, see methods in van der Kooi (2021).

Modelling achromatic contrast as a function of the amount of light scattering revealed clear differences between the type of pigment and backgrounds, though for all modelled cases the response curves are very similar in shape. There is a pattern of diminishing returns for all colours and backgrounds: the strongest changes in achromatic contrast are obtained when the scattering coefficient is low (**Figure 2**). At very high scattering coefficients (>10), the achromatic contrast curve plateaus for all colours and backgrounds. The scattering coefficients found in flowers in nature varies between ~ 1 and 10 (van der Kooi et al., 2016, 2017), presumably due to mechanical constraints associated with flower thickness and interior inhomogeneity, which are the principal factors that determine backscattering. From the modelling (**Figure 2**) it becomes clear that producing flowers with extremely high backscattering also provides little benefit in terms of achromatic contrast and visibility.

Against a green leaf background, white and yellow flowers exhibit higher achromatic contrast than blue and red flowers (**Figures 2A,C**). The achromatic contrast of yellow and white flowers is also comparatively high regardless of the amount of scattering, because their pigments absorb light over a small wavelength range. Achromatic contrast further increases with scattering but plateaus around 0.4, close to the maximum contrast that is theoretically possible (0.5). The modelled blue and red flowers exhibit low achromatic contrast (**Figures 2B,D**), although this also increases with scattering coefficient. Against a blue sky the opposite occurs:

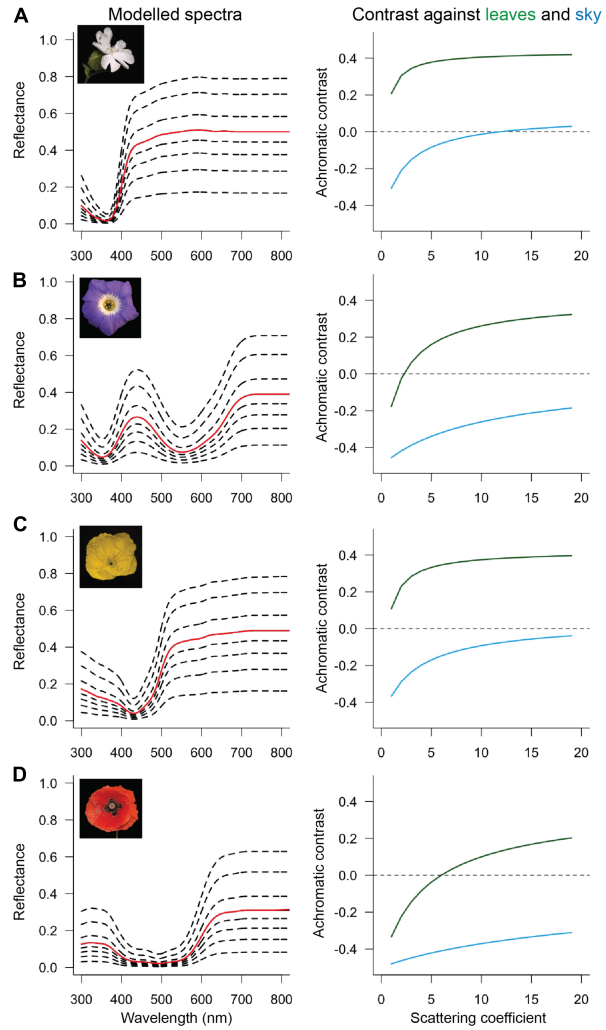

FIGURE 2 | Modelling achromatic contrast for different flower colours and scattering. The left column shows the measured reflectance spectra (red curve) and several modelled cases with higher and lower scattering coefficients (dashed curves). The right column shows the achromatic contrast of different modelled spectra against a green leaf background (green curves) and against a blue sky (blue curves). The measured reflectance is identical to the fifth modelled spectrum, so at scattering coefficient = 5. Although achromatic contrast is normally expressed in absolute values because its direction (positive or negative) is irrelevant, for visualisation purposes we did not normalise contrast values, so contrast is relative to 0. **(A)** *Silene latifolia*, **(B)** *Nolana paradoxa*, **(C)** *Oenothera macrocarpa*, and **(D)** *Papaver rhoeas*. For the background spectra please see **Supplementary Figure 1**.

