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Major Flower Pigments Originate Different Colour Signals to Pollinators

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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

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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

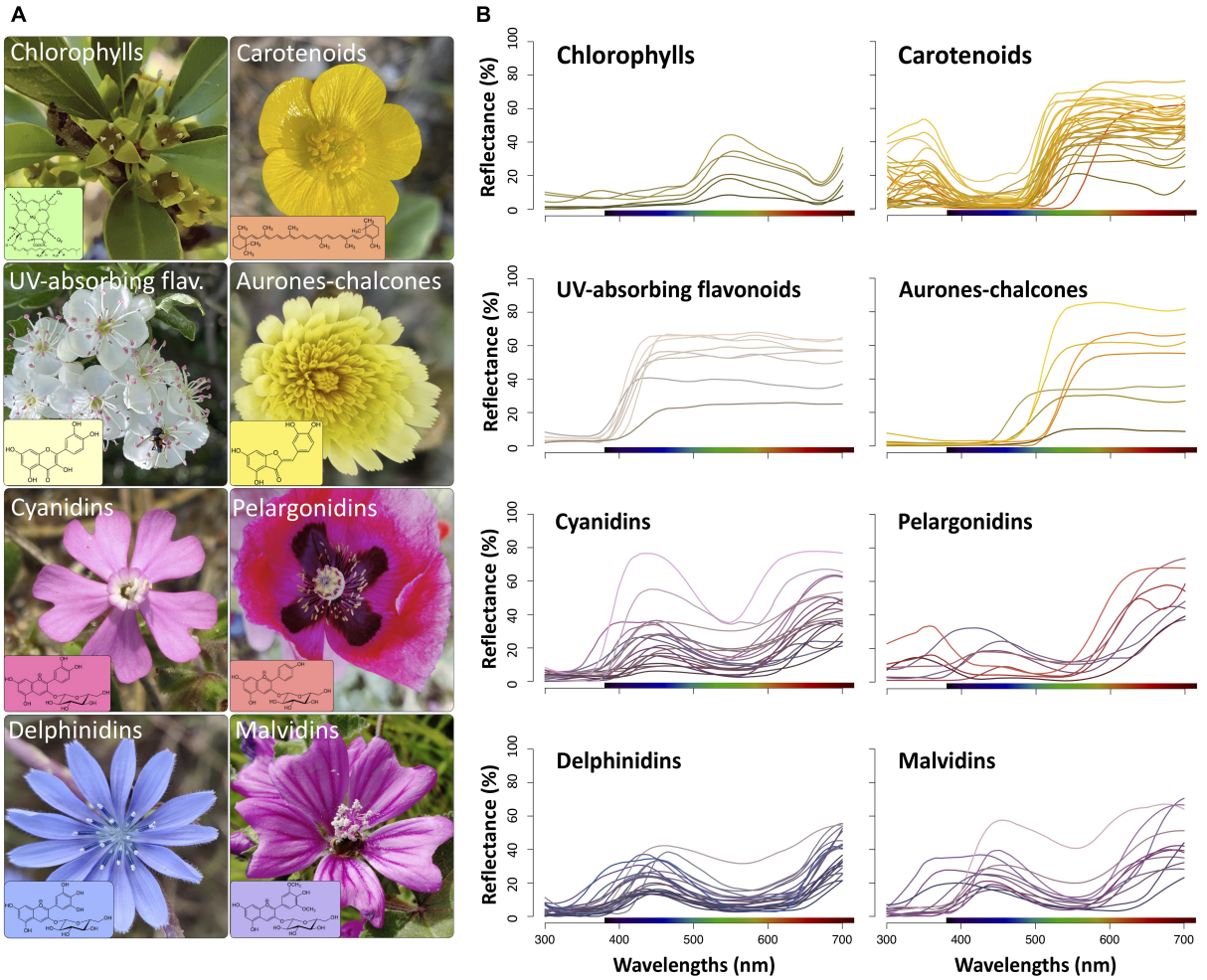

FIGURE 1 | (A) Examples of species with flowers containing one of the main groups of pigments considered in this study and its molecular structures (in the boxes). *Rhamnus lycioides* (Rhamnaceae), chlorophylls; *Ranunculus acris* (Ranunculaceae), carotenoids; *Crataegus monogyna* (Rosaceae), UV-absorbing 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