blue and red flowers exhibit a much higher achromatic contrast than white and yellow flowers, at least with low scattering coefficients, because these colours are comparatively dark against the bright sky. Nevertheless, when flowers are high up a tree or stalk and presented against the sky background, the visual signal for pollinators may also be determined by the transmission instead of the reflection, or a combination of both, which may differ in spectral composition and intensity (van der Kooi et al., 2016).

Our modelling illustrates how flower colour and structure, as well as the type of background determines a flower's achromatic contrast. Overall, the relationship between achromatic contrast and scattering coefficient mimics the response of colour contrast as a function of the amount of pigment (van der Kooi, 2021). For weakly pigmented flowers, an increase in the amount of pigmentation yields a stronger increase in visibility than in flowers that have a moderate amount of pigment (van der Kooi, 2021). Future studies could reveal whether there are trade-offs in visibility via the chromatic versus the achromatic channel, how that translates to different flower pigmentation and anatomy, as well as whether the flower's location on the plant and the type of background are driving factors for the evolution of the optical properties of flowers.

CONCLUDING REMARKS

Research on animal vision traditionally focussed more on the chromatic aspect than the achromatic aspect. Various factors contributed to this bias, e.g., the presumed dichotomy in signal processing between the achromatic and chromatic channel as well as the taxonomic bias toward honeybees as model system. In a changing light environment, chromatic information is more invariant and robust than achromatic information. On the other hand, achromatic cues have a higher signal to noise ratio, particularly in dim light. Presumably therefore, during daytime or in constantly changing illumination conditions, chromatic vision may be more reliable, whereas in dim light, achromatic vision may be more useful than chromatic vision. Our behavioural experiment suggests that naive *M. stellatarum* and *D. elpenor* hawkmoths prefer a higher intensity to a lower intensity stimulus if both stimuli have the same chromatic properties. Various studies tested the detection and discrimination of stimuli with and without achromatic contrast to the background (**Table 2**). The overall picture is that, overall, chromatic contrast largely determines a stimulus' detectability, particularly at short distances; however, achromatic contrast contributes to visibility both physiologically as well as behaviourally. Achromatic information feeds into the colour vision channel (see "Historical Perspective") and it mediates detection of stimuli by bees, flies, butterflies, and moths (**Table 2**). For several species-groups, however, it is still unknown which type of photoreceptor is most important for achromatic signal detection (**Table 1**). Presumably, the use of achromatic cues also plays a role in detecting oviposition sites for herbivorous insects (Prokopy et al., 1975; Blake et al., 2020; Yang et al., 2020).

The finding that achromatic cues are important for flower detection by bees, flies, butterflies and moths has broader relevance for flower colour evolution. Chromatic cues may be most important for diurnal flowers pollinated by bees, butterflies or birds, and achromatic cues may be particularly important for flowers that are pollinated at night. In addition to diurnal and nocturnal hawkmoths that show a preference for stimuli of high intensity (this study), bats use colour-blind rod vision at night (Borges et al., 2016) and thus rely on achromatic visual signals. It has frequently been noted that nocturnal

pollinated flowers are often white or yellow (e.g., Kevan and Baker, 1983), which both appear bright to animals that use the long-wavelength photoreceptor for achromatic vision. Our optical modelling showed that scattering properties crucially determine the achromatic contrast of flowers (section "How Do Floral Scattering and Pigmentation Properties Determine the Achromatic Contrast?"). We therefore propose that not just the pigments, but also the amount of reflected light determines the visibility and brightness of nocturnal pollinated flowers. This means that flowers pollinated at night may evolve structural features that enhance reflectance, e.g., more cell layers or more strongly scattering structures. Further, selective pressures on floral visual signals may be coupled with floral display size. Larger displays will be noticeable from longer distances – and this process is mediated particularly by achromatic vision. Small flowers may thus compensate for their limited visibility via extra reflectance and achromatic contrast. We welcome future experimental studies on pollinator vision and comparative studies on the optical properties of the flowers that they pollinate.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the experiment, wrote this study, and approved the submitted version.

FUNDING

CJvdK was funded by the Dutch Science Foundation (016.Veni.181.025) and by AFOSR/EOARD (FA9550-19-1-7005).

ACKNOWLEDGMENTS

The authors thank Doekele Stavenga and two referees for commenting on an earlier version of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.819436/full#supplementary-material

Supplementary Figure 1 | The blue and green background spectra used for the model calculations.

REFERENCES

An, L., Neimann, A., Eberling, E., Algora, H., Brings, S., and Lunau, K. (2018). The yellow specialist: dronefly *Eristalis tenax* prefers different yellow colours for landing and proboscis extension. *J. Exp. Biol.* 221:jeb184788. doi: 10.1242/jeb.184788

Arikawa, K. (2003). Spectral organization of the eye of a butterfly, *Papilio*. *J. Comp. Physiol. A* 189, 791–800. doi: 10.1007/s00359-003-0454-7

Arikawa, K., Nakatani, Y., Koshitaka, H., and Kinoshita, M. (2021). Foraging small white butterflies, *Pieris rapae*, search flowers using color vision. *Front. Ecol. Evol.* 9:178.

Ausma, T., Bansal, V., Kraaij, M., Verloop, A. C. M., Gasperl, A., Müller, M., et al. (2021). Floral displays suffer from sulphur deprivation. *Environ. Exp. Bot.* 192:104656. doi: 10.1016/j.envexpbot.2021.104656

Balamurali, G. S., Rose, S., Somanathan, H., and Kodandaramaiah, U. (2020). Complex multi-modal sensory integration and context specificity in colour preferences of a pierid butterfly. *J. Exp. Biol.* 223:jeb223271. doi: 10.1242/jeb.223271

Balkenius, A., and Kelber, A. (2004). Colour constancy in diurnal and nocturnal hawkmoths. *J. Exp. Biol.* 207, 3307–3316. doi: 10.1242/jeb.01158

Blake, A. J., Hahn, G. S., Grey, H., Kwok, S. A., McIntosh, D., and Gries, G. (2020). Polarized light sensitivity in *Pieris rapae* is dependent on both color and intensity. *J. Exp. Biol.* 223:jeb220350. doi: 10.1242/jeb.220350

Borges, R. M., Somanathan, H., and Kelber, A. (2016). Patterns and processes in nocturnal and crepuscular pollination services. *Q. Rev. Biol.* 91, 389–418. doi: 10.1086/689481

Bukovac, Z., Shrestha, M., Garcia, J. E., Burd, M., Dorin, A., and Dyer, A. G. (2017). Why background colour matters to bees and flowers. *J. Comp. Physiol. A* 203, 369–380. doi: 10.1007/s00359-017-1175-7

Chittka, L., Faruq, S., Skorupski, P., and Werner, A. (2014). Colour constancy in insects. *J. Comp. Physiol. A* 200, 435–448. doi: 10.1007/s00359-014-0897-z

Chittka, L., Shmida, A., Troje, N., and Menzel, R. (1994). Ultraviolet as a component of flower reflections, and the colour perception of Hymenoptera. *Vision Res.* 34, 1489–1508. doi: 10.1016/0042-6989(94)90151-1

Cronin, T. W., Johnsen, S., Marshall, N. J., and Warrant, E. J. (2014). *Visual ecology*. Princeton: Princeton University Press.