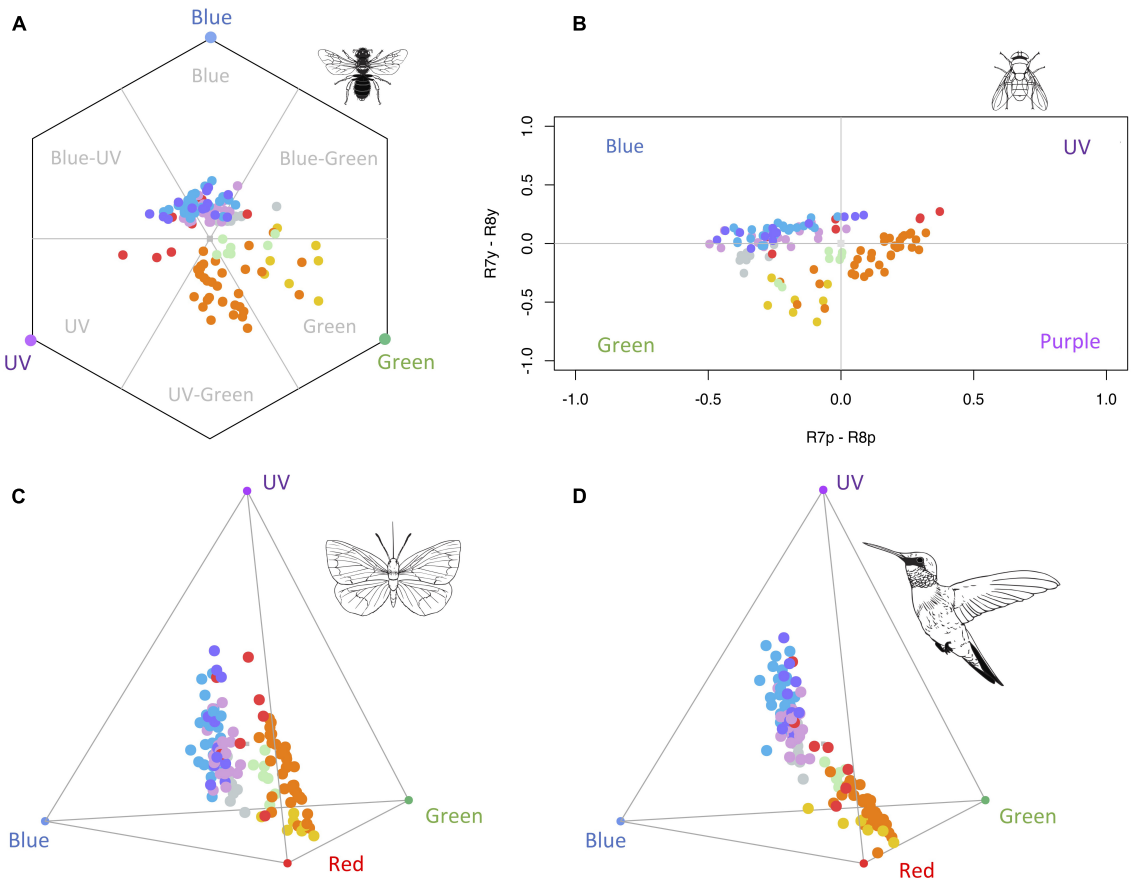

FIGURE 2 | Colour loci of species used in the study according to different colour vision models. The 123 colour loci are represented in the hexagon colour space for the trichromatic vision of bees **(A)**, the categorical colour space for the tetravariant visual system of flies **(B)**, the tetrachromatic 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