Dyer, A. G., Spaethe, J., and Prack, S. (2008). Comparative psychophysics of bumblebee and honeybee colour discrimination and object detection. *J. Comp. Physiol. A* 194, 617–627. doi: 10.1007/s00359-008-0335-1

Dyer, A. G., Streinzer, M., and Garcia, J. (2016). Flower detection and acuity of the Australian native stingless bee *Tetragonula carbonaria* Sm. *J. Comp. Physiol. A* 202, 629–639. doi: 10.1007/s00359-016-1107-y

Ellis, A. G., Anderson, B., and Kemp, J. E. (2021). Geographic mosaics of fly pollinators with divergent color preferences drive landscape-scale structuring of flower color in daisy communities. *Front. Plant Sci.* 12:617761. doi: 10.3389/fpls.2021.617761

Finnell, L. M., and Koski, M. H. (2021). A test of Sensory Drive in plant–pollinator interactions: heterogeneity in the signalling environment shapes pollinator preference for a floral visual signal. *New Phytol.* 232, 1436–1448. doi: 10.1111/nph.17631

Giurfa, M., and Vorobyev, M. (1998). The angular range of achromatic target detection by honey bees. *J. Comp. Physiol. A* 183, 101–110. doi: 10.1007/s003590050238

Giurfa, M., Vorobyev, M., Brandt, R., Posner, B., and Menzel, R. (1997). Discrimination of coloured stimuli by honeybees: alternative use of achromatic and chromatic signals. *J. Comp. Physiol. A* 180, 235–243. doi: 10.1007/s003590050044

Giurfa, M., Vorobyev, M., Kevan, P., and Menzel, R. (1996). Detection of coloured stimuli by honeybees: minimum visual angles and receptor specific contrasts. *J. Comp. Physiol. A* 178, 699–709.

Goyret, J. (2010). Look and touch: multimodal sensory control of flower inspection movements in the nocturnal hawkmoth *Manduca sexta*. *J. Exp. Biol.* 213, 3676–3682. doi: 10.1242/jeb.045831

Goyret, J., and Kelber, A. (2012). Chromatic signals control proboscis movements during hovering flight in the hummingbird hawkmoth *Macroglossum stellatarum*. *PLoS One* 7:e34629. doi: 10.1371/journal.pone.0034629

Goyret, J., Pfaff, M., Raguso, R. A., and Kelber, A. (2008). Why do *Manduca sexta* feed from white flowers? Innate and learnt colour preferences in a hawkmoth. *Naturwissenschaften* 95, 569–576. doi: 10.1007/s00114-008-0350-7

Hardie, R. C. (1986). The photoreceptor array of the dipteran retina. *Trends Neurosci.* 9, 419–423. doi: 10.1016/0166-2236(86)90136-0

Hempel de Ibarra, N., and Giurfa, M. (2003). Discrimination of closed coloured shapes by honeybees requires only contrast to the long wavelength receptor type. *Anim. Behav.* 66, 903–910. doi: 10.1006/anbe.2003.2269

Hempel de Ibarra, N., Giurfa, M., and Vorobyev, M. (2001). Detection of coloured patterns by honeybees through chromatic and achromatic cues. *J. Comp. Physiol. A* 187, 215–224. doi: 10.1007/s003590100192

Hempel de Ibarra, N., Vorobyev, M., Brandt, R., and Giurfa, M. (2000). Detection of bright and dim colours by honeybees. *J. Exp. Biol.* 203, 3289–3298.

Hempel de Ibarra, N., Vorobyev, M., and Menzel, R. (2014). Mechanisms, functions and ecology of colour vision in the honeybee. *J. Comp. Physiol. A* 200, 411–433. doi: 10.1007/s00359-014-0915-1

Herrera, C. M. (1992). Activity pattern and thermal biology of a day-flying hawkmoth *Macroglossum stellatarum* under Mediterranean summer conditions. *Ecol. Entomol.* 17, 52–56. doi: 10.1111/j.1365-2311.1992.tb01038.x

Jezeera, M., Tichit, P., Balamurali, G. S., Baird, E., Kelber, A., and Somanathan, H. (2021). Spatial resolution and sensitivity of the eyes of the stingless bee, *Tetragonula iridipennis*. *J. Comp. Physiol. A*. Epub online ahead of print. doi: 10.1007/s00359-021-01521-2

Johnsen, S., Kelber, A., Warrant, E., Sweeney, A. M., Widder, E. A., Lee, R. L., et al. (2006). Crepuscular and nocturnal illumination and its effects on color perception by the nocturnal hawkmoth *Deilephila elpenor*. *J. Exp. Biol.* 209, 789–800. doi: 10.1242/jeb.02053

Kelber, A. (2005). Alternative use of chromatic and achromatic cues in a hawkmoth. *Proc. R. Soc. London B* 272, 2143–2147. doi: 10.1098/rspb.2005.3207

Kelber, A., Balkenius, A., and Warrant, E. J. (2002). Scotopic colour vision in nocturnal hawkmoths. *Nature* 419, 922–925. doi: 10.1038/nature01065

Kelber, A., and Henze, M. J. (2013). Colour vision: parallel pathways intersect in *Drosophila*. *Curr. Biol.* 23, R1043–R1045. doi: 10.1016/j.cub.2013.10.025

Kelber, A., and Osorio, D. (2010). From spectral information to animal colour vision: experiments and concepts. *Proc. R. Soc. B* 277, 1617–1625. doi: 10.1098/rspb.2009.2118

Kelber, A., Vorobyev, M., and Osorio, D. (2003). Animal colour vision–behavioural tests and physiological concepts. *Biol. Rev.* 78, 81–118. doi: 10.1017/s1464793102005985

Kemp, D. J., Herberstein, M. E., Fleishman, L. J., Endler, J. A., Bennett, A. T. D., Dyer, A. G., et al. (2015). An integrative framework for the appraisal of coloration in nature. *Am. Nat.* 185, 705–724. doi: 10.1086/681021

Kevan, P., Giurfa, M., and Chittka, L. (1996). Why are there so many and so few white flowers? *Trends Plant Sci.* 1, 252–527. doi: 10.1016/1360-1385(96)20008-1

Kevan, P. G., and Baker, H. G. (1983). Insects as flower visitors and pollinators. *Annu. Rev. Entomol.* 28, 407–453.

Kinoshita, M., Takahashi, Y., and Arikawa, K. (2012). Simultaneous brightness contrast of foraging *Papilio* butterflies. *Proc. R. Soc. London B Biol. Sci.* 279, 1911–1918. doi: 10.1098/rspb.2011.2396

Kinoshita, M., Yamazato, K., and Arikawa, K. (2011). Polarization-based brightness discrimination in the foraging butterfly, *Papilio xuthus*. *Philos. Trans. R. Soc. B Biol. Sci.* 366, 688–696. doi: 10.1098/rstb.2010.0200

Koethe, S., Bossems, J., Dyer, A. G., and Lunau, K. (2016). Colour is more than hue: preferences for compiled colour traits in the stingless bees *Melipona mondury* and *M. quadrifasciata*. *J. Comp. Physiol. A* 202, 615–627. doi: 10.1007/s00359-016-1115-y

Koshitaka, H., Arikawa, K., and Kinoshita, M. (2011). Intensity contrast as a crucial cue for butterfly landing. *J. Comp. Physiol. A* 197, 1105–1112. doi: 10.1007/s00359-011-0671-4

Kuenzinger, W., Kelber, A., Weesner, J., Travis, J., Raguso, R. A., and Goyret, J. (2019). Innate colour preferences of a hawkmoth depend on visual context. *Biol. Lett.* 15:20180886. doi: 10.1098/rsbl.2018.0886

Lehrer, M., and Bischof, S. (1995). Detection of model flowers by honeybees: the role of chromatic and achromatic contrast. *Naturwissenschaften* 82, 145–147.