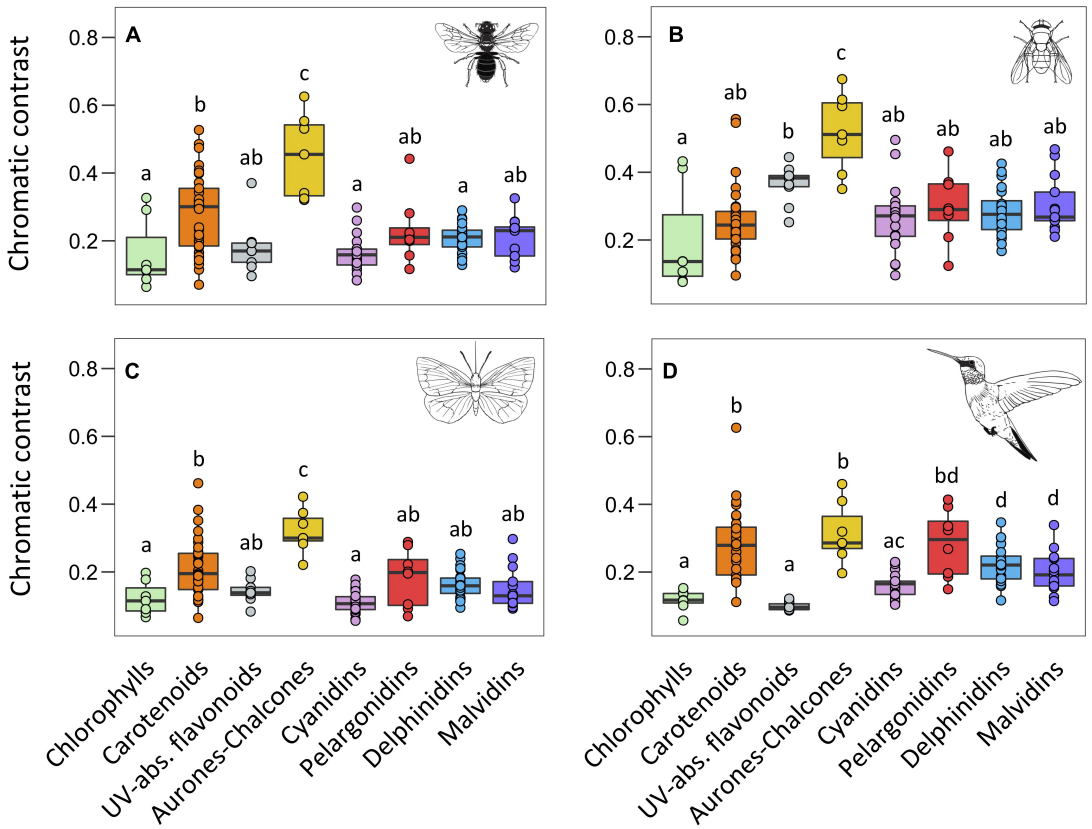

FIGURE 3 | Boxplots representing the distribution of chromatic contrast values for each type of pigment obtained from the vision models of bees **(A)**, flies **(B)**, butterflies **(C)**, and birds **(D)**. The central line displays the median, the bottom and top of the box are the first and third quartiles, 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).

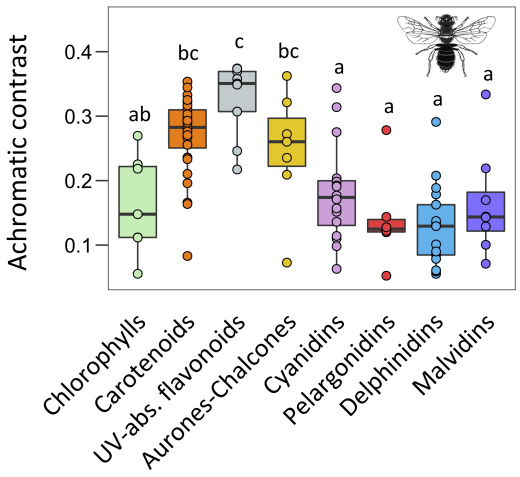

FIGURE 4 | Boxplots representing the distribution of achromatic contrast values for each type of pigment calculated in the hexagonal colour model for bee vision. The central line displays the median, the bottom and top of the box are the first and third quartiles, and dots represent sample values. We 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.

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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).

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