Lehrer, M., Srinivasan, M. V., and Zhang, S. W. (1990). Visual edge detection in the honeybee and its chromatic properties. *Proc. R. Soc. B* 238, 321–330. doi: 10.1098/rspb.1990.0002

Lunau, K., and Maier, E. J. (1995). Innate colour preferences of flower visitors. *J. Comp. Physiol. A* 177, 1–19.

Martínez-Harms, J., Vorobyev, M., Schorn, J., Shmida, A., Keasar, T., Homberg, U., et al. (2012). Evidence of red sensitive photoreceptors in *Pygopleurus israelitus* (Glaphyridae: Coleoptera) and its implications for beetle pollination in the southeast Mediterranean. *J. Comp. Phys. A* 198, 451–463. doi: 10.1007/s00359-012-0722-5

Meena, A., Kumar, A. M. V., Balamurali, G. S., and Somanathan, H. (2021). Visual detection thresholds in the Asian honeybee, *Apis cerana*. *J. Comp. Physiol. A* 207, 553–560. doi: 10.1007/s00359-021-01496-0

Menzel, R. (1981). Achromatic vision in the honeybee at low light intensities. *J. Comp. Phys. A* 141, 389–393. doi: 10.1007/bf00609941

Menzel, R., Gumbert, A., Kunze, J., Shmida, A., and Vorobyev, M. (1997). Pollinators' strategies in finding flowers. *Isr. J. Plant Sci.* 45, 141–156.

Menzel, R., and Shmida, A. (1993). The ecology of flower colours and the natural colour vision of insect pollinators: the Israeli flora as a study case. *Biol. Rev.* 68, 81–120.

Narbona, E., Del Valle, J. C., Arista, M., Buide, M. L., and Ortiz, P. L. (2021). Major flower pigments originate different colour signals to pollinators. *Front. Ecol. Evol.* 9:743850. doi: 10.3389/fevo.2021.743850

Ng, L., Garcia, J. E., and Dyer, A. G. (2018). Why colour is complex: evidence that bees perceive neither brightness nor green contrast in colour signal processing. *Facets* 3, 800–817.

Niggebrügge, C., and Hempel de Ibarra, N. (2003). Colour-dependent target detection by bees. *J. Comp. Physiol. A* 189, 915–918. doi: 10.1007/s00359-003-0466-3

Nilsson, D.-E. (2021). The diversity of eyes and vision. *Annu. Rev. Vis. Sci.* 7, 19–41. doi: 10.1146/annurev-vision-121820-074736

Osorio, D., and Vorobyev, M. (2005). Photoreceptor sectral sensitivities in terrestrial animals: adaptations for luminance and colour vision. *Proc. R. Soc. London B Biol. Sci.* 272, 1745–1752. doi: 10.1098/rspb.2005.3156

Pagni, M., Haikala, V., Oberhauser, V., Meyer, P. B., Reiff, D. F., and Schnaitmann, C. (2021). Interaction of "chromatic" and "achromatic" circuits in Drosophila color opponent processing. *Curr. Biol.* 31, 1687–1698.e4. doi: 10.1016/j.cub.2021.01.105

Peitsch, D., Fietz, A., Hertel, H., Desouza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based color-vision. *J. Comp. Physiol. A* 170, 23–40. doi: 10.1007/BF00190398

Prokopy, R. J., Economopoulos, A. P., and McFadden, M. W. (1975). Attraction of wild and laboratory-cultured *Dacus oleae* flies to small rectangles of different hues, shades, and tints. *Entomol. Exp. Appl.* 18, 141–152. doi: 10.1111/j.1570-7458.1975.tb02364.x

Reisenman, C. E., and Giurfa, M. (2008). Chromatic and achromatic stimulus discrimination of long wavelength (red) visual stimuli by the honeybee *Apis mellifera*. *Arthropod. Plant. Interact.* 2, 137–146.

Rusanen, J., Frolov, R., Weckström, M., Kinoshita, M., and Arikawa, K. (2018). Non-linear amplification of graded voltage signals in the first-order visual interneurons of the butterfly *Papilio xuthus*. *J. Exp. Biol.* 221:jeb179085. doi: 10.1242/jeb.179085

Satoh, A., Kinoshita, M., and Arikawa, K. (2016). Innate preference and learning of colour in the male cotton bollworm moth, *Helicoverpa armigera*. *J. Exp. Biol.* 219, 3857–3860. doi: 10.1242/jeb.148064

Schnaitmann, C., Garbers, C., Wachtler, T., and Tanimoto, H. (2013). Color discrimination with broadband photoreceptors. *Curr. Biol.* 23, 2375–2382. doi: 10.1016/j.cub.2013.10.037

Somanathan, H., Borges, R. M., Warrant, E. J., and Kelber, A. (2008). Nocturnal bees learn landmark colours in starlight. *Curr. Biol.* 18, R996–R997. doi: 10.1016/j.cub.2008.08.023

Spaethe, J., Streinzer, M., Eckert, J., May, S., and Dyer, A. G. (2014). Behavioural evidence of colour vision in free flying stingless bees. *J. Comp. Physiol. A* 200, 485–496. doi: 10.1007/s00359-014-0886-2

Spaethe, J., Tautz, J., and Chittka, L. (2001). Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proc. Natl. Acad. Sci.* 98, 3898–3903. doi: 10.1073/pnas.071053098

Sprengel, C. K. (1793). *Das Entdeckte Geheimniss der Natur im Bau und in der Befruchtung der Blumen*. Germany: Friedrich Vieweg.

Srinivasan, M. V. (1985). Shouldn't directional movement detection necessarily be "colour-blind"? *Vision Res.* 25, 997–1000. doi: 10.1016/0042-6989(85)90210-x

Srinivasan, M. V., and Lehrer, M. (1988). Spatial acuity of honeybee vision and its spectral properties. *J. Comp. Physiol. A* 162, 159–172. doi: 10.1007/bf00606081

Stavenga, D. G., Staal, M., and van der Kooi, C. J. (2020). Conical epidermal cells cause velvety colouration and enhanced patterning in: *Mandevilla* flowers. *Faraday Discuss.* 223, 98–106. doi: 10.1039/d0fd00055h

Stavenga, D. G., and van der Kooi, C. J. (2016). Coloration of the Chilean Bellflower, *Nolana paradoxa*, interpreted with a scattering and absorbing layer stack model. *Planta* 243, 171–181. doi: 10.1007/s00425-015-2395-0

Stewart, F. J., Kinoshita, M., and Arikawa, K. (2015). The butterfly *Papilio xuthus* detects visual motion using chromatic contrast. *Biol. Lett.* 11:20150687. doi: 10.1098/rsbl.2015.0687

Stöckl, A. L., and Kelber, A. (2019). Fuelling on the wing: sensory ecology of hawkmoth foraging. *J. Comp. Physiol. A* 205, 399–413. doi: 10.1007/s00359-019-01328-2

Streinzer, M., Paulus, H. F., and Spaethe, J. (2009). Floral colour signal increases short-range detectability of a sexually deceptive orchid to its bee pollinator. *J. Exp. Biol.* 212, 1365–1370. doi: 10.1242/jeb.027482

Telles, F. J., Lind, O., Henze, M. J., Rodríguez-Gironés, M. A., Goyret, J., and Kelber, A. (2014). Out of the blue: the spectral sensitivity of hummingbird hawkmoths. *J. Comp. Physiol. A* 200, 537–546. doi: 10.1007/s00359-014-0888-0

Tinbergen, J., and Abeln, R. G. (1983). Spectral sensitivity of the landing blowfly. *J. Comp. Physiol. A* 150, 319–328. doi: 10.1007/bf00605021

Turner, C. H. (1910). Experiments on color-vision of the honey bee. *Biol. Bull.* 19, 257–279. doi: 10.2307/1536088

Ushimaru, A., Rin, I., and Katsuhara, K. R. (2021). Covering and shading by neighbouring plants diminish pollinator visits to and reproductive success of a forest edge specialist dwarf species. *Plant Biol.* 23, 711–718. doi: 10.1111/plb.13267

Van Breugel, F., and Dickinson, M. H. (2012). The visual control of landing and obstacle avoidance in the fruit fly *Drosophila melanogaster*. *J. Exp. Biol.* 215, 1783–1798. doi: 10.1242/jeb.066498

van der Kooi, C. J. (2021). How much pigment should flowers have? Flowers with moderate pigmentation have highest colour contrast. *Front. Ecol. Evol.* 9:731626. doi: 10.3389/fevo.2021.731626

van der Kooi, C. J., Dyer, A. G., Kevan, P. G., and Lunau, K. (2019). Functional significance of the optical properties of flowers for visual signalling. *Ann. Bot.* 123, 263–276. doi: 10.1093/aob/mcy119

van der Kooi, C. J., Elzenga, J. T. M., Dijksterhuis, J., and Stavenga, D. G. (2017). Functional optics of glossy buttercup flowers. *J. R. Soc. Interface* 17:20160933. doi: 10.1098/rsif.2016.0933

van der Kooi, C. J., Elzenga, J. T. M., Staal, M., and Stavenga, D. G. (2016). How to colour a flower: on the optical principles of flower coloration. *Proc. R. Soc. B* 283:20160429. doi: 10.1098/rspb.2016.0429

van der Kooi, C. J., and Stavenga, D. G. (2019). Vividly coloured poppy flowers due to dense pigmentation and strong scattering in thin petals. *J. Comp. Physiol. A* 205, 363–372. doi: 10.1007/s00359-018-01313-1

van der Kooi, C. J., Stavenga, D. G., Belusic, G., Arikawa, K., and Kelber, A. (2021). Evolution of insect color vision: from spectral sensitivity to visual ecology. *Annu. Rev. Entomol.* 66, 435–462. doi: 10.1146/annurev-ento-061720-071644

Vasas, V., Hanley, D., Kevan, P. G., and Chittka, L. (2017). Multispectral images of flowers reveal the adaptive significance of using long-wavelength-sensitive receptors for edge detection in bees. *J. Comp. Physiol. A* 203, 301–311. doi: 10.1007/s00359-017-1156-x

von Frisch, K. (1914). Der Farbensinn und Formensinn der Biene. *Zool. Jahrbücher. Abteilung für Allg. Zool. und Physiol. der Tiere* 35, 1–188.

Wardill, T. J., List, O., Li, X., Dongre, S., McCulloch, M., Ting, C.-Y., et al. (2012). Multiple spectral inputs improve motion discrimination in the *Drosophila* visual system. *Science* 336, 925–931. doi: 10.1126/science.1215317

Werner, A., Menzel, R., and Wehrhahn, C. (1988). Color constancy in the honeybee. *J. Neurosci.* 8, 156–159. doi: 10.1523/JNEUROSCI.08-01-00156.1988

Wertlen, A. M., Niggebrügge, C., Vorobyev, M., and de Ibarra, N. H. (2008). Detection of patches of coloured discs by bees. *J. Exp. Biol.* 211, 2101–2104. doi: 10.1242/jeb.014571

Yang, X., Li, M., Fan, F., An, L., Li, J., and Wei, G. (2020). Brightness mediates oviposition in crepuscular moth, *Grapholita molesta*. *J. Pest Sci.* 93, 1311–1319.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership