THE ECOLOGY OF PLANT CHEMISTRY AND HOW IT DRIVES MULTI-SPECIES INTERACTIONS

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THE ECOLOGY OF PLANT CHEMISTRY AND HOW IT DRIVES MULTI-SPECIES INTERACTIONS

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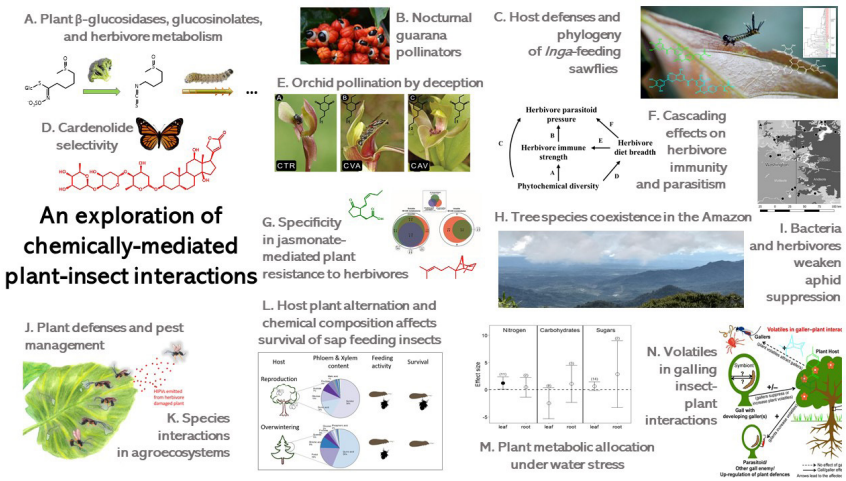

An exploration of chemically-mediated plant-insect interactions. Figures from (A) Vassão et al. (2018) 10.3389/fpls.2018.01389; and Jeschke et al. (2017) 10.3389/fpls.2017.01995. (B) Krug et al. (2018) 10.3389/fpls.2018.01072. (C) Endara et al. (2018) 10.3389/fpls.2018.01237. (D) Petschenka et al. (2018) 10.3389/fpls.2018.01424. (E) Wong et al. (2018) 10.3389/fpls.2018.00839. (F) Slinn et al. (2018) 10.3389/fpls.2018.00656. (G) Schuman et al. (2018) 10.3389/fpls.2018.00787. (H) Vleminckx et al. (2018) 10.3389/fpls.2018.00836. (I) Blubaugh et al. (2018) 10.3389/fpls.2018.01239. (J) Furlong et al. (2018) 10.3389/fpls.2018.01436. (K) Silva et al. (2018) 10.3389/fpls.2018.01713. (L) Gallinger and Gross (2018) 10.3389/fpls.2018.00484. (M) Mundim and Pringle (2018) 10.3389/fpls.2018.00852. (N) Borges (2018) 10.3389/fpls.2018.01139.

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EDITORIAL
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Editorial: The Ecology of Plant Chemistry and How it Drives Multi-Species Interactions

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Keywords: chemical ecology, plant secondary metabolites, plant volatiles, pollination, structure activity analysis, multi-trophic interactions, biodiversity, specificity

Editorial on the Research Topic

The Ecology of Plant Chemistry and How it Drives Multi-Species Interactions

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In this Research Topic issue, we are pleased to present a diverse collection of papers illustrating the importance of chemical ecology for our understanding of ecosystem functioning in both natural and agricultural settings. The field of chemical ecology is quickly advancing as new tools in chemical analyses and molecular biology allow us to better appreciate the degree to which plant chemistry influences interspecific interactions. The papers presented here are an excellent representation of the application of these techniques and demonstrate how plant chemistry shapes interspecific interactions across different scales.

Several papers in this issue represent a field experiencing exciting developments—research on the biochemical mechanisms of the action of defense compounds and their fates within herbivores. Chemical structure is critical to the function of secondary metabolites, even within broadly defined groups of compounds. Monarch butterflies, for example, are shown in fascinating work by Petschenka et al. to have Na^+/K^+-ATPases with broader and overall higher resistance to a suite of cardenolides compared to the enzymes of less specialized herbivores, but the biological effects of cardenolides from *Asclepias* host-plants do not simply reflect their inhibitory activities on monarch enzymes *in vitro*. The effects of different side-chain structures within another class of chemical defenses, glucosinolates, were studied by Jeschke et al. The authors used isotopes to demonstrate that specialist herbivores avoid proper activation of these defenses, while these same compounds were fully activated in generalist herbivores and differentially inhibited their development. Vassão et al. further show that plant glycosidases, including some responsible for the activation of glucosinolates and of a diversity of other plant defenses from a wide variety of species, remain undigested by generalist insects, indicating strong selective pressures on plants to promote the continuous activation of their defense compounds.

A mechanistic understanding of plant defense and of the specificity with which plants respond to different herbivores was addressed by Schuman et al. The authors used transgenic lines of *Nicotiana attenuata* with reduced production of jasmonoyl isoleucine, a jasmonic acid (JA) derivative that regulates many plant responses to herbivory, to test the specificity of plant resistance mechanisms. Elicitation by JA and its derivatives allows for plasticity in direct and indirect responses of plants to damage, as indicated by metabolomic analyses of volatiles and water-soluble compounds from experimentally treated plants. Moreover, a meta-analysis in this issue (Mundim and Pringle) examines above- and belowground changes in primary and secondary metabolites in response to water stress and herbivory, addressing long-debated questions

about growth-defense trade-offs with the fine-grained detail offered by metabolomics. They further suggest more realistic studies of the effects of multiple, co-occurring stressors on plants are needed.

Plant volatiles are deservedly highlighted in this issue, reflecting their considerable interest to chemical ecology as we continue to learn about the roles they play as the first line of communication between plants and insects. Borges discusses research on volatiles and galling insects, stressing that the topic is understudied and requires further attention. New discoveries in pollination biology are brought to light by Wong et al. and Wong et al. whose careful study of orchid mimicry of female thynnine wasp sex pheromones demonstrates how developmental and tissue-specific transcriptional changes in orchid flowers allow orchids to successfully lure male pollinators to their flowers at just the right time. Suggestions for studies on the evolution and biochemical production of orchid scents used in deceptive pollination that can be applied to other non-model organisms are also presented by Wong et al. In addition, a study describing guarana flower pollination by Krug et al. shows that nocturnal bees are attracted by volatile blends that differ in composition from those released during the day.

At a larger scale, our understanding of tropical diversity is increasingly improved by chemical ecology, and some excellent papers in this issue demonstrate the importance of plant chemical diversity for tropical forest diversity. Vleminckx et al. show that secondary metabolites are less similar than expected by chance in co-occurring congeners, suggesting herbivory selects for chemical diversity. Endara et al. further show that a phylogeny of specialist sawflies matches plant chemotypes more closely than host plant phylogeny. The response of the third trophic level to chemical diversity was analyzed by Slinn et al. These authors used an invaluable 19-year-long database to determine that phytochemical diversity affects larval immunity against parasitoids in specialist but not generalist herbivores. This result differed between two tropical forests, however, and the authors open the door to further questions about the scale of these patterns and how the degree of herbivore specialization affects them.

Several of the articles in this edition highlight the value of considering chemically mediated interactions in applied arenas and indicate directions for future research that examines chemical ecology and tri-trophic interactions in complex agroecosystems. Addressing applications of chemical ecology to agriculture from the bottom-up, Blubaugh et al. report that rhizobium-inducing soil bacteria affect aphid growth and parasitism, underscoring the complexity of chemically-mediated trophic interactions. Furlong et al. and Silva et al. both discuss the role plant volatiles could play in improving agriculture. Silva et al. note that volatiles should receive more attention in studies targeting pollination and biocontrol, while Furlong et al. suggest that the failure to successfully translate laboratory tests of volatiles that attract parasitoids to field-level applications for improved biocontrol is due to the complexity of these interactions and the ability of parasitoids to discern between reliable and unreliable signals. Lastly, herbivore host-use is analyzed by Gallinger and Gross who show that psyllid agricultural pests that host-switch during their life cycle from fruit crops to pines are able to feed on but not complete their development on pines due to their phytochemistry.

We are excited to share this representative selection of fascinating work in the growing body of research linking plant chemistry to the ecology and evolution of multi-trophic interactions.

We hope you will enjoy these articles and that they will inspire further advances in the study of chemical ecology.

AUTHOR CONTRIBUTIONS

MK coordinated the editing for the Research Topic, contributed to the proposal of the Research Topic subject, and edited manuscripts. TM, DV, MS, and LY contributed to the proposal of the Research Topic subject and edited manuscripts.

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ORIGINAL RESEARCH
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Relative Selectivity of Plant Cardenolides for Na^+/K^+-ATPases From the Monarch Butterfly and Non-resistant Insects

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A major prediction of coevolutionary theory is that plants may target particular herbivores with secondary compounds that are selectively defensive. The highly specialized monarch butterfly (*Danaus plexippus*) copes well with cardiac glycosides (inhibitors of animal Na^+/K^+-ATPases) from its milkweed host plants, but selective inhibition of its Na^+/K^+-ATPase by different compounds has not been previously tested. We applied 17 cardiac glycosides to the *D. plexippus*-Na^+/K^+-ATPase and to the more susceptible Na^+/K^+-ATPases of two non-adapted insects (*Euploea core* and *Schistocerca gregaria*). Structural features (e.g., sugar residues) predicted *in vitro* inhibitory activity and comparison of insect Na^+/K^+-ATPases revealed that the monarch has evolved a highly resistant enzyme overall. Nonetheless, we found evidence for relative selectivity of individual cardiac glycosides reaching from 4- to 94-fold differences of inhibition between non-adapted Na^+/K^+-ATPase and *D. plexippus*-Na^+/K^+-ATPase. This toxin receptor specificity suggests a mechanism how plants could target herbivores selectively and thus provides a strong basis for pairwise coevolutionary interactions between plants and herbivorous insects.

Keywords: monarch butterfly, Na^+/K^+-ATPase, cardenolide, cardiac glycoside, phytochemical diversity, structure–activity relationship, toxin–receptor interaction, resistance

INTRODUCTION

It is widely recognized that coevolution between plants and herbivores occurs in a community context (Agrawal, 2005; Johnson and Stinchcombe, 2007; Poelman and Kessler, 2016), but the mechanisms and consequences of such complex interactions are unclear. Most plants must defend against multiple herbivores, often from different feeding guilds (Maddox and Root, 1990; Ali and Agrawal, 2014), which poses a challenge for the plant. In some cases, a single defense trait may be effective against a multitude of herbivores. Alternatively, different defenses may defend against alternative herbivores (Linhart and Thompson, 1999; Züst et al., 2012). However, even for relatively well-characterized types of defenses such as certain classes of plant toxins, it is largely unclear

whether distinct chemical forms have selective biological activities against different herbivores (Kim and Jander, 2007).

The extend of phytochemical diversity, even within a single compound class and in an individual plant, has long bewildered chemical ecologists (Berenbaum et al., 1991; Jones and Firn, 1991; Berenbaum and Zangerl, 1996; Richards et al., 2015). One potential explanation for this diversity is compound selectivity, that is, individual plant compounds are targeted at distinct herbivores. Selectivity in plant defense has mainly been demonstrated ecologically for induced plant defenses (Agrawal, 2000), and is often driven by trade-offs between signaling cascades and insect feeding guilds (Ali and Agrawal, 2012; Erb et al., 2012; Castillo et al., 2014). Across populations, coevolutionary studies have found some evidence that different herbivores can select for specific defensive traits (Mithen et al., 1995; Züst et al., 2012; Castillo et al., 2014) and it was demonstrated that selective interactions between plant defensive compounds and herbivores exist, i.e., that the same substance can have different effects on different herbivores (Ayres et al., 1997; Linhart and Thompson, 1999). Nonetheless, the underlying mechanisms of such interactions have never been revealed and previous work on natural plant–herbivore interactions has not identified selective toxin–target site interactions. As a first step to address the potential for coevolution between species at the interface of toxins and receptors, one must demonstrate that individual toxins act selectively on targets from different species, i.e., a specific plant toxin is affecting one herbivore's physiological target relatively stronger or weaker compared to that of another herbivore species.

In this study, we used the specific interaction of cardenolide toxins from plants and insect Na^+/K^+-ATPases as a model to test for structure–activity relationships as well as selective interactions as a potential mechanism involved in insect plant coevolution. Due to the ease of interpreting the directly quantified inhibition of Na^+/K^+-ATPases *in vitro*, the use of structurally diverse plant toxins which act on the same insect target site represents an unprecedented opportunity to test a set of mechanistic hypotheses. Cardenolides are classic plant toxins in the context of multi-trophic interactions and form a structurally diverse group of compounds which are found in at least 12 plant families (Agrawal et al., 2012). They consist of a steroid core linked to a five-membered lactone ring at C17 and exist either as glycosides or as free genins (Malcolm, 1991). Cardenolides, together with the bufadienolides which carry a six-membered lactone ring, have been termed cardiac glycosides, a name based on their pronounced action on the human heart. In their glycosidic form, one or more sugar molecules bind to position C3. Cardiac glycosides are specific inhibitors of the ubiquitous animal enzyme Na^+/K^+-ATPase, which maintains the cellular membrane potential (Yatime et al., 2011).

Cardenolide-producing plants are characterized by the production of diverse cardenolide structures, with foxglove known to produce >100 distinct forms (Luckner and Wichtl, 2000) and many milkweeds producing >20 compounds within individuals of a single species (Agrawal et al., 2012). Comparative studies of structure–activity relationships between cardenolides and the Na^+/K^+-ATPase have revealed substantial variation, but have been carried out only in the biomedical context of the vertebrate Na^+/K^+-ATPases (Schönfeld et al., 1985; Farr et al., 2002; Paula et al., 2005). Although cardenolides have attracted attention as plant defense compounds against herbivorous insects (Agrawal and Fishbein, 2008; Rasmann et al., 2009), the effect of structural variation on insect Na^+/K^+-ATPases has not been previously investigated.

Despite the potent toxicity of cardenolides, many insects have colonized cardenolide-producing plants (Agrawal, 2017) and have evolved mechanisms to protect their Na^+/K^+-ATPase from these dietary toxins. Caterpillars of the monarch butterfly (*Danaus plexippus*), for example, are specialized to feed on milkweeds (*Asclepias* spp., Apocynaceae) and sequester plant cardenolides into their hemocoel to store them as a defense against predators (Brower et al., 1967; Reichstein et al., 1968). The monarch butterfly Na^+/K^+-ATPase is highly resistant to the standard cardenolide ouabain (Vaughan and Jungreis, 1977), and this resistance is conferred by two amino acid substitutions (L111V and N122H, Holzinger et al., 1992; Aardema et al., 2012). Such target site insensitivity has evolved convergently in at least five insect orders that use cardiac glycoside-producing plants as hosts (Al-Robai et al., 1990; Dobler et al., 2012, 2015; Zhen et al., 2012; Petschenka et al., 2017). Nonetheless, basal milkweed butterfly species in the Danaini, which are adapted to feed on cardenolide-containing plants, maintain a sensitive Na^+/K^+-ATPase and have evolved alternative mechanisms to cope with cardenolides (Petschenka et al., 2013a; Petschenka and Agrawal, 2015).

Although monarchs are exposed to a great variety of cardenolides naturally, all previous research on the monarch butterfly Na^+/K^+-ATPase is based on the non-milkweed cardenolide ouabain, and no other cardenolide has previously been applied to an insect Na^+/K^+-ATPase (Holzinger et al., 1992; Vaughan and Jungreis, 1977; Petschenka et al., 2012). To test the prediction that structurally diverse compounds exert differential pharmacological activities and also may target different herbivore species selectively, here we report on studies where we applied 16 different cardenolides and one bufadienolide to the Na^+/K^+-ATPase of the monarch butterfly, a closely related basal milkweed butterfly *Euploea core*, as well as to the Na^+/K^+-ATPase of an insect not adapted to dietary cardiac glycosides (the desert locust, *Schistocerca gregaria*). *S. gregaria* Na^+/K^+-ATPase likely represents ancestral insect Na^+/K^+-ATPase, *E. core*, also maintains a highly sensitive Na^+/K^+-ATPase (Petschenka and Agrawal, 2015), and as discussed above, monarchs possess a highly resistant form.

In addition to 11 commercially available cardenolides from non-milkweed sources (which have *cis–trans–cis* configuration of A/B, B/C, and C/D rings), we also used five *Asclepias* cardenolides (*trans–trans–cis* configuration) including calactin and calotropin, which are preferentially sequestered by monarchs (Cheung et al., 1988; Groeneveld et al., 1990). We further tested Na^+/K^+-ATPase inhibition by eight pregnane glycosides, related steroids occurring alongside cardenolides in *Asclepias* (Araya et al., 2012a) and also *Digitalis* (Teuscher and Lindequist, 2010), but with unknown ecological function. Specifically, we tested if: (1) structural variation of cardenolides causes differences in

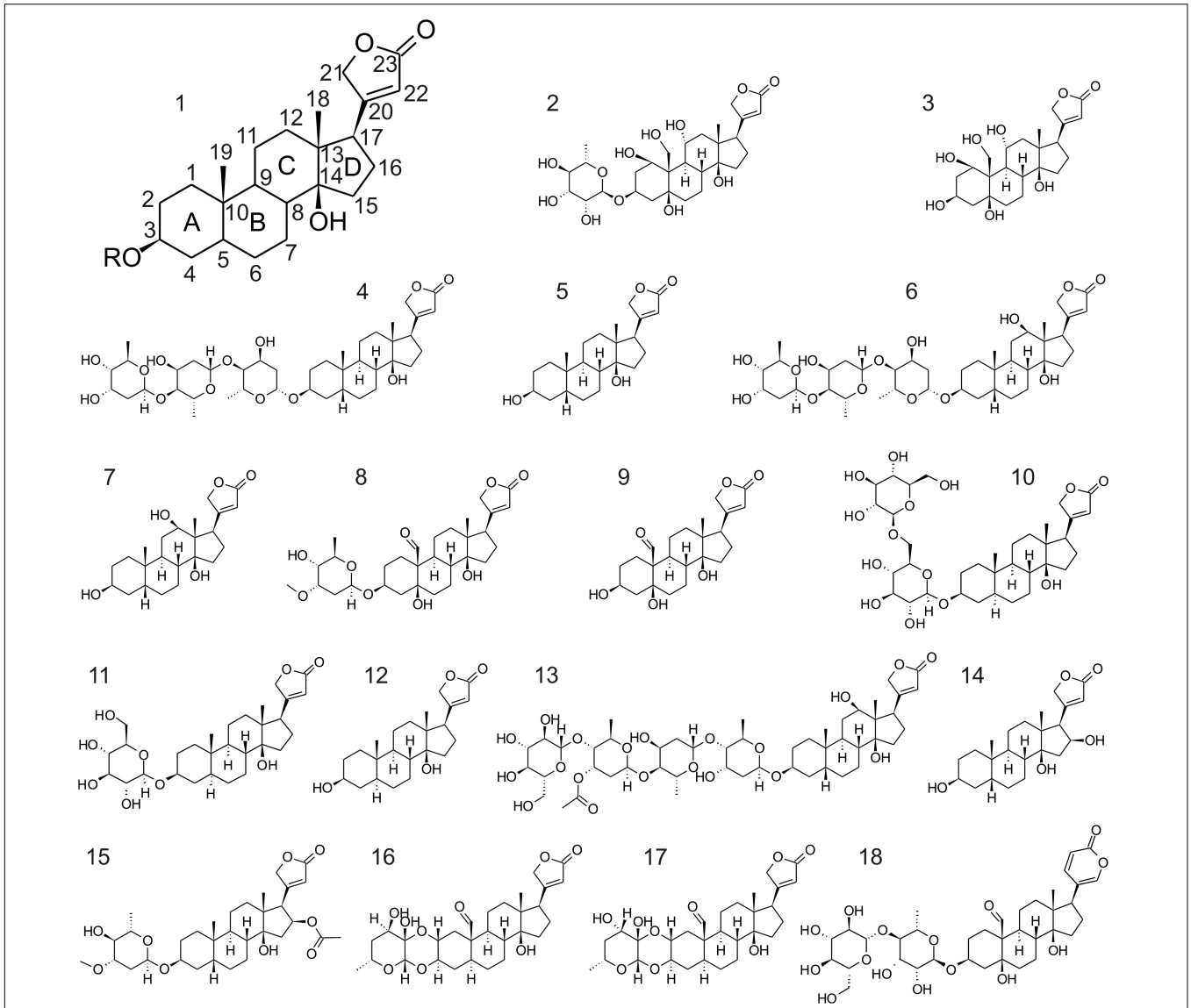

FIGURE 1 | Structural formulas of the cardiac glycosides used in this study. 1, structure of the genin of cardiac glycosides of the cardenolide type (bufadienolides have a six-membered lactone ring as present in 18); 2, ouabain; 3, ouabagenin; 4, digitoxin; 5, digitoxigenin; 6, digoxin; 7, digoxigenin; 8, cymarin; 9, strophanthidin; 10, uzarin; 11, desglucouzarin; 12, uzarigenin; 13, lanatoside C; 14, gitoxigenin; 15, oleandrin; 16, calactin; 17, calotropin; 18, hellebrin.

inhibition of insect Na^+/K^+-ATPases, (2) the sugar moiety of cardenolides influences their pharmacodynamic properties, (3) 5β-cardenolides act differently from 5α-cardenolides, the latter of which are typical for *Asclepias* and related genera, (4) individual cardenolides interact selectively with different insect Na^+/K^+-ATPases, and (5) if pregnane glycosides have any inhibitory effect on insect Na^+/K^+-ATPase.

MATERIALS AND METHODS

Cardenolides and Bufadienolides

Most cardiac glycosides used in this study (**Figure 1**) were purchased commercially. Digitoxigenin, digitoxin, digoxigenin, digoxin, gitoxigenin, lanatoside C, ouabagenin, ouabain, and strophanthidin were from Sigma-Aldrich (Taufkirchen, Germany), oleandrin and cymarin were from Phytolab (Vestenbergsgreuth, Germany), uzarigenin from Chem Faces (Wuhan, China), hellebrin from Enzo Life Sciences (Lörrach, Germany), and adynerin from Chromadex (LGC Standards, Wesel, Germany). Uzarin, desglucouzarin as well as the eight pregnane glycosides (verticilloside A-H) were purified from *Asclepias syriaca* and *A. verticillata* previously (Araya et al., 2012a,b).

Calactin and calotropin were purified from caterpillars of *D. plexippus* raised on *A. curassavica* in the course of this study. For this purpose, 15 caterpillars (fifth instar) were frozen in liquid N_2, freeze-dried, and subsequently dissected to remove

gut contents. Dissected tissues were pooled and dried at 50°C overnight in a drying oven. Roughly 1 g of material was extracted with methanol (3 ml × 50 ml) and combined extracts were filtered. After addition of 50 ml deionized water, the solution was extracted with petrol ether (8 ml × 50 ml). Due to poor phase separation, we added additional 50 ml of water and centrifuged. The next day, the recovered methanol–water layer was concentrated in a rotary evaporator until evaporation was very low at 200 mbar (water bath at 60°C). The remaining aqueous phase was extracted five times with ca. 30 ml chloroform, dried with Na_2SO_4, filtered, and then evaporated to dryness. The sample was dissolved in methanol and subjected to semi-preparative HPLC. Seventy-five microliters of the extract were injected into an Agilent 1100 series HPLC and compounds were separated on a Nucleodur C18 reversed phase column (5 μm, 250 mm × 10 mm, Macherey-Nagel, Düren, Germany). Cardenolides were eluted on a constant flow of 3 ml/min with an acetonitrile–H_2O gradient as follows: 0–2 min 16% acetonitrile, 10 min 30% acetonitrile, 25 min 50% acetonitrile, 30 min 50% acetonitrile, and 10 min reconditioning at 1% acetonitrile. UV absorbance spectra were recorded from 200 to 400 nm by a diode array detector. Peaks with symmetrical absorption maxima between 218 and 220 nm at 22.4 and 24.3 min retention time were collected individually. Acetonitrile–water mixtures were dried down to provide crystalline cardenolides. The structural identity of calactin and calotropin was validated by NMR spectroscopy.

In vitro Inhibition Assay of Insect Na^+/K^+-ATPase

Danaus plexippus was caught in the field or purchased; *E. core* and *S. gregaria* were obtained commercially, with additional *E. core* from a laboratory colony. Na^+/K^+-ATPase extractions from butterfly brains or locust recta were prepared as described previously (Petschenka et al., 2013a). Briefly, brains dissected from butterflies stored at −80°C or recta from locusts were homogenized (individually or pooled) with an all glass grinder (Wheaton) in deionized water, frozen at −80°C, freeze-dried, and stored frozen until use. For *in vitro* assays, lyophilisates were dissolved in water, sonicated in an ultrasonic bath, and centrifuged to remove undissolved matter. All cardiac glycosides were tested at least with three biological replicates of Na^+/K^+-ATPase (i.e., Na^+/K^+-ATPase from genetically different specimens or pools of specimens). For pregnane glycosides, only one biological replicate was collected due to the general lack of pronounced effects.

For estimating the effects of ouabain, ouabagenin, digitoxin, digitoxigenin, digoxin, digoxigenin, cymarin, strophanthidin, lanatoside C, gitoxigenin, oleandrin, and hellebrin on Na^+/K^+-ATPase of *D. plexippus* and *S. gregaria*, all Na^+/K^+-ATPase inhibition assays were carried out in duplicate (i.e., three biological and six technical replicates per cardiac glycoside tested). Means of duplicate determinations (i.e., technical replicates) were used as data points. In consequence, each data point of a dose–response curve is the mean of at least three biological replicates based on two technical replicates, each. In a few cases (digitoxigenin, lanatoside C, oleandrin, and hellebrin on *D. plexippus*-Na^+/K^+-ATPase) more than six technical replicates were collected. Here, technical replicates collected on the same 96-well plate were averaged and used as data points. According to our previous work on cardiac glycoside resistance of Na^+/K^+-ATPase from different insect orders, we do not expect intraspecific variation. Therefore, all variation observed within a species should represent technical noise and discrimination between biological and technical replication will not influence the results. Technical replication was omitted for determination of the effects of uzarin, desglucouzarin, uzarigenin, digitoxigenin, calactin, and calotropin on *D. plexippus* and *E. core*-Na^+/K^+-ATPase.

Na^+/K^+-ATPase activity was quantified by photometric determination of inorganic phosphate released from enzymatic ATP hydrolysis compared to a non-inhibited control (Petschenka et al., 2013a). The sensitive insect Na^+/K^+-ATPases of *S. gregaria* and *E. core* showed no differences based on inhibition by ouabain ($F_{2,7}$ = 0.29, p = 0.75) and digitoxigenin ($F_{2,6}$ = 3.29, p = 0.11). As both enzymes also are identical at positions 111 and 122 (S. Dobler, personal communication, April 2017) known to be critical for cardenolide resistance, results from both Na^+/K^+-ATPases were combined for some analyses (**Figures 2** and **3**; **Supplementary Figures S3–S5**). For **Figure 2** and **Supplementary Figures S3–S5**, the IC_{50} values for digitoxigenin and ouabain obtained with *S. gregaria*-Na^+/K^+-ATPase were used.

Preparation of Inhibitors (Cardiac Glycosides and Pregnane Glycosides)

Inhibitors were typically dissolved in 10% dimethyl sulphoxide (DMSO) in water resulting in a final concentration of 2% DMSO in the assay. For the 5α-cardenolide uzarigenin, we used 25% DMSO in water (final concentration in the assay 5%) as we observed at least three to four times lower solubility of uzarigenin compared to its 5-β-isomer digitoxigenin under ambient conditions. For the comparison of uzarigenin and digitoxigenin, the digitoxigenin was also dissolved in 25% DMSO in water. Inhibitor concentrations typically ranged from 10^{-4} to 10^{-8} M with the exception of digitoxin, oleandrin, and uzarigenin whose solubility was too low to achieve 10^{-4} M in our solvent system. Stock solutions of cardiac glycosides, as well as pregnane glycosides, were prepared from crystalline compounds in DMSO. While concentrations of all other compounds were based on mass, calactin, and calotropin stock solutions were adjusted with HPLC using digitoxin as a reference. Diluted inhibitor solutions of uzarin, desglucouzarin, calactin, and calotropin (5×10^{-4} and 5×10^{-5} M) were additionally surveyed with HPLC (using digitoxin as a reference).

In vitro Inhibition Assay of Porcine Na^+/K^+-ATPase

For comparison with insect Na^+/K^+-ATPase, we also tested inhibition of the cardenolides ouabain, desglucouzarin, and uzarin on the porcine Na^+/K^+-ATPase (Sigma-Aldrich, St. Louis, MO, United States) which was purified from pig brain. Lyophilized Na^+/K^+-ATPase was dissolved in water to a

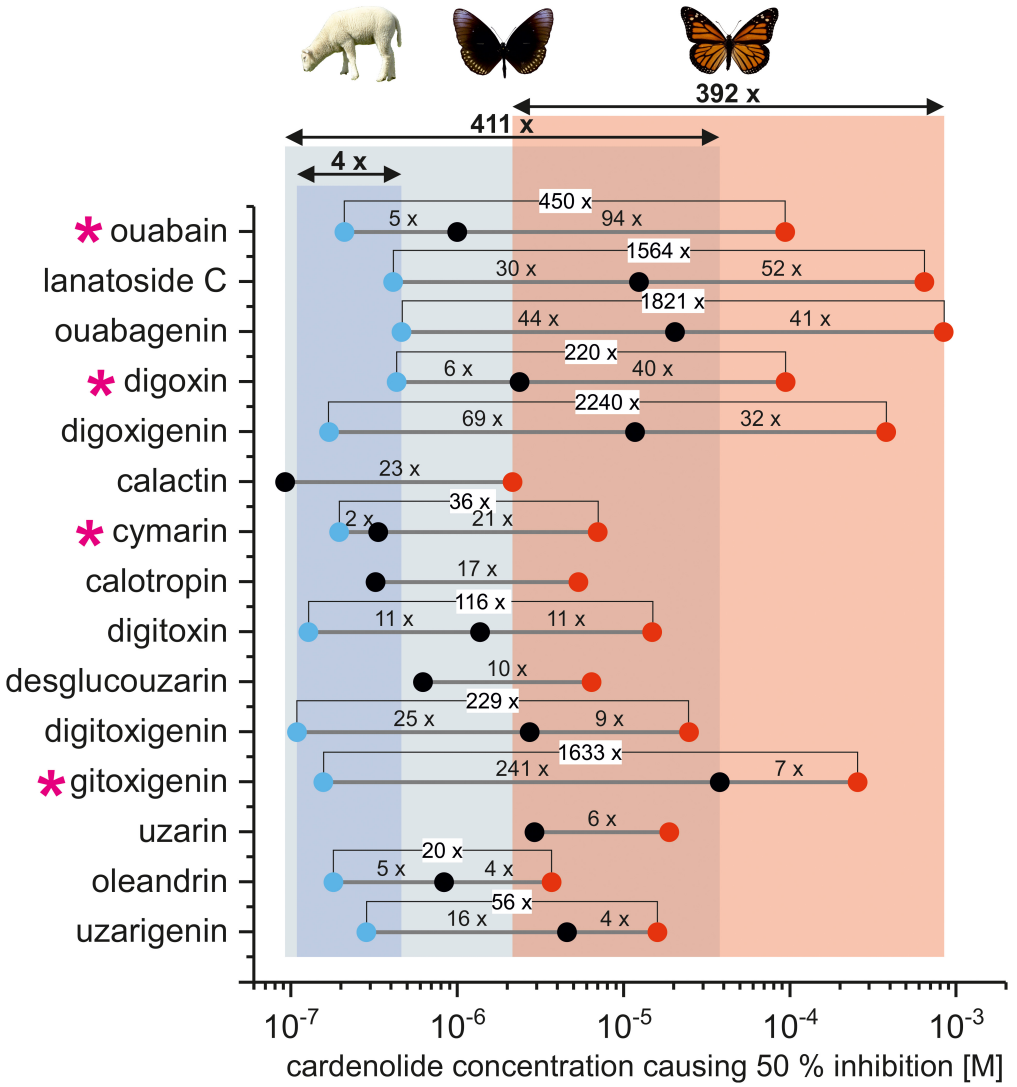

FIGURE 2 | IC_{50} values of individual cardiac glycosides applied to lamb [blue dots, based on (Paula et al., 2005)], non-adapted (black dots), and adapted insect Na^+/K^+-ATPase (red dots). Numbers above gray lines indicate the fold difference between the IC_{50} values of individual cardiac glycosides for all three forms of Na^+/K^+-ATPase. The overall variation of IC_{50} values for individual cardenolides across each form of Na^+/K^+-ATPase is indicated by columns (blue = lamb, gray = *E. core/S. gregaria*, and red = *D. plexippus*) and double arrows (numbers above arrows indicate fold differences). Pink asterisks highlight cardenolides which show especially high discrimination between lamb, non-adapted, and adapted insect Na^+/K^+-ATPase. Note that for calactin, calotropin, desglucouzarin, and uzarin no data for lamb Na^+/K^+-ATPase were available.

concentration of 1 U/ml, stored at −80°C in single-use aliquots and diluted with H_2O to 0.05 U/ml for use in the *in vitro* assay (final concentration in the assay 0.01 U/ml). The data on inhibition of porcine Na^+/K^+-ATPase are presented in **Supplementary Figure S7** and **Supplementary Table S1**.

Data Evaluation

Non-linear curve fitting of dose–response curves was carried out using OriginPro 2016 (OriginLab Corporation, Northampton, MA, United States) with top and bottom asymptotes set to 100 and 0, respectively. Pairwise statistical comparisons of dose-response curves were carried out using the "compare datasets" option in OriginPro, which is based on an *F*-Test evaluating if the two curves are identical. Correlation analyses using log10-transformed IC_{50} values was also carried out with OriginPro. To illustrate selective effects of individual cardiac glycosides on different forms of Na^+/K^+-ATPase (adapted or non-adapted), inhibition was compared to a reference cardenolide (**Supplementary Figures S3**, **S4**). For this purpose, we computed the log10 of the ratio (IC_{50} reference compound)/(IC_{50} test compound). We used ouabain as a reference as it is the most widely used cardenolide in research on Na^+/K^+-ATPase (Paula et al., 2005). This procedure results in negative values for compounds inhibiting Na^+/K^+-ATPase stronger than ouabain and positive values for compounds inhibiting Na^+/K^+-ATPase weaker than ouabain. The results of these comparisons strongly

depend on the cardiac glycoside used as a reference. Therefore, in addition to ouabain, we used one of the least toxic cardiac glycosides for both forms of Na^+/K^+-ATPase (ouabagenin), an intermediate inhibitor (digitoxigenin), and the most toxic cardiac glycoside (calactin) as reference toxins (**Supplementary Figure S4**). To analyze relative selectivity without a reference, we also plotted the fold differences between the IC_{50} values of individual cardenolides for non-adapted and adapted insect Na^+/K^+-ATPase and compared them to mammalian (lamb) Na^+/K^+-ATPase (**Figure 2**). IC_{50} values for lamb Na^+/K^+-ATPase were inferred from the study of Paula et al. (2005). We excluded seven outliers out of 1,700 absorbance measurements which were clearly due to technical errors. The data reported in this paper are available from the Dryad Digital Repository (Petschenka et al., 2018).

RESULTS

Structural Variation Causes Differential Inhibition of Adapted and Non-adapted Na^+/K^+-ATPases

Pairwise comparisons of all cardenolide glycosides (**Figure 1**) applied to both the monarch- and one of the non-adapted insect Na^+/K^+-ATPases (*S. gregaria* or *E. core*) revealed that the *D. plexippus*-Na^+/K^+-ATPase was 4- to 94-fold more resistant (based on IC_{50} values, **Figure 2**, please see **Supplementary Table S1** for a complete list of IC_{50} values), and the level of enhanced resistance depended strongly on the specific compound tested (**Figure 2** and see below).

When effects of the same cardenolides were compared on Na^+/K^+-ATPases from a vertebrate species (lamb, Paula et al., 2005), *S. gregaria* or *E. core*, and monarchs, we observed much less variation (fourfold) in the IC_{50} values of the more sensitive mammalian enzyme compared to either of the insect Na^+/K^+-ATPases (>100-fold variation, **Figure 2**). Inclusion of additional cardenolides for which no data on lamb Na^+/K^+-ATPase were available revealed roughly 400-fold variation in IC_{50} values (**Figure 2**) for both insect Na^+/K^+-ATPases.

Overall, the IC_{50} values ranged from 8.42×10^{-7} (hellebrin) to 8.41×10^{-4} M (ouabagenin) for the *D. plexippus*-Na^+/K^+-ATPase and from 9.20×10^{-8} M (calactin) to 3.78×10^{-5} M (gitoxigenin) for non-adapted insect-Na^+/K^+-ATPases (**Supplementary Table S1** and **Figure 2**). Although the bufadienolide hellebrin was the most toxic cardiac glycoside for the *D. plexippus*-Na^+/K^+-ATPase overall, the most toxic cardenolide for both Na^+/K^+-ATPases tested was calactin (IC_{50} *E. core*-Na^+/K^+-ATPase = 9.20×10^{-8} M, IC_{50} *D. plexippus*-Na^+/K^+-ATPase = 2.15×10^{-6} M). The eight pregnanes applied on *E. core*- and *D. plexippus*-Na^+/K^+-ATPase (**Supplementary Figure S1**) resulted in only slight inhibition (ca. 90% remaining activity at 10^{-4} M in both species) which is in agreement with an observed lack of activity on porcine Na^+/K^+-ATPase (Komarnytsky et al., 2013). Furthermore, there was no difference in the effect of pregnanes between the Na^+/K^+-ATPases of *E. core* and *D. plexippus* (**Supplementary Figure S1**). One cardenolide, adynerin, which is known to lack cardiotonic activity in vertebrates (Imai et al., 1965) also failed to inhibit the *S. gregaria*-Na^+/K^+-ATPase (**Supplementary Figure S2**).

Different Cardiac Glycosides Show Relative Selectivity for Different Insect Na^+/K^+-ATPases

Comparison of the IC_{50} values of individual cardiac glycosides for lamb, non-adapted, and adapted insect Na^+/K^+-ATPases (**Figure 2**) revealed strong relative selectivity exerted by some of the inhibitors tested. Between non-adapted insect and *D. plexippus*-Na^+/K^+-ATPase, the polar cardenolide ouabain showed the strongest relative selectivity with a 94-fold difference between the two IC_{50} values. By contrast, IC_{50} values for oleandrin and uzarigenin differed only by fourfold indicating much lower discrimination between adapted and non-adapted insect Na^+/K^+-ATPase. Across lamb, non-adapted and adapted insect Na^+/K^+-ATPase, cardenolides such as ouabagenin or digitoxin discriminated more or less equally between the three forms of Na^+/K^+-ATPase. Ouabain and cymarin, on the other hand, discriminate strongly between non-adapted and *D. plexippus*-Na^+/K^+-ATPase but to a much lesser extent between lamb and non-adapted insect Na^+/K^+-ATPase. This effect seems not to be related to the overall level of Na^+/K^+-ATPase resistance as indicated by the pronounced selectivity of gitoxigenin between lamb and non-adapted insect Na^+/K^+-ATPase and the relatively low discrimination of this compound between non-adapted insect and *D. plexippus*-Na^+/K^+-ATPase.

To illustrate selective interactions on the level of toxins and receptors, we plotted full dose–response curves for adapted vs. non-adapted Na^+/K^+-ATPases and different cardiac glycosides relative to a standard, ouabain (**Figure 3**). Calactin, for example, inhibited *D. plexippus*- as well as *E. core*-Na^+/K^+-ATPase more potently than ouabain (**Figure 3A**). By contrast, the diglucoside uzarin (**Figure 3B**) caused a substantially stronger inhibition than ouabain on *D. plexippus* Na^+/K^+-ATPase, but far weaker inhibition than ouabain on *E. core* Na^+/K^+-ATPase while desglucouzarin (**Figure 3C**) which has the same genin, but linked to only one glucose, had a strongly inhibitory effect on *D. plexippus*-Na^+/K^+-ATPase compared to ouabain, and had nearly the same inhibitory effect as ouabain on the *E. core*-Na^+/K^+-ATPase. The application of uzarigenin, the genin of this series, and also its 5β isomer digitoxigenin again resulted in countervailing effects on the two insect Na^+/K^+-ATPases compared to ouabain (**Figures 3D,E**). In contrast to desglucouzarin, gitoxigenin inhibited the non-adapted locust-Na^+/K^+-ATPase to a much lower extent compared to ouabain, whereas the inhibition of *D. plexippus*-Na^+/K^+-ATPase was similar to the effect caused by ouabain (**Figure 3F**).

Across the 14 comparisons made in total (**Supplementary Figure S3** and **Supplementary Table S2**), 10 compounds caused a unidirectional effect, i.e., the effect of the compound on the enzyme compared to ouabain was similar when tested on both forms of Na^+/K^+-ATPase. The remaining four toxins, however, produced countervailing effects, e.g., stronger than ouabain on

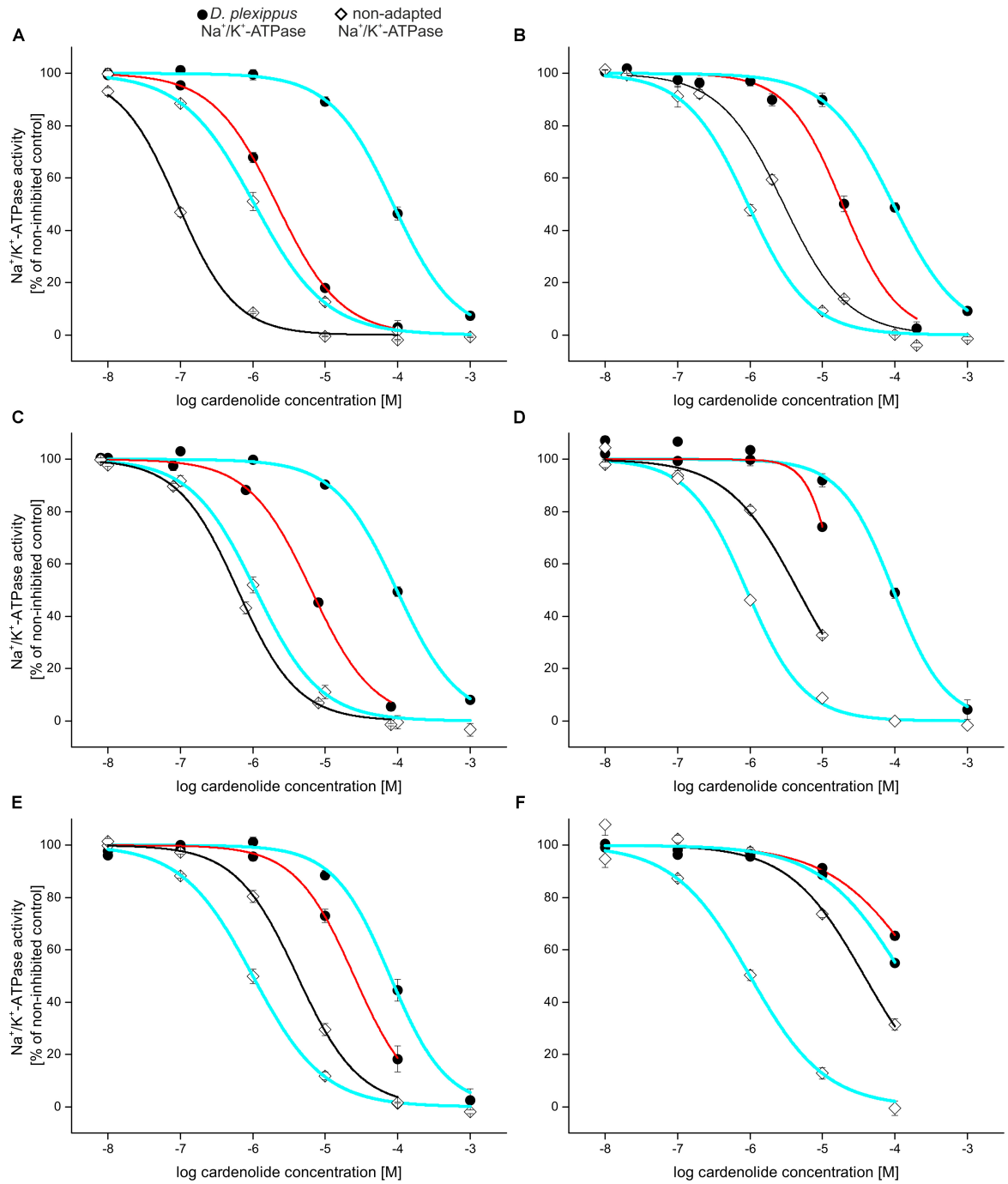

FIGURE 3 | Relative selectivity of inhibition visualized by dose–response curves: inhibition of Na^+/K^+-ATPases of *D. plexippus* (circles), *E. core* [diamonds **(A–E)**], and *S. gregaria* [diamonds, **(F)**] by selected cardenolides compared to ouabain (bold blue line; left curve = *S. gregaria*/*E. core*-Na^+/K^+-ATPase, black; right curve = *D. plexippus*-Na^+/K^+-ATPase, red). **(A)** Calactin, **(B)** uzarin, **(C)** desglucouzarin, **(D)** uzarigenin, **(E)** digitoxigenin, and **(F)** gitoxigenin. Note that adapted and non-adapted Na^+/K^+-ATPases are differentially impaired relative to ouabain.

the adapted Na^+/K^+-ATPase but weaker (or equal) to ouabain on the non-adapted Na^+/K^+-ATPase. We emphasize that the observed patterns (i.e., stronger or weaker toxicity compared to the reference) strongly depend on the reference compound used and cannot be viewed in absolute terms. Using ouabagenin, digitoxigenin, or calactin as a reference in place of ouabain shifted the direction of the effects but nonetheless supports visualization of relative selectivity exposed by individual cardiac glycosides on the two different forms of insect Na^+/K^+-ATPase (**Supplementary Figure S4**).

To test for the occurrence of such specific interactions more broadly, we carried out correlation analysis between inhibition caused by the same 11 cardiac glycosides on the adapted *D. plexippus*-Na^+/K^+-ATPase, the non-adapted Na^+/K^+-ATPase of *E. core* and *S. gregaria*, and mammalian Na^+/K^+-ATPase from lamb (Paula et al., 2005). Along this gradient of resistance (*D. plexippus* > *E. core*/*S. gregaria* > lamb, **Figure 2**), we observed a positive correlation between the IC_{50} values for cardenolides applied to non-adapted insect- and *D. plexippus*-Na^+/K^+-ATPase (**Supplementary Figure S5A**, Pearson's $r = 0.82$, $n = 11$, $p = 0.002$), but no correlation between the IC_{50} values for cardenolides applied to lamb Na^+/K^+-ATPase (Paula et al., 2005) and either of the insect-ATPases (**Supplementary Figures 5B,C**, EC/SG: $r = 0.28$, $n = 11$, $p = 0.401$; DP: $r = 0.49$, $n = 11$, $p = 0.125$). These results indicate that, on a broad scale, the extent of inhibition is not predictable between the vertebrate and insect Na^+/K^+-ATPases, but there is greater concordance among insect Na^+/K^+-ATPases, even with clear differences in the enzyme.

Comparison of Structural Attributes and Na^+/K^+-ATPase Inhibition

We next compared the inhibition of Na^+/K^+-ATPase using five pairs of cardenolide glycosides and their corresponding genins, clearly demonstrating that glycosides are consistently more potent inhibitors of the enzyme (**Figures 4A–E**, see **Supplementary Table S3** for IC_{50} values and statistics). The comparison of the uzarin–desglucouzarin–uzarigenin series of identical compounds with varying numbers of sugar groups revealed that *E. core* and *D. plexippus* Na^+/K^+-ATPases were most strongly inhibited by the monoglucoside desglucouzarin (**Figure 4E** and **Supplementary Table S3**). The biglucoside uzarin caused weaker inhibition than desglucouzarin (DP: $F_{2,6} = 27.9$, $p < 0.001$; EC: $F_{2,6} = 71.371$, $p < 0.001$) and was not statistically different from the genin uzarigenin (**Supplementary Table S3**). Thus, the number of sugar groups attached to a cardenolide does not necessarily correspond to the extent of Na^+/K^+-ATPase inhibition.

Most *Asclepias* cardenolides have an A/B-*trans*-steroid skeleton while cardenolides from other genera typically have an A/B-*cis* configuration (Seiber et al., 1983; Cheung et al., 1988; Malcolm, 1991). We compared the inhibition of *E. core* and *D. plexippus* Na^+/K^+-ATPases by the 5α-cardenolide uzarigenin and its 5β equivalent, digitoxigenin. As for the other cardiac glycosides described above, we found that both, uzarigenin and digitoxigenin, inhibited *E. core* Na^+/K^+-ATPase more strongly than *D. plexippus* Na^+/K^+-ATPase. However, we observed no difference between uzarigenin and digitoxigenin in terms of their ability to inhibit either of the Na^+/K^+-ATPases (**Figure 4F**, DP: $F_{2,4} = 0.898$, $p = 0.476$, EC: $F_{2,4} = 1.942$, $p = 0.257$).

DISCUSSION

Here, we have shown that structural variation in plant toxins, belonging to the same chemical class, strongly influences the strength of inhibition of the specific target site. Differential activity of structurally diverse defensive metabolites and selective impairment of different herbivores is a major prediction of coevolutionary theory and has been demonstrated on the level of whole organisms (Ayres et al., 1997; Linhart and Thompson, 1999; Müller et al., 2010; Kleine and Müller, 2011). The underlying mechanisms, however, have rarely been revealed, especially on divergent targets of adapted and non-adapted herbivore species. Monarch butterflies have been assumed to be highly resistant to cardenolides by means of an altered Na^+/K^+-ATPase, although the effects are dose-dependent and were only previously tested with ouabain, a cardenolide that does not occur in the monarch's host plants (Vaughan and Jungreis, 1977; Holzinger et al., 1992; Petschenka et al., 2012). Our comparison of 16 cardiac glycosides (including *Asclepias* cardenolides) on *D. plexippus*-Na^+/K^+-ATPase is thus a critical step to approach the complex and evolutionarily relevant scenario monarchs and other milkweed herbivores are exposed to naturally.

While the *D. plexippus*-Na^+/K^+-ATPase is consistently more resistant than the non-adapted insect Na^+/K^+-ATPases of *E. core* and *S. gregaria* (**Figure 2** and **Supplementary Table S1**), 11 out of 16 cardenolides tested acted stronger on its Na^+/K^+-ATPase than ouabain. Strikingly, calactin and calotropin, which are preferentially sequestered by monarchs from *Asclepias* spp. (Cheung et al., 1988; Groeneveld et al., 1990; **Supplementary Figure S6**), were the most toxic cardenolides and inhibited *D. plexippus*-Na^+/K^+-ATPase up to 60 times more strongly than ouabain (based on IC_{50} values). As *A. curassavica* is a rich source of calactin and calotropin (**Supplementary Figure S6**) and also a very good dietary resource for monarch caterpillars (Petschenka and Agrawal, 2015), it is unlikely that this observed *in vitro* enzymatic toxicity causes a substantial physiological burden. As these compounds can be detected in the hemolymph, the monarch probably has additional mechanisms of resistance (e.g., barrier tissues preventing contact with the *D. plexippus*-Na^+/K^+-ATPase, Petschenka et al., 2013b). Monarch caterpillars sequester calactin and calotropin in high amounts and additionally derive these compounds enzymatically from other milkweed cardenolides such as uscharidin (Seiber et al., 1980). Moreover, monarchs only seem to sequester cardenolides within a certain polarity range and compounds such as calactin and calotropin may be easier to store in the body tissues compared to more lipophilic cardenolides (Roeske et al., 1976). Duffey (1977) stated that while non-polar cardenolides are absorbed more easily via the gut relatively more polar cardenolides seem to cause emesis in vertebrates at lower doses. While the moderately non-polar cardenolides calactin and calotropin are easily absorbed across the gut they also have low-dose requirements to cause emesis after reaching the blood stream of a vertebrate (see also Parsons and Summers, 1971). Thus, calactin and calotropin seem to represent the ideal substrate for sequestration, which is supported by observations on the grasshopper *Poekilocerus bufonius* that also selectively sequesters calactin and calotropin (Roeske et al., 1976). It is unknown whether these compounds are highly bitter or easily detected by taste for would-be predators.

The different cardiac glycosides tested produced much higher variation of IC_{50} values when applied to adapted and non-adapted insect Na^+/K^+-ATPases (**Figure 2**) compared to the

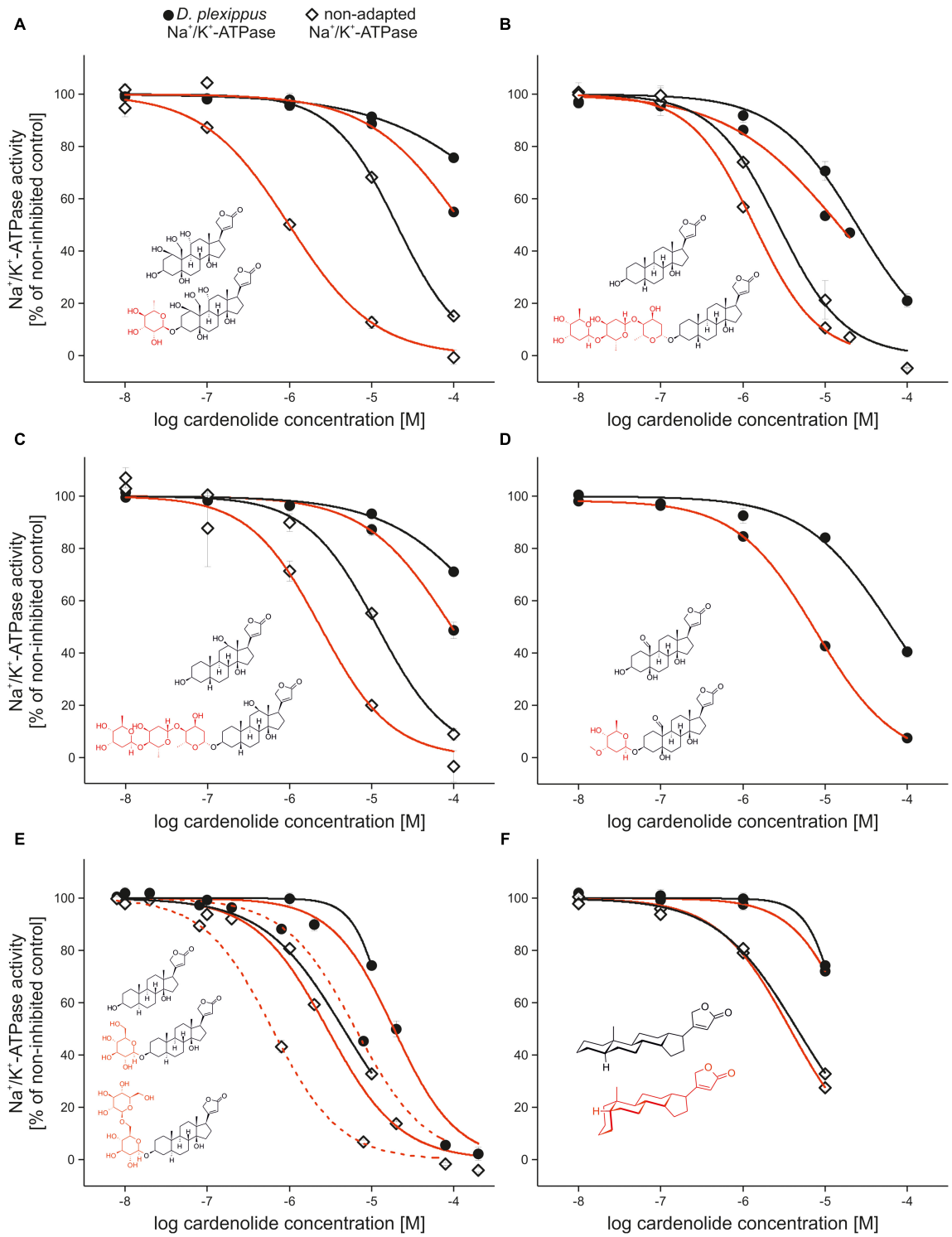

FIGURE 4 | Comparison of the inhibition caused by cardiac glycosides and corresponding genins and the effect of 5α vs. 5β configuration. **(A–E)** Inhibition of Na^+/K^+-ATPases of *Danaus plexippus* (circles), *Euploea core* and *Schistocerca gregaria* (diamonds) by cardenolide glycosides (black lines), and their corresponding genins (red lines). Inserted structural formulas show the glycosides and genins used for comparisons. Sugar moieties of glycosides are labeled in red. **(A)** Ouabain vs. ouabagenin, **(B)** digitoxin vs. digitoxigenin, **(C)** digoxin vs. digoxigenin, and **(D)** cymarin vs. strophanthidin. All comparisons are made between *D. plexippus* and *S. gregaria*-Na^+/K^+-ATPase. **(E)** Effect of uzarin (solid red line) vs. desglucouzarin (dashed line) vs. uzarigenin (solid black line) on *D. plexippus*- (circles) and *E. core*-Na^+/K^+-ATPase (diamonds). **(F)** Inhibition of *D. plexippus* (circles) and *E. core*-Na^+/K^+-ATPase (diamonds) by digitoxigenin (red line) and its 5α-isomer uzarigenin (black line). The insert shows the steroidal backbone plus lactone of cardiac glycosides with A/B rings in *trans* (black, as realized in uzarigenin) and A/B rings in *cis* configuration (red, as realized in digitoxigenin).

more sensitive lamb-Na^+/K^+-ATPase which showed "little fine-specificity of binding" (Farr et al., 2002). While our data do not allow for drawing specific conclusions about monarch–milkweed coevolution, our results demonstrate that the more resistant insect Na^+/K^+-ATPases respond variably to structurally distinct cardiac glycosides, opening the potential for coevolution mediated by toxin–receptor interactions. While synergistic effects of different compounds on herbivores were suggested to select for phytochemical diversity (Berenbaum et al., 1991; Dyer et al., 2003), adapted insect target sites could also impose natural selection on the plant to maintain structurally different toxins. This increased diversity of compounds would then raise the probability to possess a toxin which is an especially potent inhibitor for this particular form of the enzyme target. Given the dramatically higher absolute amounts of toxins necessary to inhibit an adapted Na^+/K^+-ATPase, using the most potent cardiac glycosides for defense may be especially critical to save costs of production for the plant (Züst et al., 2015).

It was historically shown that cardenolide glycosides are more toxic than corresponding genins in whole organism vertebrate assays (Hoch, 1961). Sugar residues of cardiac glycosides have been shown to stabilize the Na^+/K^+-ATPase-inhibitor complex by interactions of hydroxyl groups of the sugar molecule with proton-donating, as well as proton-accepting, groups on the Na^+/K^+-ATPase (Yoda, 1973) and sugars can prevent reactivation of the enzyme after cardiac glycoside inhibition (Cornelius et al., 2013). Furthermore, fixation of cardiac glycosides to the heart muscle depends on the sugar moiety (Hoch, 1961). In general, the effect of sugars on any given Na^+/K^+-ATPase seem to depend on the number and chemical identity of the sugar molecules (Farr et al., 2002). For the vertebrate Na^+/K^+-ATPase, responses were heterogeneous and the removal of sugar is reported to decrease or increase strength of inhibition (O'Brien et al., 1993; Farr et al., 2002; Paula et al., 2005; Cornelius et al., 2013). Ouabain, in agreement with our dataset on insect Na^+/K^+-ATPase, had a higher inhibitory potency than ouabagenin on lamb-, shark-, and human Na^+/K^+-ATPase (Farr et al., 2002; Paula et al., 2005; Katz et al., 2010; Cornelius et al., 2013). As for adapted and non-adapted insect Na^+/K^+-ATPases, digoxin and digitoxin had a higher inhibitory potency on shark and human Na^+/K^+-ATPase (Cornelius et al., 2013) compared to digoxigenin and digitoxigenin, but the reverse was found for lamb Na^+/K^+-ATPase (Farr et al., 2002; Paula et al., 2005). Shark and lamb-Na^+/K^+-ATPase also showed opposing results for the comparison between gitoxin and gitoxigenin (Farr et al., 2002; Paula et al., 2005; Cornelius et al., 2013). Surprisingly, the glycoside cymarin did not cause differential inhibition from its corresponding genin strophanthidin on lamb-Na^+/K^+-ATPase (Farr et al., 2002), but was ninefold more inhibiting on *D. plexippus*-Na^+/K^+-ATPase (but see Paula et al., 2005). These comparisons indicate that the effect of the cardiac glycoside sugar moiety on inhibition depends on the specific biochemical properties of the receptor Na^+/K^+-ATPase. Additionally, by conducting the analyses of compound impacts on Na^+/K^+-ATPases in a single study, our results are more easily interpretable because of consistency in purity of compounds and methods employed. Across the five pairs of glycosides and genins, glycosides were on average sixfold more potent in their inhibition of insect Na^+/K^+-ATPases, including the specialized monarch.

The unusual features of sugars present in cardiac glycosides suggest an adaptive significance for plants, but the mechanisms of natural selection have not been revealed. Apart from absorption, these sugars could contribute to hydrolytic stability of cardiac glycosides which could be an anti-predator strategy (e.g., prevention of hydrolysis in the animal gut). In this regard, cardenolides from the Asclepiadoideae (the milkweeds), which have cyclic bridges, are highly resistant to acid hydrolysis (Seiber et al., 1983) and should thus receive specific future attention. Although some insects attach sugar moieties to toxic aglycones (genins) in the course of detoxification (Heckel, 2014), it is unclear whether this could be important for insects adapted to cardiac glycosides. Given the increase of Na^+/K^+-ATPase inhibition in glycosides vs. genins, we speculate that glycosylation of cardiac glycoside genins could lead to metabolic activation instead of detoxification.

Another remarkable feature of milkweed cardenolides is the *trans*-bent junction of rings A and B in the steroid (Seiber et al., 1983; Malcolm, 1991) which is *cis*-bent in *Digitalis* and other medicinally relevant cardenolides. Such *trans*-bent 5α-cardiac glycosides were speculated to have a highly reduced biological potency based on their toxicity (Brown and Thomas, 1984) and show low affinity to mammalian Na^+/K^+-ATPase (Paula et al., 2005; Katz et al., 2010). Although the direct comparison of uzarigenin with its 5β-isomer digitoxigenin revealed the A/B *trans*-ring junction as being causal for lower binding affinity and reduced inhibition on the mammalian enzyme (Farr et al., 2002; Paula et al., 2005), we found no differences in inhibitory strength between the two isomers, neither for non-adapted nor for adapted insect Na^+/K^+-ATPases. Moreover, the most toxic cardenolides observed, calactin and calotropin, also are 5α cardenolides. The putatively different effect of the A/B *trans*-ring junction on insect vs. vertebrate Na^+/K^+-ATPase should be addressed in future studies. Our observation that uzarigenin has a strongly reduced water solubility compared to digitoxigenin points to the importance of the configuration of the A/B ring junction for basic physicochemical properties and thus could easily influence absorption and sequestration of milkweed cardenolides in herbivorous insects.

How plants can respond selectively to attacks with defenses specific for particular antagonists is a major unanswered question in the study of plant–insect coevolution. It has been shown that structurally different plant defenses (e.g., monoterpenes from thyme, Linhart and Thompson, 1999) or condensed tannins from several plant families (Ayres et al., 1997) can affect different herbivores with different strength, but the underlying mechanisms have never been revealed. In his book on cardiac glycosides, Hoch (1961) states that "an arrangement of cardiac glycosides in the order of their relative toxicities would not be the same for different animal species." Here, we show in a detailed mechanistic way, that structurally different cardiac glycosides can affect specific animal Na^+/K^+-ATPases in highly distinct ways. Our observation thus suggests a mechanistic basis for how such selectivity could be achieved on the level of plant toxins and their physiological targets. Moreover, the

concept of coevolution predicts reciprocal escalation between plants and insects, and cardiac glycosides like uzarin which show comparatively little relative selectivity between the adapted *D. plexippus*-Na^+/K^+-ATPase and the non-adapted Na^+/K^+-ATPase, could be the plants' response to the adapted insect's target site (Farrell and Mitter, 1998). Indeed, there is evidence that isoforms of human Na^+/K^+-ATPase are also differentially inhibited by individual cardiac glycosides (Katz et al., 2010), which might be related to the importance of cardiac glycosides as endogenously occurring hormones and fine-scale regulation of tissue-specific isoforms of Na^+/K^+-ATPase. We speculate that the dramatically different effects which structurally diverse cardiac glycosides have on the more resistant insect Na^+/K^+-ATPases might be a driving force selecting for structural diversity of defense chemicals. For interactions involving sequestration, such as that between milkweeds and monarchs, both the plant and the animal antagonists must manage toxicity, not only in the pair but also to the third trophic level.

AUTHOR CONTRIBUTIONS

GP, CF, and SS collected data. AA, JA, and BT provided materials. GP and AA designed research, analyzed data, and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01424/full#supplementary-material

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How Glucosinolates Affect Generalist Lepidopteran Larvae: Growth, Development and Glucosinolate Metabolism

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Multiple lepidopteran larvae feed successfully on plants containing glucosinolates despite the diverse array of toxic and deterrent breakdown products, such as isothiocyanates (ITCs), formed upon plant damage. While much is known about how specialist lepidopterans metabolize and tolerate glucosinolates, there is little information about the metabolic fate of these plant defense compounds in specialized herbivores. Employing ^{13}C- and ^{14}C-labeled 4-methylsulfinylbutyl glucosinolate (glucoraphanin), we identified and quantified the major detoxification products of glucosinolates and ITCs in selected specialized and generalist larvae. While specialists prevented glucosinolate hydrolysis or diverted hydrolysis to form nitriles, hydrolysis in generalists proceeded to toxic ITCs, of which a portion were conjugated to glutathione. However, a large amount of ITCs remained unmodified, which may have led to the observed negative effects on growth and development. The performance of two generalist-feeding caterpillars, *Spodoptera littoralis* (African cotton leafworm) and *Mamestra brassicae* (cabbage moth) on *Arabidopsis thaliana* Col-0 and various glucosinolate-deficient mutants was investigated from hatching until pupation. We found that glucosinolates negatively affected larval growth and development, but not survival, with aliphatic glucosinolates having stronger effects than indolic glucosinolates, and the combination of the two glucosinolate types being even more detrimental to growth and development. Curiously, last instar larvae grew better on wild type than on non-glucosinolate-containing plant lines, but this could not be attributed to a change in detoxification rate or feeding behavior. Glucosinolates thus appear to be effective defenses against generalist lepidopteran herbivores at least during most stages of larval development. Nevertheless, the reversal of negative effects in the oldest instar is intriguing, and further investigation of this phenomenon may shed light on how generalists adjust their physiology to feed on diets with many different types of plant defense compounds.

Keywords: ***Spodoptera littoralis, Mamestra brassicae, Arabidopsis thaliana*, glucoraphanin, glucobrassicin, isothiocyanate, detoxification, Lepidoptera**

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INTRODUCTION

In their struggle against herbivores and pathogens, plants rely on a large arsenal of defense metabolites to protect their tissues (Wittstock and Gershenzon, 2002; Hartmann, 2007). Herbivores have evolved an equally extensive suite of behavioral, physiological and molecular mechanisms to circumvent plant defenses (Heckel, 2014; Heidel-Fischer and Vogel, 2015). While many previous studies have quantified the effects of plant chemical defenses on lepidopteran herbivores after short-term feeding, few have explored how development is affected by feeding on chemically well-defended plant material for an extended period. More knowledge about whether long-term feeding on defense compounds influences growth and survival will help clarify the linkages between the presence of chemical defense compounds and the evolution of detoxification mechanisms in herbivores.

Among plant defense compounds, glucosinolates (GLSs) are part of the "mustard oil bomb" of the Brassicaceae and related families, a group that includes agriculturally important crops such as cabbage and rapeseed. GLSs are a chemically diverse group composed of a sulfur- and nitrogen-containing glucosidic core attached to a variable, amino acid-derived side-chain R (**Figure 1A**). They are commonly grouped into three classes based on their precursors: aliphatic, derived from methionine or other aliphatic amino acids; indolic, derived from tryptophan; and benzenic, derived from phenylalanine or tyrosine (Fahey et al., 2001; **Figure 1A**). Intact GLSs are not toxic themselves, but only after their glucose moiety is hydrolyzed by plant myrosinase enzymes (ß-thioglucoside glucohydrolases, EC 3.2.1.147) upon tissue damage resulting in a profusion of potentially toxic products (Wittstock and Burow, 2010). Herbivores specialized on brassicaceous plants detoxify GLSs by well-known mechanisms that prevent GLS hydrolysis or the formation of toxic products (**Figure 1A**; reviewed in Jeschke et al., 2016a). In contrast, generalist herbivores are reported to suffer from GLS hydrolysis and only detoxify the resulting isothiocyanate (ITC) products (Schramm et al., 2012; Zou et al., 2016). However, only few generalist species have been studied and it is not clear what proportion of GLSs are metabolized in this way and whether other modes of detoxification occur in generalist herbivores.

The Brassicaceae model plant, *Arabidopsis thaliana* Col-0, is an ideal organism for GLS studies as it contains a varied assortment of more than 20 different GLSs throughout its development (Brown et al., 2003), and genetically engeneered genotypes are available that do not produce particular classes of GLSs. In the vegetative stage of *A. thaliana* Col-0, the predominant classes in rosette leaves are aliphatic and indolic GLSs, which account for ∼85% and ∼15% of total GLSs, respectively (Brown et al., 2003). Aliphatic GLSs are a large, structurally diverse group in *A. thaliana* with a composition that varies strongly among ecotypes (Kliebenstein et al., 2001). In contrast, indolic glucosinolates consist of a small group of compounds with low structural diversity that is widely distributed in all *A. thaliana* ecotypes. Their biosynthesis and abundance are strongly influenced by environmental factors, such as herbivory (Textor and Gershenzon, 2009). *A. thaliana* Col-0 aliphatic GLSs are hydrolyzed by myrosinase to predominantly form ITCs (Wittstock and Burow, 2010), while indolic GLSs can break down independently of myrosinase activation (Pedras et al., 2002) to form the corresponding nitriles or ITCs, with the latter further decomposing to the carbinol and conjugating to nucleophiles (Agerbirk et al., 2009). In spite of their different properties, the effects of aliphatic vs. indolic GLSs have not always been clearly distinguished, especially for generalist herbivores.

Ingestion of GLSs and their corresponding breakdown products impairs the growth of a wide range of herbivores, and different GLSs have contrasting effects depending on the herbivore studied (reviewed in Jeschke et al., 2016a). Furthermore, quantitative variation of the different GLSs in the host plant significantly affects the insect herbivore diversity and resulting plant fitness (Mithen et al., 1995; Mosleh Arany et al., 2008; Santolamazza-Carbone et al., 2015). As structural and regulatory genes of the GLS biosynthetic pathway have been identified, genetically manipulated plants with altered GLS profiles have become available (Benderoth et al., 2009; Geu-Flores et al., 2009; Sønderby et al., 2010). Short-term feeding studies using such plant lines showed that aliphatic and indolic GLSs have differential effects on the growth of chewing herbivores, with aliphatic GLSs generally having stronger detrimental effects than indolic GLSs (reviewed in Jeschke et al., 2016a). However, how the effects of different GLS classes interact to influence the long-term development of generalist herbivores has not yet been examined.

To study the effects of GLSs on generalist herbivores, we begin by comparing the metabolism of an isotopically labeled GLS in both generalist and specialist lepidopteran caterpillars. The compound fed, 4-methylsulfinylbutyl glucosinolate (4msob-GLS), is the most abundant GLS in the rosette leaves of the Columbia-0 ecotype of *A. thaliana*. Since considerable amounts of free 4msob-ITC were found in the frass of generalists, but not specialists tested, we then investigated the long-term effects of GLS feeding on lepidopteran larvae of two generalist species with different preferences for plants of the Brassicaceae over a long developmental period from neonate to pupation. *Spodoptera littoralis*, the African cotton leafworm, is an extremely polyphagous herbivore infesting plants from 40 different families, including the Brassicaceae (Brown and Dewhurst, 1975). *Mamestra brassicae*, the cabbage moth, is a generalist feeder but one that prefers GLS-containing plants, including several Brassicaceae crop plants (Popova, 1993). Four *A. thaliana* plant lines were used to dissect the effects of aliphatic vs. indolic GLSs in a natural background: (1) Columbia-0 wild type, (2) the double mutant of *cyp79B2* and *cyp79B3* that contains only aliphatic GLSs (Zhao et al., 2002), (3) the double mutant of the *myb28* and *myb29* transcription factors that regulate biosynthesis of aliphatic GLSs, which contains only indolic GLSs (Sønderby et al., 2007; Beekwilder et al., 2008), and (4) the quadruple mutant *myb28myb29cyp79B2cyp79B3* which does not contain detectable levels of either aliphatic or indolic GLSs (Sun et al., 2009). The results give a detailed overview of the effects of GLS

feeding on the metabolism, growth and development of generalist lepidopteran insects.

MATERIALS AND METHODS

Plants, Insects, and Chemicals

Arabidopsis thaliana was cultivated in a controlled–environment chamber under short-day conditions (10:14 h, light:dark) at 21°C and 50–60% relative humidity (RH). The lines used are the Col-0 wild type, and the following mutants: *cyp79B2 cyp79B3* (aliphatic GLS only) (Zhao et al., 2002), *myb28 myb29* (indolic GLS only) (Sønderby et al., 2007), and *cyp79B2 cyp79B3 myb28 myb29* (no GLS) (Sun et al., 2009). Broccoli (*Brassica oleracea* cv. "Broccocress") seeds were a generous gift from Discover Freshhh (Monster, The Netherlands), and were germinated and grown under controlled light and temperature conditions (16:8 h, light:dark, day-time temperature 22°C, night-time 20°C). Cabbage plants used for rearing specialist lepidopterans were grown in glasshouses at 22–28°C, with light supplementation as needed for 16:8 h light:dark conditions.

Egg clutches of *Spodoptera littoralis* Boisduval (African cotton leafworm) were a generous gift from Syngenta Crop Protection (Stein, Switzerland), and reared on an artificial diet based on white beans (as described in Jeschke et al., 2016b) at 18–20°C under natural light. Larvae of *Helicoverpa armigera* Hübner (cotton bollworm, Toowoomba strain, generously provided by the Dept. of Entomology, MPI-CE) were reared on a pinto bean diet (Perkins et al., 1973) at 26°C, 16:8 h, light:dark, and 60% RH. Larvae of *Mamestra brassicae* Linnaeus (cabbage moth, provided by the Laboratory of Entomology, Wageningen University of Plant Sciences, The Netherlands) and *Trichoplusia ni* Hübner (cabbage looper, purchased from Benzon Research, Carlisle, PA, United States) were reared on a diet based on wheat germ (Burton, 1969) at room temperature under natural light. Larvae of *Plutella xylostella* (diamondback moth, generously provided by the Dept. of Entomology, MPI-CE) were maintained as in Badenes-Perez et al., 2014. Larvae of *Pieris rapae* (small cabbage white, provided by the Laboratory of Entomology, Wageningen University of Plant Sciences, The Netherlands) were maintained on cabbage plants under short-day conditions (10:14 h, light:dark) at 21°C and 50–60% RH. For the long-term feeding study, larvae were kept at 19°C and under a light cycle of 12:12 h.

Of the chemicals used, 4-methylsulfinylbutyl isothiocyanate (1-isothiocyanato-4-methylsulfinylbutane, sulforaphane), reduced L-glutathione (GSH), D-(+)-glucose, D-(-)-fructose and albumin from bovine serum (BSA) were obtained from Sigma–Aldrich (Munich, Germany). D-(+)-sucrose, Tris, acetonitrile, chloroform, and formic acid (LC grade) were obtained from Roth (Karlsruhe, Germany). Bradford reagent was obtained from Serva (Heidelberg, Germany) and the $^{13}C/^{15}N$ labeled amino acid standard mix was obtained from Isotec (Miamisburg, OH, United States). Conjugates of 4msob-ITC (4msob-GSH, 4msob-Cys and 4msob-NAC) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, United States). 4msob-CysGly was synthesized as described in (Schramm et al., 2012). Methanol was purchased from Merck (Darmstadt, Germany). All chemicals were obtained in the highest available grade and solvents were in the analytical grade.

Administration of Isotopically Labeled GLSs to Lepidopteran Larvae

The production of isotopically labeled [^{13}C]- and [^{14}C]-4msob-GLS, introduction into detached leaves of the *A. thaliana myb28myb29* double knock-out mutant, feeding set-up, feces collection, extraction and HPLC analysis were performed as described in Schramm et al. (2012). The remaining solid feces material was then additionally extracted with Me_2CO and hexanes sequentially to check for the presence of less polar radioactive compounds via scintillation counting. An aliquot of the final solid residue, as well as crushed insect tissues, were also analyzed by scintillation counting for quantification of radioactivity. ^{13}C-Labeled and unlabeled metabolites were identified by LC-MS as previously described (Schramm et al., 2012; Jeschke et al., 2016b; Malka et al., 2016).

Long-Term Feeding of *S. littoralis* and *M. brassicae* on *A. thaliana* of Varying GLS Content

Larvae were offered leaves of a single line of *A. thaliana* under no-choice conditions starting from when they were newly hatched. Larvae were fed *ad libitum* on leaves detached daily from 6 to 7 week-old plants at the pre-bolting stage. The experiment was run in two different phases: (1) early development (from hatching until 3rd instar) in which larvae were measured (weights, instar changes, and feces collections) in groups of ten, and (2) late development (3rd instar until pupation) in which larvae were measured as individuals. Larvae from different feeding groups were reared concurrently, and the placement of the Solo® cups among trays was randomized daily. Fresh larval weights were recorded in mg with two decimal digits with a Mettler-Toledo XP26 microbalance (Giessen, Germany), at approximately the same time each morning. *Early development:* Neonate larvae of *S. littoralis* and *M. brassicae* were placed in groups of 10 into Solo® cups that were lined with moist filter paper. The cups were then randomly assigned to plant lines with ten replicates per line. Larvae were counted, weighed and checked for instar change every day until all larvae reached the 3rd instar. *Late development:* Neonate larvae of both species were separated in small Solo® cups in random groups of 40, with six replicates per plant line. They were fed exclusively with the designated plant line, but no data were collected until the later stages of development. With the onset of the 3rd instar (on average on the 7th day for *S. littoralis* and the 10th day *M. brassicae*), three larvae out of each of the six group cups were each transferred singly into an individual Solo® cup for a total number of 18 larvae per plant line. This marked the start of the individual phase. The weight of each larvae was recorded every second day and the instar of the larvae was recorded

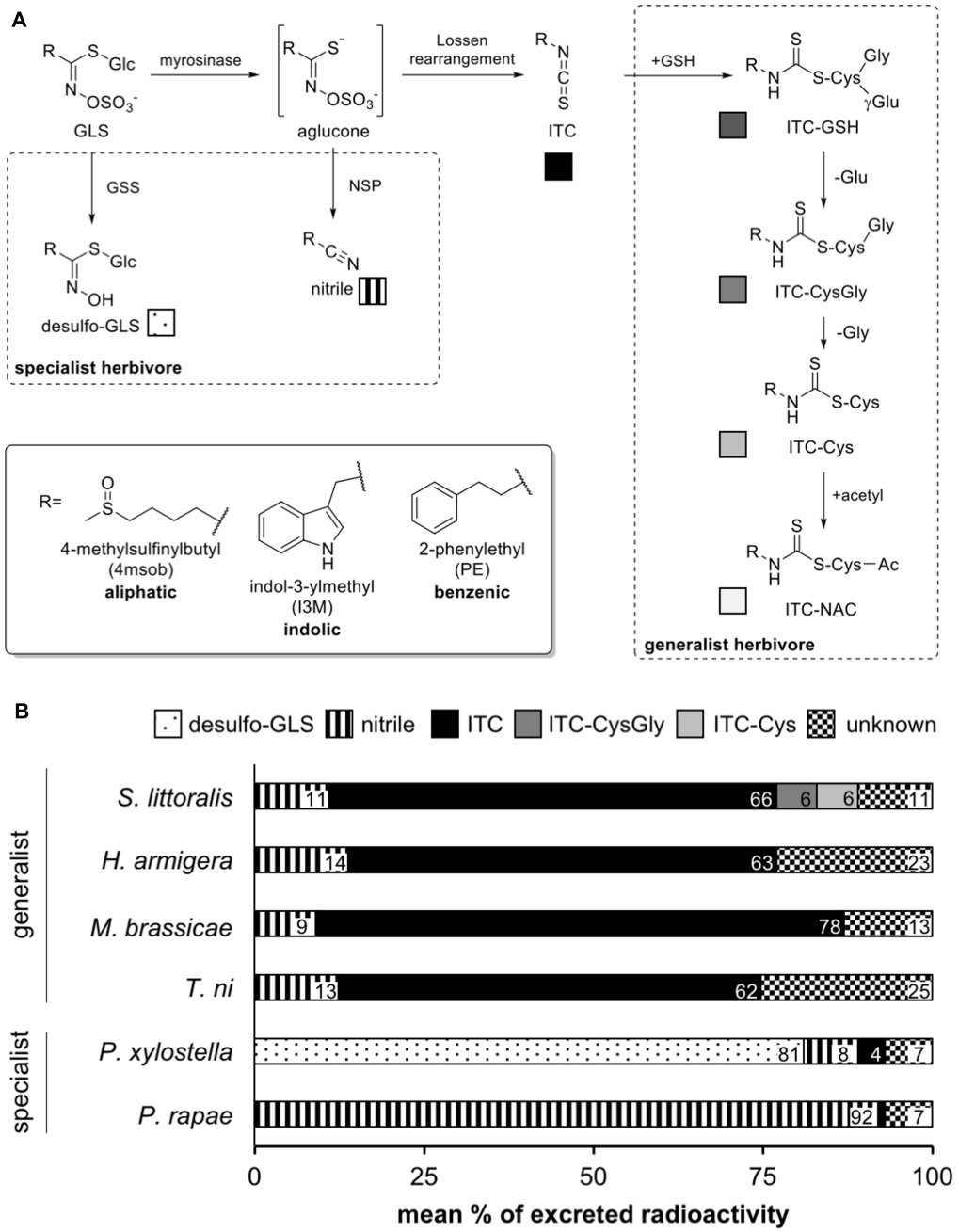

FIGURE 1 | Metabolism of glucosinolates (GLSs) by lepidopteran herbivores. **(A)** In dashed boxes, known pathways of specialist and generalist herbivores are depicted. Specialist adaptations include the production of glucosinolate sulfatases (GSS) to generate desulfo-GLS that cannot be hydrolysed, or the use of nitrile specifier proteins (NSP) to generate nitriles instead of the more toxic isothiocyanates (ITCs). Generalist caterpillars detoxify the ITCs formed upon GLS hydrolysis via conjugation to glutathione (GSH) which is further metabolized via the mercapturic acid pathway. **(B)** The profile of radioactively labeled 4-methylsulfinylbutyl (4msob)-GLS-derived metabolites in the feces of specialist and generalist caterpillars. Intact GLS were not detected. ITC-GSH, -CysGly, -Cys and -NAC: ITC conjugates with glutathione (absent), cysteinylglycine, cysteine and *N*-acetylcysteine (absent), respectively.

daily. Two days after molting into the 6th instar, potting soil was added to the cups to provide a suitable substrate in which the larvae could pupate. Date of pupation was recorded as the day in which the larva was no longer visible on top of the soil.

The feces were collected from the cups starting on day 3 and thereafter every second day into Eppendorf tubes for metabolite analysis. Feces preparation, extraction and metabolite analysis were carried out as described in Jeschke et al. (2016b).

Chemical Analysis of *A. thaliana* Lines of Varying GLS Content

Whole rosettes of all four Arabidopsis lines were collected thrice weekly for plant quality control. GLSs were analyzed with *p*-hydroxybenzyl GLS (sinalbin) as internal standard as described in Schramm et al., 2012. The contents of protein, sugar, and amino acids were determined by extracting 10 mg freeze-dried ground plant material in 100 µL aqueous buffer (Tris, 50 mM, pH 7.5). Protein and sugar were determined as described in Jeschke et al. (2016b). Amino acids were analyzed as described in Docimo et al. (2012).

Determination of Leaf Fragment Sizes in Insect Feces

Spodoptera littoralis larvae of the 4th and 6th instars were allowed to feed *ad libitum* for 24 h on detached leaves of *A. thaliana* WT or the no-GLS line ($N = 5$). The feces were collected, resuspended in 1 mL H_2O and three drops of the suspension were plated on individual microscope slides. Using a Zeiss Axiovert 200 microscope connected to an AxioCam MRc 5 digital camera, we took five pictures of different areas of the slides and determined the perimeter of all fragments in each area using Adobe Photoshop®. Pictures derived from one caterpillar were treated as technical replicates in the analysis and averaged.

Statistical Analysis

All statistical testing was performed using the software R 3.0.2 (R Core Team, 2017) unless otherwise noted. Data are presented as mean ± standard error. Data were controlled for statistical prerequisites such as homogeneity of variances and normality. For differences in survival of the young larvae within the first seven days, Kaplan–Meier curves were generated using a right-censored regression analysis (R survival package) (Therneau, 2015). Models with various distributions were conducted and compared with an analysis of deviance to find the model with the best fit. In case of significant difference a Tukey test (multicomp package, Hothorn et al., 2008) was performed. Weight data was analyzed using generalized least squares models (R nlme package (Pinheiro et al., 2017); general formula: Weight ∼ Treatment). To account for the variance heterogeneity of the residuals between feeding treatments (except for weight gain in the fifth instar of *S. littoralis*) the varIdent variance structure was used to allow the variance of the residuals of each feeding treatment to vary independently. Pairwise comparisons between treatments were performed using least squares means with the lsmeans package (Lenth, 2016) with a Tukey HSD adjustment of the *p*-values. The average day of an instar change was determined by plotting the % of caterpillars that had changed into the new instar each day. A sigmoidal curve (Boltzmann function) was then fitted and the inflection point (x_0) represents the day at which 50% of caterpillars had reached the new instar. The fitting of the sigmoidal function and calculation of the inflection point (with standard error) was performed in Origin 8 SR2 (v8.0891, OriginLab Corporation, United States) with further statistical analysis (ANOVA and Tukey *post hoc* test) using SigmaPlot 11 (v11.2.0.5, Systat Software, Inc.). Larval weight gain per instar was calculated as the difference of the weight at the onset of two sequential instars. Statistical testing was performed with ANOVA. In case of inhomogeneity of variance (Fligner–Kileen test, $P < 0.05$) a Kruskal–Wallis test was used. Percentages of survival to day 6 were calculated per cup (survival of original 40 neonate larvae) and the percentages of 10 cups per treatment were averaged. Statistical testing was performed with an ANOVA. Percentages of survival to 6th instar and pupation were compared with a test of proportions (prop.test). The ITC detoxification products in the feces were analyzed for the early developmental phase (1st and 2nd instar) per days and for the later developmental phase (3rd to 6th instar) per instar. In cases where several feces collections per larva were made for a certain larval stage, the mean of these measurements was used in the analyses. Detoxification products of ITC with GSH were analyzed with linear mixed models with the lme function (nlme package, Pinheiro et al., 2017) to account for the repeated measurement of feces from groups or individual larvae. Treatment and day (early phase) or instar (later phase) were treated as fixed effects. Larval group or individual larvae were treated as random effects. The data were transformed to log values to fulfill the requirements for the test. *P*-values for explanatory variables were obtained by deleting explanatory variables one after another and comparison of the likelihood of the more complex model with that of the simpler model (Zuur et al., 2009). Factor level reductions were used to reveal differences between different larval stages (Crawley, 2013).

RESULTS

Generalist Lepidopteran Herbivores Metabolized GLSs to Free ITCs and Glutathione Conjugates

To investigate the effect of glucosinolates (GLSs) on four species of generalist feeding lepidopteran larvae, we first compared their GLS metabolism by determining the metabolic fate of a dose of [^{13}C]- and [^{14}C]- 4-methylsulfinylbutyl glucosinolate (4msob-GLS, glucoraphanin). The ^{13}C label allowed identification of metabolites by LC-MS when necessary, while the ^{14}C label permitted quantitative comparison of the flux into various metabolites by direct radioactivity measurement. The four lepidopteran species studied included two showing a high natural preference for GLS-containing plants (*Mamestra brassicae* and *Trichoplusia ni*) (Popova, 1993; Chow et al., 2005) and two with little or no preference (*Spodoptera littoralis* and *Helicoverpa armigera*) (Brown and Dewhurst, 1975; Firempong and Zalucki, 1989). For contrast, we also included measurements of the GLS metabolism of two specialist lepidopterans, *Pieris rapae* and *Plutella xylostella*, whose major GLS metabolites have been described (Ratzka et al., 2002; Wittstock et al., 2004).

For all species, nearly 100% of the ingested radioactivity was recovered in the final extracts (**Figure 1B**). For the specialists, the large majority of ingested GLSs were excreted as the

nitrile for *P. rapae* and as the desulfo-GLS for *P. xylostella*, consistent with previous reports (Ratzka et al., 2002; Wittstock et al., 2004). However, in generalist species the major share of ingested 4msob-GLS (62–78%) was excreted as the free isothiocyanate (ITC), while evidence of ITC conjugation with glutathione was detected for *S. littoralis* here and in the long-term feeding experiment for *M. brassicae* (see below). Approximately 10% of the ingested GLS in each generalist species was excreted as the corresponding nitrile. Since this proportion is similar to that found in the hydrolysis products formed by *A. thaliana* leaves infused with [^{14}C]-4msob-GLS, then crushed and extracted, it may not be a consequence of insect metabolism. The remainder of the ingested GLS (up to 25%) was excreted as unidentified, very polar compound(s) with no retention on the reverse phase HPLC column used (Schramm et al., 2012).

In conclusion, all generalist species excreted large amounts of unconjugated ITCs, and thus there was no relation between their GLS metabolism and their degree of feeding preference for GLS-containing plants. The large amount of free ITCs released suggested that GLSs would have negative impacts on these insects. Hence long-term feeding studies were undertaken with one generalist with no preference for GLS-containing plants (*S. littoralis*) and one with a decided preference (*M. brassicae*) to investigate how GLSs affect insect survival, growth and development.

Mutant Lines with Altered GLS Content Did Not Differ from Wild Type in Levels of Most Sugars, Proteins, and Amino Acids

Four *A. thaliana* lines were used to examine the effects of GLSs on caterpillar development: (1) Columbia-0 wild type (WT) containing native levels of aliphatic and indolic GLSs, (2) the double mutant of *cyp79B2 cyp79B3* that contains only aliphatic GLSs and is devoid of indolic GLSs (Zhao et al., 2002), (3) the double mutant of the *myb28 myb29* transcription factors that regulate biosynthesis of aliphatic GLS, which contains only indolic and no aliphatic GLSs (Sønderby et al., 2007; Beekwilder et al., 2008), and (4) the quadruple mutant *myb28/29 cyp79B2/B3* with undetectable levels of aliphatic and indolic GLS (Sun et al., 2009). WT Col-0 rosette leaves (6–7 weeks old) contained on average 21.29 ± 2.31 μmol/g dry weight total GLSs (**Figure 2** and Supplementary Table S1). Aliphatic GLS accounted for ∼90% of the total, and were present in similar concentrations in leaves of *cyp79B2cyp79B3* (aliphatic GLS only line) as in WT Col-0. Indolic GLS (∼10% of the total in Col-0 WT) were 1.6-fold more concentrated in leaves of *myb28myb29* (indolic GLS only line) than in Col-0 WT. The quadruple mutant *myb28/29cyp79B2/3* (no GLS line) accumulated only trace levels of GLS. Additionally, we looked for differences among the lines in other metabolites that could influence insect feeding, including soluble protein, free proteinogenic amino acids and simple sugars (sucrose, fructose and glucose + galactose) (Supplementary Table S1). There were no significant differences among the four lines except for an elevated glucose + galactose content in the aliphatic GLS only line.

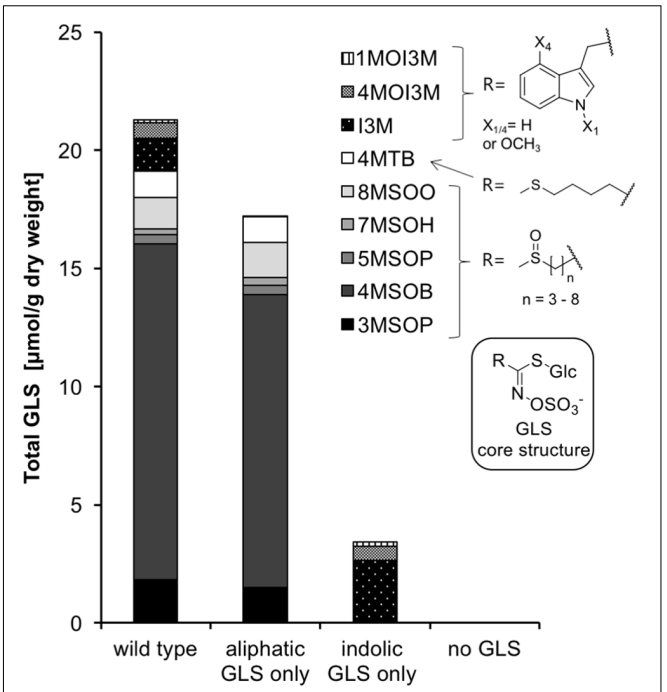

FIGURE 2 | The GLS content of 7-week-old plants of *A. thaliana* Col-0 (wild type) and the biosynthetic knock-down mutants *cyp79B2cyp79B3* (aliphatic GLS only), *myb28myb29* (indolic GLS only) and *cyp79B2/3myb28/29* (no GLS). For the exact values as well as sugar, soluble protein and amino acid content, refer to Supplementary Table S1. Abbreviations for GLSs: 1MOI3M/4MOI3M: 1- and 4-methoxyindol-3-ylmethyl, I3M: indol-3-ylmethyl, 4MTB: 4-methylthiobutyl, 8MSOO: 8-methylsulfinyloctyl, 7MSOH: 7-methylsulfinylheptyl, 5MSOP: 5-methylsulfinylpentyl, 4MSOB: 4-methylsulfinylbutyl, 3MSOP: 3-methylsulfinylpropyl.

GLSs Reduced Larval Growth Rate

The development of *S. littoralis* and *M. brassicae* larvae on the four lines of *A. thaliana* Col-0 was observed from hatching until pupation to compare the influence of different GLS classes. Developmental stages were grouped as: (1) early larval development, from hatching to the beginning of the 3rd instar, and (2) late larval development, from 3rd instar to pupation. We assessed mortality, growth rate, and the duration of development.

No significant treatment-related effects were observed in either species on mortality during early and late larval development stages and on pupation success (Supplementary Table S2). However, the different food plants significantly influenced larval growth during both early and late development in both species (**Figure 3A**, Supplementary Figure S1 and Supplementary Table S3). By 6 days after hatching (at the end of the early development phase), larvae reared on plants containing no GLSs or only indolic GLSs had grown equally well. In contrast, larvae fed on plants containing only aliphatic GLS exhibited significantly reduced growth reductions of 53 and 38% for *S. littoralis* and *M. brassicae*, respectively, compared to larvae fed on the plant line with no GLSs. This reduction was even stronger for larvae fed on WT Col-0 (66 and 56% for

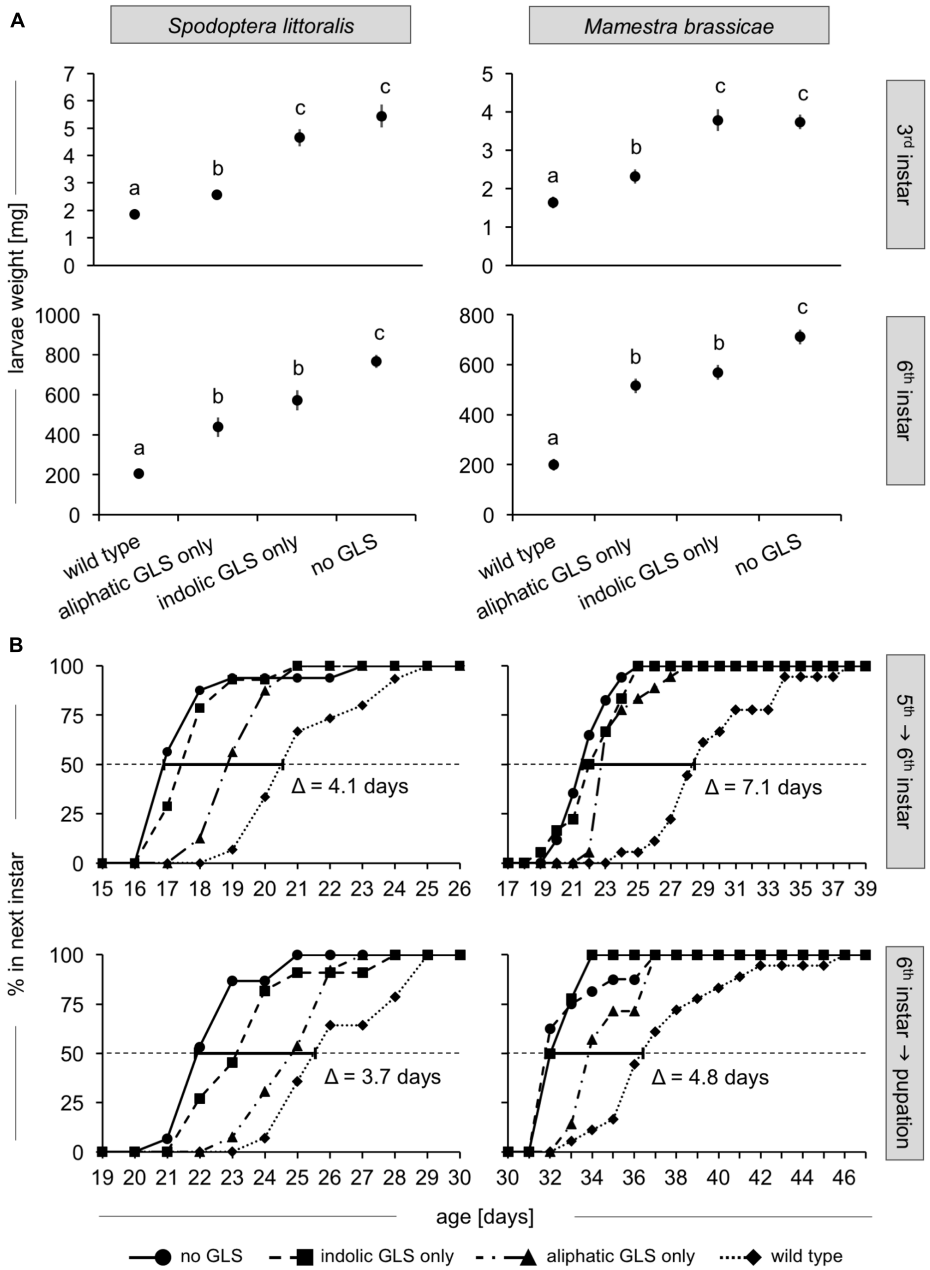

FIGURE 3 | Growth and development of *S. littoralis* and *M. brassicae* larvae on GLSs. **(A)** Larval fresh weights at the onset of the 3rd instar (upper panels, day 6 for *S. littoralis,* $P < 0.001$, $N = 10$; day 10 for *M. brassicae*; $P < 0.001$, $N = 10$), and entering 6th instar (lower panels, day 20 for *S. littoralis,* $P < 0.001$, $N = 13$–17; day 28 for *M. brassicae,* $P < 0.001$, $N = 17$–18). Data points represent the estimates of the means ± standard error from the gls model (Supplementary Table S3). Statistical analysis was performed using an *ANOVA*, and the letters denote significantly different groups based on a *Tukey post hoc test* (0.05 level). **(B)** Instar transition time for 5th to 6th instar and for 6th instar to the pupal stage. The dotted horizontal line indicates when 50% of the caterpillars had reached the next developmental stage. The bold line shows the time intervals between the transition times for larvae fed on WT and no-GLS plant lines calculated by fitting a sigmoidal curve to the data (see Materials and Methods). Data for the mean day ± standard error, and *ANOVA* and *Tukey post hoc* analysis for all instars are in Supplementary Table S4.

S. littoralis and *M. brassicae,* respectively, compared to those fed on the no-GLS line). By the onset of the 6th instar (day 20 for *S. littoralis* and day 28 for *M. brassicae* fed on no GLS-plants), larvae fed on plant lines with indolic GLS only also showed a significant reduction in growth (25 and 20% for *S. littoralis* and *M. brassicae*, respectively) compared to larvae fed on plants with no GLSs, while larvae fed on aliphatic GLS-only plants showed an even stronger reduction (43 and 27% for *S. littoralis* and *M. brassicae*, respectively). Again, larvae reared on WT Col-0 had significantly lower weights than those fed on any of

TABLE 1 | Contrasting effect of feeding on various GLS-containing plant lines on larval growth at different stages of development.

Instar		Plant line fed on					
		Wild type	**Aliphatic GLS only**	**Indolic GLS only**	**No GLS**	***P*-value**	***F*/$\chi^{2\#}$ value**
Spodoptera littoralis	3rd	3.20 ± 0.26 (a)	4.17 ± 0.34 (ab)	4.12 ± 0.42 (ab)	**4.74 ± 0.42 (b)**	0.030	3.203
	4th	18.79 ± 1.20	18.73 ± 1.81	17.96 ± 2.96	16.16 ± 1.13	n.s.$^{\#}$	1.941
	5th	69.80 ± 5.55	68.62 ± 5.24	69.06 ± 7.33	83.95 ± 5.66	n.s.	1.653
	6th	**212.05 ± 20.87 (a)**	135.88 ± 11.20 (b)	114.83 ± 8.40 (b)	146.72 ± 9.27 (b)	<0.001	8.985
Mamestra brassicae	4th	6.55 ± 0.67 (a)	8.86 ± 0.64 (ab)	8.16 ± 0.61 (ab)	**9.33 ± 0.67 (b)**	0.026	3.291
	5th	33.65 ± 2.88	34.53 ± 1.45	33.37 ± 1.61	30.51 ± 1.46	n.s.	0.874
	6th	**94.59 ± 3.73 (a)**	56.19 ± 2.41 (b)	75.22 ± 3.91 (c)	68.54 ± 7.71 (c)	<0.001$^{\#}$	35.070

The larval mass gained for each instar (in mg) is presented. Listed are the means ± standard error (N = 10–16 for S. littoralis, N = 14–18 for M. brassicae). The bold print highlights the plant treatment giving the highest mass gain when these were significantly different. Statistical analysis was performed using ANOVA or $^{\#}$Kruskal–Wallis test and the letters denote significantly different groups based on a Tukey post-hoc test (0.05 level; see Materials and Methods for details).

the other plant lines (73 and 72% reduction *S. littoralis* and *M. brassicae*, respectively) compared to larvae fed on plants without GLSs.

GLSs Extended Larval Development Time

To examine whether feeding on plants of different GLS content influenced the duration of larval instars, the time points of instar change were defined as the day on which 50% of caterpillars in a feeding treatment entered the next instar (dotted line, **Figure 3B**). Instar duration in both species was lengthened by the ingestion of GLS. Aliphatic GLS had a more pronounced effect than indolic GLS, and their combination in WT Col-0 caused an even stronger effect. First instar duration was already lengthened in WT Col-0-fed compared to no-GLS-fed larvae by 1.4 and 0.9 days for *S. littoralis* and *M. brassicae*, respectively (Supplementary Table S4). For each following instar change, the WT Col-0-fed caterpillars experienced an additional delay compared to caterpillars fed on no-GLS plants. By the last instar change, this resulted in an accumulated delay of 4.1 days for *S. littoralis* and 7.1 days for *M. brassicae* between the WT-fed and no GLS-fed treatments.

Surprisingly, the cumulative developmental time difference between wild type and no-GLS-fed caterpillars decreased at the time of pupation. That is, WT-fed insects "caught up" during their last instar, reducing the gap between groups: treatment-associated gaps measured during molting into the last larval instar were shortened by 0.4 days for *S. littoralis* and 2.3 days for *M. brassicae* on pupation. A comparison of the growth rates in each instar during late development stages was therefore performed (**Table 1**). The growth rates (mg/instar) were 48 and 42% higher for the no-GLS-fed larvae than those fed WT Col-0 in the 3rd and 4th instar for *S. littoralis* and *M. brassicae*, respectively. However, this pattern was reversed during the 6th instar, when larvae fed on WT Col-0 leaves grew significantly faster (by 1.4-fold for both species) than larvae fed on no-GLS plants. Examination of pre-molt larval weights (Supplementary Table S5) showed that the average weight before the 4th instar in *M. brassicae* reflects the trends observed during development: Larvae fed on wild type plants are significantly lighter than larvae that were challenged with only one GLS class or none (Supplementary Figure S1). The weights before the last instar change did not significantly differ between treatments. Similarly, the average weight before each molt did not differ between treatments for *S. littoralis* larvae. However, *S. littoralis* larvae fed on GLS-containing plants showed a trend in the 5th and 6th instar toward higher pre-molting weight than no GLS-fed larvae. Both of these developments reflect the trends in growth rate in the later instars.

ITC Detoxification Rate, But Not Leaf Fragment Size Changed during Larval Development

To try to account for the changing growth rates of caterpillars on different plant lines during development, we quantified the detoxification products found in the feces at different developmental stages from the major aliphatic GLS, 4msob-GLS (**Figure 2**), which is hydrolyzed to 4msob-ITC. Both *S. littoralis* and *M. brassicae* detoxified 4-msob-ITC by conjugation to GSH and then metabolized the initial conjugate further via the mercapturic acid pathway (**Figure 1A**). We measured the feces of larvae that had fed on plants containing 4msob-GLS, i.e., those fed on WT plants and those fed on plants with aliphatic GLS only (**Figure 3**). The total amounts of detoxification products excreted by *S. littoralis* were higher than those found in the feces of *M. brassicae* and depended significantly on the stage of larval development, decreasing with age during both early and late development phases. In contrast, *M. brassicae* larvae showed a trend toward increased detoxification during development with a significant increase at day 10 (Supplementary Table S6). The relative distribution of detoxification products changed over time for both species, with increasing proportions of later products of the mercapturic acid pathway (NAC- and Cys-conjugates) instead of earlier ones (CysGly- and GSH-conjugates). The presence of indolic GLS (in WT Col-0) did not significantly influence the total amount of these 4msob-ITC detoxification products in either species, with larvae reared on plants with aliphatic GLS only not excreting more detoxification

products than larvae reared on WT Col-0 (Supplementary Tables S6, S7).

Another way to account for changing growth or detoxification rates of larvae during development would be if insects changed their feeding behavior. Lepidopteran larvae feeding on cyanogenic glucoside-containing plants have been reported to snip off relatively large leaf fragments and so cause less tissue damage and reduced formation of toxic hydrolysis products (Pentzold et al., 2014). Production of larger leaf fragments might results from larger mandible size or a change in feeding behavior. We hypothesized that larger *S. littoralis* larvae might encounter fewer hydrolysis products than smaller larvae due to the decrease observed in metabolites of 4msob-ITC in frass. However, the size of leaf fragments in the feces of 4th and 6th instar *S. littoralis* caterpillars fed on WT plants did not differ from those fed on no-GLS plants (Supplementary Table S8). Neither instar nor plant treatment influenced the average leaf fragment sizes in frass.

DISCUSSION

GLS Metabolism by Generalist Lepidopterans Is Completely Different from That of Specialists

Despite the potential for GLSs to be hydrolyzed on plant damage and form toxic products, many insects feed and perform well on GLS-containing species. This has been attributed to several well-described detoxification processes (reviewed in Jeschke et al., 2016a). For instance, among specialist lepidopteran feeders on GLS-containing plants, *P. rapae* diverts GLS hydrolysis to form nitriles instead of the more toxic ITCs (Wittstock et al., 2004), while *P. xylostella* modifies GLSs by desulfation, which prevents myrosinase-catalyzed hydrolysis altogether (Ratzka et al., 2002), but information is lacking on other possible routes of GLS metabolism in these insects. Five species of generalist feeding lepidopterans have been reported to deactivate ITCs by conjugation to GSH (Schramm et al., 2012), a general detoxification pathway for nucleophilic toxins in many organisms, but most of these insects have not been systematically analyzed for other potential GLS metabolites. The feeding of radiolabeled 4msob-GLS in this study to both specialists and generalists has now put their GLS metabolism on a firmer quantitative basis.

Specialist lepidopteran herbivores were found to metabolize 4msob-GLSs principally to specialized detoxification products, including over 80% conversion to desulfo-GLSs in *P. xylostella* and over 90% conversion to nitriles in *P. rapae*, with no evidence of ITC conjugation. On the other hand, metabolism in the generalist lepidopterans studied gave rise principally to GSH conjugates of 4msob-ITC or free 4msob-ITC (60–80%) without any detection of desulfo-GLSs (**Figures 1**, **3**). A background level of 8–15% nitrile formation was found in all feeding experiments, probably a consequence of *in planta* nitrile formation after activation of labeled glucosinolates in this experiment. Thus there are fundamental differences in the way that specialist and generalist lepidopteran larvae process dietary glucosinolates. Among the four generalists investigated, there were no differences in the gross patterns of GLS metabolism despite the fact that their frequency of feeding on GLS-containing plants varies. *M. brassicae* and *T. ni* use Brassicaceae frequently as host plants, while *S. littoralis* and *H. armigera* are seldom reported on GLS-containing plants (Brown and Dewhurst, 1975; Firempong and Zalucki, 1989; Popova, 1993; Chow et al., 2005). The relatively high proportion of free ITCs observed in the feces of generalists (up to 78%) was surprising. The detoxification of ITCs with via conjugation to GSH may not be very efficient, or alternatively these toxic GLS hydrolysis products could have arisen from ITCs initially conjugated with GSH but then dissociated. This process could reduce the nitrogen and sulfur cost of conjugation by recovering cysteine and other amino acids but could also increase the risk of toxicity.

GLSs, Especially Aliphatic GLSs, Reduce Generalist Lepidopteran Performance over Much of the Larval Life Span

The abundance of free ITCs as GLS metabolites in generalist lepidopterans motivated us to explore GLS effects on larval performance in detail. While most previous studies of this type have been of short duration (reviewed in Jeschke et al., 2016a), we investigated larval survival, growth and development from neonate to pupation employing *A. thaliana* lines knocked out in aliphatic or indolic GLS biosynthesis, the same lines previously used in many herbivore feeding studies (Zhao et al., 2002; Beekwilder et al., 2008; Sun et al., 2009). Chemical analysis of these plant lines confirmed the GLS composition previously reported. In addition, we found no major differences among the lines in sugar, protein or free amino acid content making it likely that GLS are responsible for any differences in insect performance observed.

The presence of aliphatic or indolic GLSs or both significantly reduced the larval growth rate of the two lepidopteran species studied (**Figure 3A**) leading to longer development times and a delay in pupation (**Figure 3B**, Supplementary Figure S1 and Supplementary Table S4). The effect of both GLS classes together was stronger than either one alone. Both classes of GLSs have been previously demonstrated to reduce the growth of generalist lepidopteran larvae (Beekwilder et al., 2008; Schlaeppi et al., 2008; Müller et al., 2010). The longer larval development could be detrimental in a natural setting due to longer exposure to predators (the slow growth-high mortality hypothesis) and may result in other fitness costs such as fewer generations per season (Clancy and Price, 1987; Benrey and Denno, 1997). In fact, *S. littoralis* larvae raised on cabbage plants gave rise to adult moths that avoided Brassicaceae as host plants for oviposition (Thöming et al., 2013; Proffit et al., 2015) demonstrating that – presumably negative – larval experiences on brassicaceous plants later influence adult choices. In contrast to the effects on growth and development, the presence of GLSs had no effect on larval survival. However, some previous studies have indeed

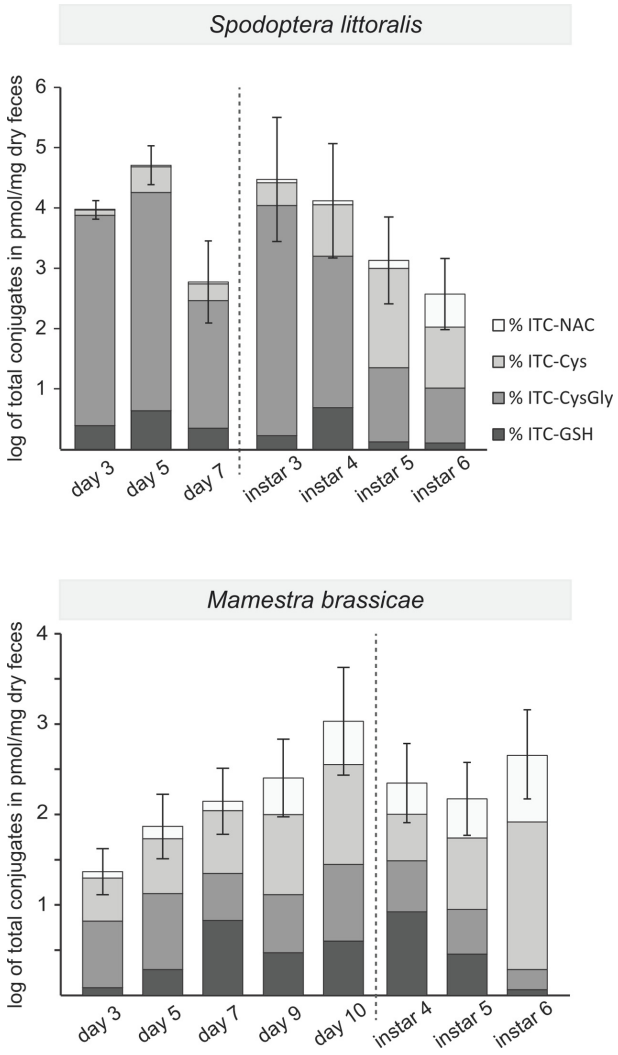

FIGURE 4 | Excretion of 4msob-ITC detoxification products in the feces at different stages of larval development. The sum of the ITC conjugates is plotted in log scale (mean ± standard error) with a division (dotted line) between early development (1st to 3rd instar, listed by day) and late development (listed by instar). Relative amounts of the individual metabolites within each bar are shaded in gray tones (expressed as percentages of the total). The total amounts of conjugates and percentages are averaged for larvae fed on the two plant lines containing aliphatic GLS (WT Col-0 and aliphatic GLS only). *S. littoralis*, early development: factor (day): $P = 0.003$, late development: factor (day): $P < 0.001$; *M. brassicae,* early development: factor (day): $P = 0.020$, late development: factor (day): P n.s (see Supplementary Table S6 for detailed information).

found higher mortality of lepidopteran larvae when fed on GLS-containing diets (Lichtenstein et al., 1962; Li et al., 2000; Agrawal and Kurashige, 2003; Ahuja et al., 2010; Santolamazza-Carbone et al., 2015).

In general, aliphatic GLSs seem to be more detrimental to lepidopteran larvae performance than indolic GLSs (Schlaeppi et al., 2008; Müller et al., 2010; Jeschke et al., 2016a), as found in this study also. The weight reduction caused by feeding on WT Col-0 was greater than that of either GLS class alone, suggesting synergistic or additive effects between two classes as previously proposed for *Spodoptera exigua* (Müller et al., 2010). However, synergism cannot be rigorously tested here since the indolic GLS-only plant line contained significantly more of this class than the WT line (**Figure 2**). Nevertheless, we can exclude the possibility that the presence of indolic GLS inhibits the detoxification of aliphatic GLS and vice versa (**Figure 4**).

Older Larvae May Grow Better on GLS-Containing vs. Non-GLS Plant Lines

Despite the overall negative impact of GLSs on the growth of both lepidopteran species during larval development, GLS-fed insects seemed paradoxically to "catch up" to non-GLS-fed controls during the last instar. For example, *S. littoralis* larvae fed on WT plants grew significantly faster in their final instar than those fed on the plant line without GLSs (**Table 1**). There are several possible explanations for this unexpected reversal of GLS impact in older larvae. One is that a change in the direction of GLS metabolism occurred allowing older insects to degrade GLSs and exploit the nitrogen and sulfur for their own growth and development. However, no major changes in the course of metabolism were evident between the penultimate and ultimate instars (**Figure 4**).

Another possible explanation for the improved ability of older larvae to grow on GLS-containing diets is that the rate of detoxification increased with age. This is true in *M. brassicae* at some early stages in development, perhaps because of increases in the efficiency of ITC conjugation due to more GSH or higher amounts of glutathione *S*-transferase activity. However, in *S. littoralis*, the rate of GLS detoxification actually declined with development. Less ITC might be reaching the midgut epithelial tissue in older larvae due to the larger volume of the gut, or insects retain the food bolus for a smaller timespan. Alternatively, previously conjugated ITCs may undergo more complete dissociation to free ITCs in older larvae resulting in fewer conjugates being excreted. This would benefit larvae by salvaging cysteine residues, which are limiting for GSH biosynthesis (Jeschke et al., 2016b). Or, older larvae may bite off and ingest larger leaf fragments due to their larger mandibles and thus cause less cell damage and subsequently less GLS hydrolysis. Feeding behavior that gives larger leaf fragments has been observed during lepidopteran herbivory on cyanogenic glucoside-containing plants, and reduces exposure to toxic hydrogen cyanide (Pentzold et al., 2014; Pentzold et al., 2015). However, we found no evidence that *S. littoralis* increases the size of the leaf fragments it produces in the last instar or as a result of increased GLSs in the diet (Supplementary Table S8).

CONCLUSION

The metabolism of GLSs by the generalist lepidopterans studied does not block the activation of these defenses, but instead conjugates toxic ITCs to glutathione after their hydrolysis. This metabolism prevents mortality, but does not stop GLSs from

decreasing the growth and development of these insects. Thus GLSs may still function as effective defenses against generalist lepidopteran herbivores if longer development times increase the risk of predation. In addition, these compounds may act defensively as feeding deterrents, a property not tested here. Sub-lethal effects may be more typical for plant defenses than outright lethality in part because synthesis of concentrations high enough to kill feeding herbivores may be too expensive considering the actual risks of herbivory. Growth reduction, deterrency and increased predation may together be sufficient to reduce herbivore levels.

AUTHOR CONTRIBUTIONS

VJ, EK, JG, and DV conceived and designed the experiments. EK, KS, AS, and DV performed the metabolism experiment. VJ and EK performed the development experiment. VJ, EK, and GK performed statistical analysis and VJ, EK, JG, and DV interpreted the results and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.01995/full#supplementary-material

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ORIGINAL RESEARCH
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Plant Defensive β-Glucosidases Resist Digestion and Sustain Activity in the Gut of a Lepidopteran Herbivore

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Two-component activated chemical defenses are a major part of many plants' strategies to disrupt herbivory. The activation step is often the β-glucosidase-catalyzed removal of a glucose moiety from a pro-toxin, leading to an unstable and toxic aglycone. While some β-glucosidases have been well studied, several aspects of their roles *in vivo*, such as their precise sites of enzymatic activity during and after ingestion, and the importance of particular isoforms in plant defense are still not fully understood. Here, plant defensive β-glucosidases from maize, white mustard and almonds were shown to resist digestion by larvae of the generalist lepidopteran *Spodoptera littoralis*, and the majority of the ingested activities toward both general and plant pro-toxic substrates was recovered in the frass. Among other proteins potentially involved in defense, we identified specific plant β-glucosidases and a maize β-glucosidase aggregating factor in frass from plant-fed insects using proteomic methods. We therefore found that, while *S. littoralis* larvae efficiently degraded bulk food protein during digestion, β-glucosidases were among a small number of plant defensive proteins that resist insect digestive proteolysis. These enzymes remain intact in the gut lumen and frass and can therefore further catalyze the activation of plant defenses after ingestion, especially in pH-neutral regions of the digestive system. As most of the ingested enzymatic activity persists in the frass, and only particular β-glucosidases were detected via proteomic analyses, our data support the involvement of specific isoforms (maize ZmGlu1 and *S. alba* MA1 myrosinase) in defense *in vivo*.

Keywords: plant two-component defense, β-glucosidase, myrosinase, *Spodoptera*, frass proteomics, glucosinolate, benzoxazinoid, DIMBOA

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INTRODUCTION

Many plants utilize activated plant chemical defenses to fend off herbivore attacks. These small molecular weight compounds are stored as biologically inactive pro-toxins in the intact tissue, and are then enzymatically activated to form bioactive toxic compounds after tissue damage by an attacking herbivore (Halkier and Gershenzon, 2006; Morant et al., 2008). This two-component system avoids the problem of auto-toxicity, as these defensive molecules can be stored without ill

effects to the plants. A very common activation strategy is the enzymatic removal of a protecting glucose group by a β-glucosidase (Morant et al., 2008). The resulting aglycone is typically more reactive than the parent glucoside and accordingly displays increased toxicity. Furthermore, its lower polarity facilitates diffusion through cell membranes and penetration into the herbivore cells where toxicity is often exerted. In order to avoid uncontrolled and unnecessary activation, the two components of the defensive system (the glucosylated pro-toxin and the activating β-glucosidase) are kept spatially separated. These meet only when tissue integrity is lost, leading to mixing of the contents of different cells and cellular compartments. The increased water-solubility conferred by glycosylation allows for storage of millimolar concentrations of compounds such as glucosinolates, benzoxazinoids, and cyanogenic glucosides. While much is known about the chemistry and compartmentalization of activated defenses, it is not clear whether they are activated only on plant damage or also later in the herbivore gut.

Among activated plant chemical defenses, one of the best-studied systems involves glucosinolates and myrosinases that are mostly restricted to brassicaceous plants such as broccoli, mustard, and rapeseed (Halkier and Gershenzon, 2006). Hydrolysis of glucosinolates results in the liberation of glucose and an unstable *N*-(sulfooxy)alkylimidothioic acid aglycone that undergoes rearrangement in aqueous media to form compounds of varying toxicity. These products include isothiocyanates (mustard oils), nitriles, epithionitriles, and thiocyanates (**Figure 1A**) depending on the reaction conditions (e.g., pH, concentrations of metal cations and plant-derived specifier proteins) and the nature of the glucosinolate side chain (Bones and Rossiter, 2006; Wittstock and Burow, 2010; Jeschke et al., 2016b). Among those, isothiocyanates are considered particularly toxic, reacting with biological nucleophiles such as glutathione (GSH) and certain amino acid side-chains in proteins (Bruggeman et al., 1986; Mi et al., 2011; Mithöfer and Boland, 2012; Schramm et al., 2012; Jeschke et al., 2016a). The sugar moiety in glucosinolates is connected to the aglycone via an *S*-β-glucosidic bond. The β-glucosidases that activate glucosinolates are therefore β-thioglucoside glucohydrolases, commonly named myrosinases.

Cyanogenic glucosides are similarly activated by removal of their sugar group(s), leading to α-cyanohydrin aglycones. For example, one sugar molecule from the diglucoside amygdalin, found especially in fruits of the Rosaceae, is removed by amygdalin hydrolases to give prunasin, and this cyanogenic monoglucoside is further hydrolyzed into mandelonitrile and glucose by prunasin hydrolases (**Figure 1B**). The mandelonitrile aglycone thus formed may then dissociate into benzaldehyde and the very toxic hydrogen cyanide (HCN) non-enzymatically, especially under high pH (Fomunyam et al., 1985), or with enzymatic catalysis by mandelonitrile lyase (Swain and Poulton, 1994a,b). Emission of HCN, or cyanogenesis, occurs in a surprisingly large number of plant species of importance to humans, being a feature of grain crops such as oats, sorghum and maize, fruit crops such as apples and mangoes, and in the sub-Saharan staple food crop cassava.

Finally, benzoxazinoids (BXDs) are indole-derived defense compounds produced by a small number of agriculturally relevant grass (Poaceae) crops, including maize, wheat and rye (Frey et al., 1997; Niemeyer, 2009), and in scattered dicotyledonous species. They are mostly accumulated in intact plant tissues as the corresponding BXD glucosides. These lead, after glucose hydrolysis, to cyclic hemiacetal aglycones that equilibrate to α-oxoaldehydes by ring opening (**Figure 1C**) and are reactive toward a wide range of biological nucleophiles (Maresh et al., 2006; Dixon et al., 2012). The major BXD glucoside in young maize leaves is (2*R*)-2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA-Glc), whose aglycone DIMBOA is considered to be one of the primary maize defensive chemicals against generalist leaf-chewing herbivores (Cambier et al., 2000; Glauser et al., 2011).

Glycosidases are a generally well-studied class of enzymes, due in part to their industrial utility. Plant β-glucosidases are classified as part of the Family 1 of glycoside hydrolases, catalyzing the hydrolysis of a terminal β-D-glucose residue via a mechanism in which the anomeric configuration of the glucose is retained. These β-glucosidases generally have a subunit molecular mass of 55–65 kDa, slightly acidic pH optima (pH 5–6), and an absolute specificity toward β-glucosides (Fowler, 1993). The tertiary structure of plant β-glucosidases belonging to family 1 glycoside hydrolases is highly conserved, as are the active site amino acids involved in binding of the sugar moiety. The residues that define the aglycone binding pocket are found at conserved positions in the active site, but are variable. The regions involved in oligomerization also vary among β-glucosidases. This is reflected in the diversity of quaternary structures observed in plant β-glucosidases, where the different active enzymes can be monomers, dimers, tetramers, or even higher oligomers. While the glucoside pro-toxins are typically stored in the vacuole, the subcellular localization of activating β-glucosidases differs in monocotyledonous and dicotyledonous plants. Monocotyledonous β-glucosidases contain an N-terminal transit peptide leading to localization to plastids. In dicotyledons, these activating enzymes contain an N-terminal signal that results in co-translational glycosylation and secretion (Morant et al., 2008). Protein glycosylation promotes stability and solubility and mediates protein localization and protein–protein interactions (Nastruzzi et al., 1996; Burmeister et al., 1997), but is not essential for full enzymatic activity (Zhou et al., 2012).

Myrosinases (E.C. 3.2.1.147), the thioglucosidases responsible for hydrolytic activation of glucosinolates, are commonly present in multigene families, with members possibly having different roles in different tissues. The *Arabidopsis thaliana* genome encodes for six myrosinase genes (*tgg*1-6), with two apparently being pseudogenes, and two genes being expressed each in aerial parts (*tgg*1/2) and underground tissues (*tgg*4/5) (Andersson et al., 2009; Zhou et al., 2012). More recently, additional *A. thaliana* proteins (PEN2 and PYK10) have been described to have myrosinase-like activities restricted toward indolic glucosinolates (Bednarek et al., 2009; Nakano et al., 2014), but their specific functions in plant defense against herbivores are still unclear.

The widely commercially available β-glucosidase from almonds, which produce cyanogenic glycosides, has been studied

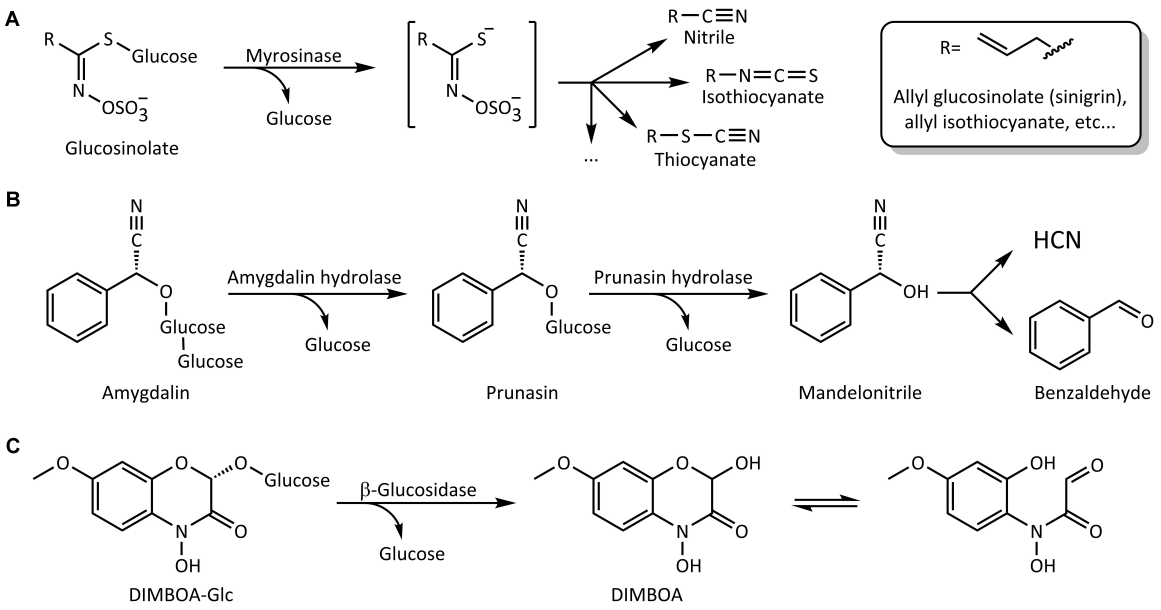

FIGURE 1 | Schematic representation of some reactions catalyzed by plant β-glucosidases. **(A)** Activation of glucosinolates by myrosinase. **(B)** Step-wise hydrolysis of the cyanogenic diglucoside amygdalin. **(C)** Hydrolytic activation of the benzoxazinoid glucoside DIMBOA-Glc and formation of the open-ring aglycone form in solution.

biochemically in great detail. While it was shown to artificially induce herbivory-like volatile emission in cabbages (Mattiacci et al., 1995), its potential roles *in planta* also remain to be better characterized. It can be isolated in large amounts from sweet almonds as a mixture of isozymes, and it has been massively used as a model enzyme in the study of β-glucosidase mechanism, kinetics, and chemical inactivation [for example (Shulman et al., 1976; Grover and Cushley, 1977; He and Withers, 1997)]. Its stability also allows its use in the synthesis of glucosides *in vitro* in high concentrations of organic solvents, exploiting the reversibility of the glucoside hydrolysis reactions [for example (Vic et al., 1995; Ducret et al., 2006)]. Nevertheless, while this enzyme accepts many compounds *in vitro*, information about whether it hydrolyzes cyanogenic glucosides *in vivo* is lacking.

In maize, BXD β-glucosidase reactions can be performed by two enzymes, ZmGlu1 and ZmGlu2 (Czjzek et al., 2000), which share 90% sequence identity. ZmGlu1 and ZmGlu2 hydrolyze a broad spectrum of artificial and natural compounds in addition to the benzoxazinoid DIMBOA-Glc (Czjzek et al., 2000, 2001). The maize β-glucosidase Zm-p60.1, of which ZmGlu1 is the corresponding allozyme, has been implicated in the release of active cytokinins from inactive stored cytokinin-*O*-glucosides (Campos et al., 1993; Zouhar et al., 1999; Zouhar et al., 2001).

Some maize proteins have been shown to interact directly with β-glucosidases resulting in the formation of large aggregates. Biochemical and immunological studies demonstrated that β-glucosidase activity could not be detected in zymograms of certain maize genotypes (so-called "nulls") due to the enzyme occurring as large insoluble or poorly soluble quaternary complexes (Esen and Blanchard, 2000). This complexation is mediated by a β-glucosidase-aggregating factor (BGAF), a chimeric maize protein consisting of an N-terminal dirigent (disease response) domain and a C-terminal jacalin-related lectin domain (Kittur et al., 2007). An analogous jacalin-like lectin protein from wheat (*Triticum aestivum*), HFR1, has direct antifeedant effects and is involved in resistance against the Hessian fly (*Mayetiola destructor*) (Subramanyam et al., 2008), but its individual protein binding partners were not examined. The enzymatic activities of ZmGlu1 and ZmGlu2 are unaffected by BGAF binding, suggesting that interaction with BGAF does not change the conformation of the enzymes (Blanchard, 2001). Similar myrosinase-binding proteins (MBP) have been found in rapeseed (Falk et al., 1995) and in *A. thaliana* (Takechi et al., 1999; Takeda et al., 2008), where they also lead to formation of higher-molecular weight active complexes. However, whether these protein aggregates are stable in herbivore guts after ingestion and whether they play any roles in the hydrolysis of activated defenses *in vivo* is not yet known.

Nitrogen in the form of proteins and essential amino acids is considered the most limiting macronutrient in herbivorous diets, justifying the evolution of efficient and flexible cocktails of digestive proteases in lepidopteran herbivores (Terra and Ferreira, 1994; Kuwar et al., 2015). Accordingly, most bulk plant protein is effectively hydrolyzed after ingestion by these insects (Chen et al., 2005) and can be used for insect growth. However, a growing number of plant proteins have been described in insect frass, several of which remaining catalytically or biologically active after passing through the insect digestive tract and acting in plant defense both during and after ingestion (Chen et al., 2005, 2007; Chuang et al., 2013; Ray et al., 2016).

In studying plant two-component activated defenses, it is important to learn if plant β-glucosidases and other hydrolyzing

enzymes critical to defense survive initial proteolysis and are active in the insect gut. Recent experiments using insects fed on maize leaves showed that maize β-glucosidases involved in benzoxazinoid metabolism are active even after going through insect digestion (Glauser et al., 2011; Wouters et al., 2014). Hence we have now examined the stability of plant defensive β-glucosidases after digestion by a generalist insect herbivore (*Spodoptera littoralis*) in order to better understand their roles in defense. We investigated three different β-glucosidases said to be involved in benzoxazinoid, glucosinolate, and cyanogenic glycoside activation, respectively, by determining their presence in protein extracts made from insect frass and comparing their catalytic activity in non-ingested material and after passage through the gut.

MATERIALS AND METHODS

Materials

Experiments with maize (*Zea mays*) used seeds of the variety Badischer Gelber from Kiepenkerl (Bad Marienberg, Germany) while white mustard (*Sinapis alba*) seeds were obtained from N. L. Chrestensen (Erfurt, Germany). Maize and white mustard were sown in plastic pots (9 cm × 9 cm) with two plants per pot under controlled light and temperature conditions (16:8 h light/dark, day-time temperature 22°C, night-time 20°C). White mustard thioglucosidase (180 U/g toward sinigrin), almond (*Prunus dulcis*) β-glucosidase (5.2 U/mg toward salicin), sinigrin (allyl glucosinolate), amygdalin and *p*-nitrophenyl-β-D-glucoside (pNPG) were obtained from Sigma-Aldrich (Munich, Germany). Larvae of *S. littoralis* (Lepidoptera: Noctuidae) were reared from eggs kindly provided by Syngenta Crop Protection (Stein, Switzerland) on artificial diet modified from Bergomaz and Boppré (1986) at 18°C under 12:12 h light cycle, and used at the fourth instar. The diet was prepared as follows: 2.4 g agar-agar was boiled with 80 mL tap water and added to a mixture of 25 g white bean flour, 450 mg ascorbic acid, 250 mg 4-ethylbenzoic acid and 25 mL tap water. The diet was stirred until hand-warm (∼40°C) and then supplemented with 45 mg α-tocopherol in 625 μL canola oil and 200 μL 3.7% formaldehyde.

Maize β-Glucosidase Extraction

About 100 maize kernels were washed with 80% ethanol for 2 min and 3% sodium hypochlorite with 0.5% Tween 20 for 15 min, then left in a Petri dish with water for 2 days to germinate, and sown as described above. Six- to seven-day-old seedlings were harvested, frozen and ground in liquid nitrogen and stored at −80°C prior to extraction. Enzyme extraction and cryoprecipitation were performed according to (Esen, 1992) with the final supernatant concentrated with Centricon Plus-70 regenerated cellulose membrane 30,000 NMWL ultracentrifugation filters (Merck Millipore, Hessen, Germany) for about 40 min. All operations were carried out between 0 and 4°C. The enzymatic activity of the extract was determined using the non-specific substrate pNPG as described below. The final enzymatic extract was adjusted to 1 U/mL.

DIMBOA-Glc Extraction

About 70 maize kernels were germinated as above and grown in the dark. Seven-day-old seedlings were harvested, weighed, and quickly frozen and ground in liquid nitrogen. Plant material was extracted with 3 mL of methanol per gram of ground plant material, quickly mixing the ground material in the solvent to avoid degradation. The suspension was centrifuged at 3,400 *g* for 10 min and the supernatant was collected. The extraction and centrifugation steps were repeated two more times, and the combined supernatants were filtered. Ammonium sulfate was added to the filtrate and left overnight. The supernatant was then filtered and evaporated under vacuum, and the solution was lyophilized. After resuspension at 200 mg/mL in 0.5% aqueous formic acid (FA)/MeOH (1:1) the compound was fractionated and purified using semi-preparative HPLC. Purification of DIMBOA-Glc used a Supelcosil LC-18-DB semi-preparative column (25 cm × 10 mm I.D., 5 μm particle size, Sigma-Aldrich, maintained at 25°C) with a flow rate of 4 mL/min, using FA (0.005%) in water and acetonitrile as mobile phases A and B, respectively. The gradient was as follows: 10–20% B (10 min), 100% B (3 min), 10% B (3 min) with detection by UV absorption at 254 nm. The solvents where evaporated partially by using a rotary evaporator, while the remaining water was removed by lyophilization, with 37.2 mg of DIMBOA-Glc obtained as a white powder.

Caterpillar Feeding and Collection of Diet and Frass Samples

In artificial diet feeding experiments, *S. littoralis* caterpillars previously fed on artificial diet were switched to a cube of artificial diet (0.5 cm × 0.5 cm) to which pure enzyme (10 μL 0.026 U/mL *P. dulcis* β-glucosidase or 10 μL 10 U/mL *S. alba* myrosinase) or the semi-purified maize enzyme extract (10 μL 1 U/mL) was applied (**Figure 2**). Negative controls used 10 μL of the corresponding buffer. Each caterpillar was kept in an individual plastic cup and allowed to feed until the cube was consumed. An additional feeding experiment used bovine β-lactoglobulin (20 μL 10 mg/mL) added to the diet. Non-ingested samples used diet cubes with added enzyme kept under identical temperature conditions but in the absence of *S. littoralis* larvae. The resulting diet and frass samples were collected and extracted by adding two metal beads (diameter 3 mm) and 500 μL of the respective buffer used for each assay, followed by vigorous agitation with a paint shaker for 4 × 4 min, and centrifugation at 4,300 *g* at 4°C for 40 min. The supernatants were collected and assayed for enzymatic activity.

Feeding experiments with leaves instead of artificial diets were performed with maize and white mustard. For maize, one piece (about 3 cm × 1.5 cm) from a half of a leaf (divided longitudinally) from a 2-week-old plant was fed, with the other corresponding half frozen and used to determine non-ingested enzyme activity (**Figure 2**). For white mustard, five leaves from a 1-week-old plant were fed per caterpillar, with a set containing another five leaves used to quantify the initial myrosinase activity being fed. Six replicates were made for each feeding experiment. After complete ingestion of the glucosidase-containing diet, a

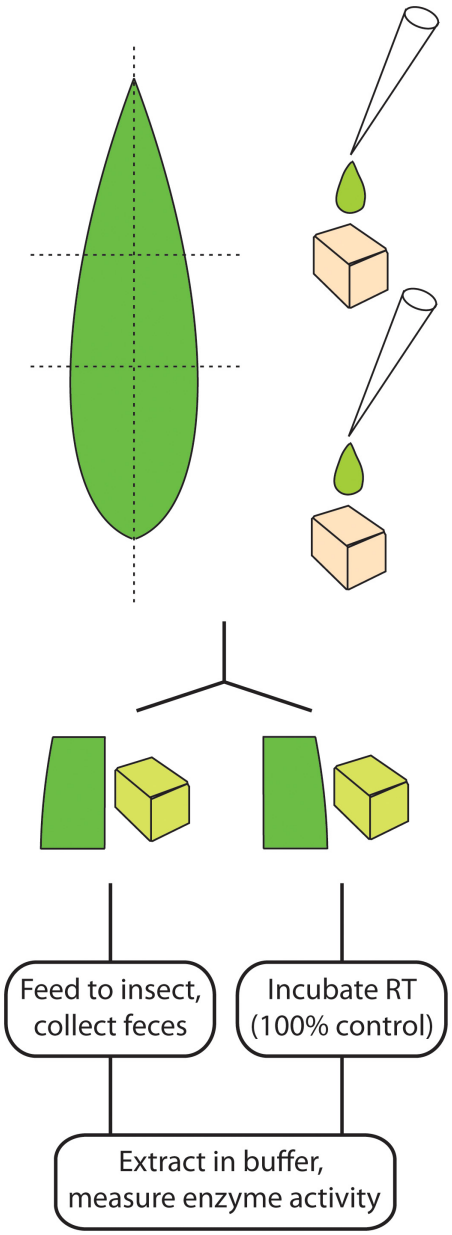

FIGURE 2 | Schematic representation of enzyme feeding strategies from divided leaves (left) and in artificial diet cubes (right).

small amount of control diet was offered to the insect, to allow for excretion of the material still present in the insect gut.

Maize β-Glucosidase Activity Assay

In initial assays the non-specific β-glucosidase substrate *p*-nitrophenyl-β-D-glucopyranoside (pNPG) was used. For that, 500 μL of enzyme extract in 10 mM phosphate buffer at pH 7.1 were incubated with 500 μL 5 mM aqueous pNPG at 25°C for 5 min. The reaction was stopped by adding 1 mL 400 mM aqueous Na_2CO_3 and freezing in liquid nitrogen until further use, and the *p*-nitrophenol liberated was measured at 400 nm in a UV-Vis spectrophotometer shortly after thawing. Other enzyme assays used DIMBOA-Glc as substrate. The standard assay mixture for DIMBOA-Glc consisted of 5 μL enzyme extract (1 U/mL), 2 μL DIMBOA-Glc in DMSO (200 μM), and 43 μL 10 mM MOPS buffer pH 7.0. After incubation at 25°C for 20 min, 50 μL MeOH/FA (1:1) were added, and the samples were analyzed by LC-MS/MS as in (Wouters et al., 2014). Assays were performed in triplicate. Statistical testing for all enzyme activity results was performed with unpaired *t*-tests using the software GraphPad (La Jolla, CA, United States). β-Glucosidase activity was evident from both the decrease of the glucoside and concomitant appearance of the corresponding aglycone, which were quantified using external calibration curves.

Almond β-Glucosidase Enzymatic Assay

The assay mixture consisted of 250 μL enzyme extract, 500 μL 0.1 M acetate buffer (pH 5.0) and 250 μL 20 mM aqueous pNPG. The reaction mixture was incubated at 37°C for 15 min and analyzed as above. Assays were performed in triplicate.

S. alba Myrosinase Enzymatic Assay

The standard assay mixture consisted of 30 μL protein extract, 20 μL 50 mM aqueous sinigrin, and 200 μL of 50 mM citrate buffer (pH 5.5). After incubation for 60 min at 37°C, the reaction was stopped by addition of 25 μL glacial acetic acid and cooling on ice. The mixture was extracted with 250 μL CH_2Cl_2 containing 0.01% (v/v) hexyl-isothiocyanate as internal standard. A 100 μL aliquot of the CH_2Cl_2 fraction was removed and left overnight on 20 mg ammonium sulfate. Assays were performed in sextuplicate. The samples were analyzed by GC-MS, with enzymatic activities measured by quantification of released isothiocyanates using an external calibration curve of allyl-isothiocyanate.

Analysis of Assay Products

LC-MS/MS analyses of DIMBOA-Glc β-glucosidase activity were performed as described in (Wouters et al., 2014). GC-MS analyses were performed in an Agilent 6890 series gas chromatograph (Agilent Technologies, Waldbronn, Germany) using an Agilent 19091S-433 capillary column (30 m × 0.25 mm × 0.25 μm), using 2 μL injection on splitless mode at 200°C. The temperature program was: 40°C for 3 min, a $10°C*min^{-1}$ ramp to 130°C, a $60°C*min^{-1}$ ramp to 300°C, held for 3 min. The total running time was 17.83 min.

Spectrophotometry Analysis

A double-beam UV-Vis spectrophotometer (UV-2401PC, Shimadzu Corporation, Kyoto, Japan) equipped with integrating sphere was used for all spectrophotometry assays.

Proteomic Analysis of Gel-Separated Proteins

Frass extracts for proteomic analyses were prepared as described above. Protein was quantified using the Bradford method, and about 200 μg of protein per sample were concentrated by lyophilization and separated using an Any kD Mini-Protean TGX Gel (Bio-Rad).

In-Gel Digestion of Proteins

Protein bands between 20 and 120 kDa were cut out from the Coomassie-stained gels, cut into small pieces, washed several times with 25 mM NH_4HCO_3 and destained with 50% ACN/25 mM NH_4HCO_3. The proteins were then reduced in-gel with 10 mM dithiothreitol at 50°C for 1 h and alkylated with 55 mM iodoacetamide at room temperature in the dark for 45 min. Next, destained, washed, dehydrated gel pieces were rehydrated for 60 min in 0.5 μM solution of porcine trypsin (Promega) in 25 mM NH_4HCO_3 at 4°C and incubated overnight at 37°C. The tryptic peptides were extracted from gel pieces using 75% ACN/5% FA, and dried down in a SpeedVac. For LC-MS, analysis samples were reconstructed in 10 μL aqueous 1% FA (Shevchenko et al., 2007).

Nano-UPLC-MS^E Analysis of Peptides

One to 8 μL of the peptide mixture was injected onto a nanoAcquity nanoUPLC system (Waters) online coupled to a Q-ToF HDMS mass spectrometer (Waters). Samples were initially transferred with 0.1% aqueous FA for desalting onto a Symmetry C18 trap-column (20 mm × 0.18 mm, 5 μm particle size) at a flow rate of 15 μL/min (0.1% aqueous FA), and peptides were subsequently eluted onto a nanoAcquity C18 analytical column (200 mm × 75 μm I.D., BEH 130 material, 1.7 μm particle size) at a flow rate of 350 nL/min with the following gradient: 1–30% B over 13 min, 30–50% B over 5 min, 50–95% B over 5 min, 95% B for 4 min, and a return to 1% B over 1 min (phases A and B composed of 0.1% aqueous FA and 100% acetonitrile containing 0.1% FA, respectively). The analytical column was re-equilibrated for 9 min prior to the next injection. LC-MS data were acquired in positive ESI mode under data-independent acquisition (MSE) controlled by MassLynx v4.1 software. The collision energy was set at 4 eV in low energy (MS) scans, and ramped from 15 to 40 eV in elevated energy (MSE) scans. The mass range (m/z) for both scans was 300–1900 and 50–1700 Da, respectively. The scan time was set at 1.5 s for both modes of acquisition with an inter-scan delay of 0.2 s. A reference compound, human glu-fibrinopeptide B [650 fmol/mL in 0.1% aqueous FA/acetonitrile (v/v, 1:1)], was infused continuously through a reference sprayer for external calibration.

Data Processing and Protein Identification

ProteinLynx Global Server (PLGS) version 2.5.2 (Waters) was used for processing of raw files and for database searching. The continuum LC-MSE data were lock-mass corrected, smoothed, background subtracted, centered, deisotoped, and charge state reduced. Thresholds for low/high energy scan ions and peptide intensity were set at 150, 30, and 750 counts, respectively. Processed data were searched against the Swissprot database[1] (downloaded on July 27, 2011) combined with protein sequences of *Z. mays*[2] (downloaded on June 11, 2013) according to the described Ion Accounting Algorithm (Li et al., 2009). Database searches were performed at 4% FDR, using the following parameters: minimum number of product ion matches per peptide (5), minimum number of product ion matches per protein (7), minimum number of peptide matches (2), and maximum number of missed tryptic cleavage sites (1). Searches were restricted to tryptic peptides with a fixed carbamidomethyl modification for Cys residues, along with variable oxidation of Met. To avoid protein interference, identified hits were classified using a script based on the classification described for PAnalyzer software (Prieto et al., 2012). According to this algorithm, proteins were divided into four groups: conclusive, non-conclusive, indistinguishable, and ambiguous. Only proteins classified as conclusive hits (proteins with at least one unique peptide) were considered as confident matches.

Estimation of Gene Expression Levels

Gene expression was estimated using the maize eFP browser of the Bio-Analytic Resource at the University of Toronto[3] using the Sekhon et al. atlas setting (Winter et al., 2007; Sekhon et al., 2011) referring to data from B73 maize. Absolute expression values in the topmost leaf at growth stage V3 ["V3_Topmost leaf" (Sekhon et al., 2011)] are reported.

RESULTS

Plants produce β-glucosidases that activate anti-herbivore defenses by cleaving various β-glucoside pro-toxins. In order to analyze the stability of these β-glucosidases during digestion by larvae from a generalist lepidopteran pest, we fed known quantities of these enzymes to *S. littoralis* caterpillars (**Figure 2**) and quantified the enzymatic activities in non-ingested material and post-ingestion. Initial *in vitro* β-glucosidase assays using the general substrate *p*-nitrophenyl-β-D-glucoside (pNPG) revealed that non-specific β-glucosidases are present in frass from untreated larvae that arise from their diet and from the insect's own endogenous activities, but these enzymes were not capable of cleaving specific plant defense compounds [such as DIMBOA-Glc (Glauser et al., 2011)]. Therefore, when using pNPG, the non-specific activities were measured (and corresponded to 5–30% of the added enzyme activities, depending on the enzyme being combined) and subtracted from activities measured in samples containing the added β-glucosidases.

Maize β-Glucosidase (Involved in Benzoxazinoid Hydrolysis) Was Active After Insect Digestion

Previous studies had shown that maize BXD-Glc β-glucosidases are active throughout the insect gut (Glauser et al., 2011), including within the hindgut and frass (Wouters et al., 2014). In order to quantify these observations, we first fed caterpillars on an artificial diet to which we applied a maize β-glucosidase enzyme extract in a similar amount/concentration to that in leaves. The

[1] http://www.uniprot.org/

[2] http://www.phytozome.net/

[3] http://bar.utoronto.ca/efp_maize

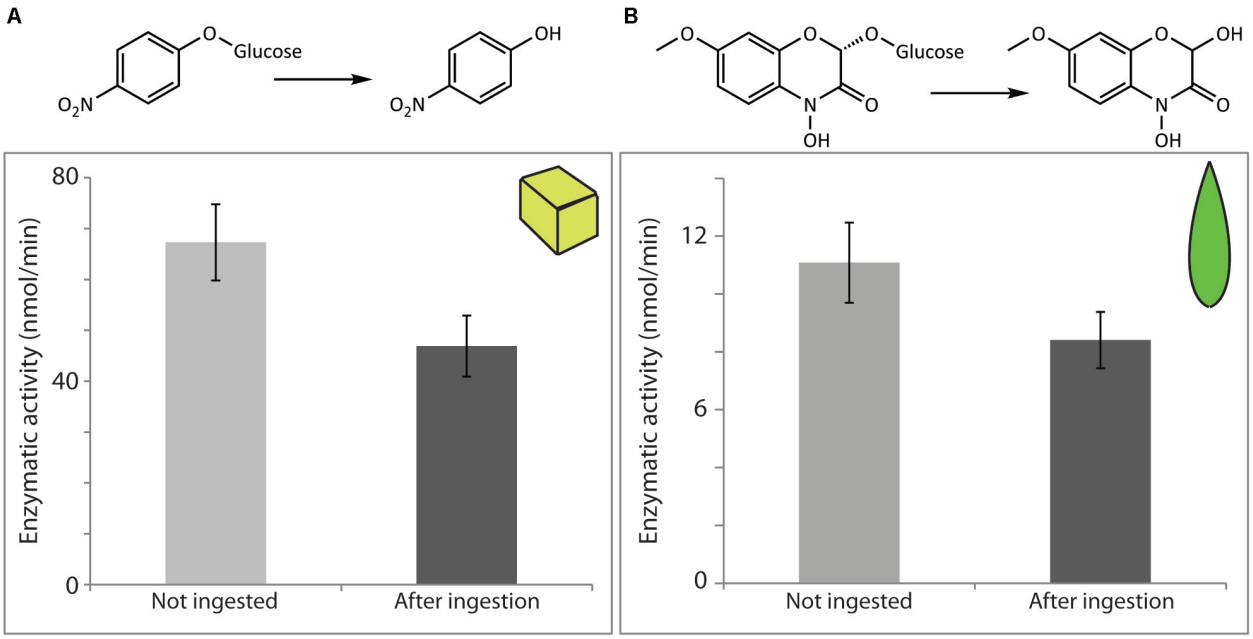

FIGURE 3 | Maize β-glucosidases were recovered in active form after digestion by *S. littoralis* larvae. Enzymatic activity levels were determined in non-ingested material and after digestion of diet cubes spiked with semi-purified maize β-glucosidase **(A)**, or after feeding on maize leaf tissue **(B)**. Activities were measured either using the non-specific β-glucosidase substrate pNPG **(A)** or the endogenous plant substrate DIMBOA-Glc **(B)**. Shown are the means ± standard errors [$N = 3$ in both analyses; $P = 0.2$ **(A)** and $P = 0.3$ **(B)**].

resulting frass was then collected and extracted. Assays using pNPG indicated that maize β-glucosidases added to the diet were indeed excreted in active form, with 69.6% of the initial activity fed to the larvae being detected in the frass extracts (**Figure 3A**).

To assess how other plant-derived proteins might influence the digestion of these maize defensive β-glucosidases, caterpillars were subsequently fed a controlled amount of young maize leaf tissue. Leaves were divided along the mid-vein, and the matching leaf pieces (i.e., from the opposing sides of the leaf blade, see **Figure 2**) were (a) fed to larvae and (b) used to quantify the initial β-glucosidase activity being fed. Quantification of activity was done with DIMBOA-Glc, a more specific and biologically relevant substrate than pNPG. We recovered 75.9% of the enzyme activity initially fed to the larvae, further confirming that the maize BXD β-glucosidases maintain most of their activity throughout the caterpillar gut (**Figure 3B**).

Myrosinase (Involved in Glucosinolate Hydrolysis) Was Active After Insect Digestion

Similarly, we quantified the activity of *S. alba* myrosinase remaining after digestion by *S. littoralis* larvae, using pure enzyme added to an artificial diet and by feeding with *S. alba* leaves. In both cases, enzymatic activities in non-ingested material and post-digestion were measured using a specific myrosinase substrate, sinigrin (allyl glucosinolate), with quantification of its main hydrolysis end-product, allyl isothiocyanate.

Myrosinase was also recovered in active form after digestion by the larvae. When the pure commercial enzyme was fed in artificial diet, 92.6% of the myrosinase activity administered was successfully recovered in the insect feces (**Figure 4A**), while 59.1% was recovered when comparing the activity in frass to that present in the corresponding unfed leaf (**Figure 4B**). The potential effects of the presence of ascorbate on the measured myrosinase activities were not tested, but this compound is known to increase *S. alba* myrosinase activity (Pihakaski and Pihakaski, 1978). Variation in its concentration between non-ingested material and post-digestion samples from plants and artificial diets might therefore account for some of the contrast in the apparent recovered activities.

Almond β-Glucosidase Was Active After Insect Digestion

For quantification of the enzymatic activity of *P. dulcis* β-glucosidase after digestion by *S. littoralis*, pure commercial enzyme added to an artificial diet was used for the feeding experiment. Enzymatic assays using amygdalin did not lead to detectable amounts of its expected hydrolysis products. Enzymatic assays performed with the non-specific substrate pNPG, on the other hand, indicated that activity was quantitatively recovered after ingestion by the larvae (**Figure 5**). The measured post-digestion activities were slightly higher than those for the undigested control, but the differences were not statistically significant ($p = 0.09$) and are likely due to natural variation in levels of endogenous insect β-glucosidase activities.

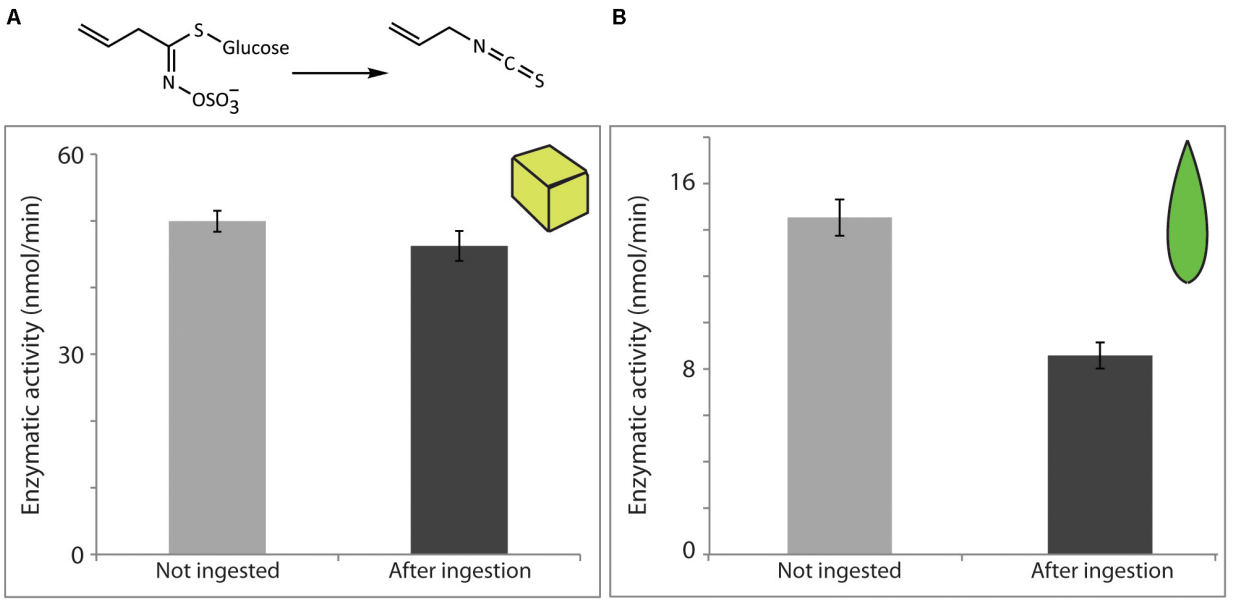

FIGURE 4 | *Sinapis alba* myrosinase activity was resistant to digestion by *S. littoralis* larvae and detected in larval frass. Enzymatic activity levels were determined in non-ingested material and after digestion of diet cubes spiked with pure enzyme **(A)** or of *S. alba* leaf material **(B)**. Activities were measured using the myrosinase-specific substrate sinigrin. Shown are the means ± standard errors [$N = 6$ in both analyses; $P = 0.2$ **(A)** and $P < 0.0001$ **(B)**].

Proteomic Analyses

Protein extracts for proteomic analyses were prepared from maize and white mustard leaves, both in non-ingested material and post-ingestion. After separation by SDS–PAGE, gel regions were excised and digested with trypsin. The resulting peptides were analyzed by LC-MS using data-independent acquisition (DIA). In this approach, referred to as MS^E, data were comprehensively acquired with an alternating mode of acquisition: a low energy (MS) mode that provided information on the mass of eluted intact peptide ions (precursors), and an elevated energy mode (MS^E) when fragmentation of these ions occurred. The ion accounting algorithm implemented in the software PLGS (Waters) was applied for searching parallel fragmentation of multiple precursor ions, matching fragment ions to their corresponding precursor peptides while taking into account their chromatographic coelution in LC-MS/MS.

These analyses led, in the case of maize, to detection of six BXD β-glucosidase-derived peptides in frass material (**Figure 6**). Two maize β-glucosidases have been identified to act on DIMBOA-Glc (ZmGlu1 and 2), but we were only able to confidently identify peptides derived from ZmGlu1. Seven peptides derived from the maize β-glucosidase aggregating factor (BGAF) protein were observed in frass extracts. Eleven peptides derived from *S. alba* myrosinase were found in frass extract samples, with unambiguous peptides indicating that the enzyme detected here is the isoform MA1. In contrast to the peptides belonging to the two maize proteins, the peptides corresponding to myrosinase were spread over a broad region of the SDS–PAGE gel (data not shown). This could result from partial deglycosylation/digestion of the proteins during passage through the gut.

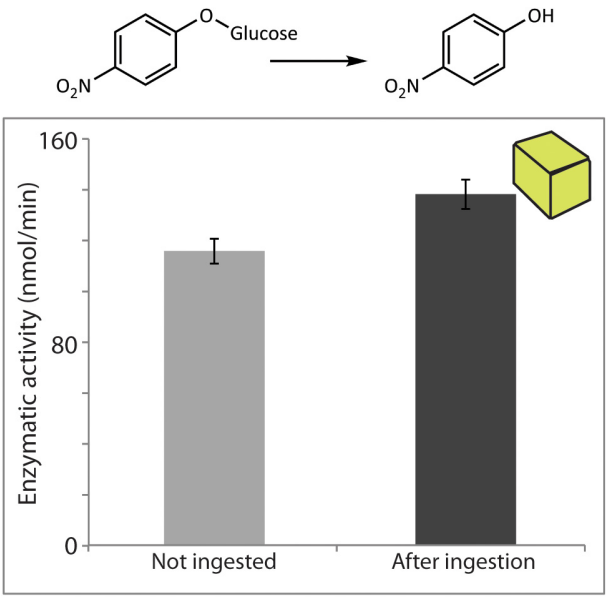

FIGURE 5 | *Prunus dulcis* glucosidase activity was resistant to digestion by *S. littoralis* larvae and detected in larval frass. Enzymatic activity levels were determined in non-ingested material and after digestion of diet cubes spiked with pure enzyme. Activities were measured using the general β-glucosidase substrate pNPG. Shown are the means ± standard errors ($N = 3$; $P = 0.09$).

Peptides belonging to 13 additional maize proteins were also detected in *S. littoralis* frass extracts (**Supplementary Table S1**), including some with predicted functions matching those previously observed in the frass of maize-fed *Spodoptera*

Maize ZmGlu1 (GRMZM2G016890)
MAPLLAAAMNHAAAHPGLRSHLVGPNNESFSRHHLPSSSPQSSKRRCNLS
FTTRSARVGSQNGVQMLSPSEIPQRDWFPSDFTFGAATSAYQIEGAWNED
GKGESNWDHFCHNHPERILDGSNSDIGANSYHMYKTDVRLLKEMGMDAYR
FSISWPRILPKGTKEGGINPDGIKYYRNLINLLLENGIEPYVTIFHWDVP
QALEEKYGGFLDKSHKSIVEDYTYFAKVCFDNFGDKVKNWLTFNEPQTFT
SFSYGTGVFAPGRCSPGLDCAYPTGNSLVEPYTAGHNILLAHAEAVDLYN
KHYKRDDTRIGLAFDVMGRVPYGTSFLDKQAEERSWDINLGWFLEPVVRG
DYPFSMRSLARERLPFFKDEQKEKLAGSYNMLGLNYYTSRFSKNIDISPN
YSPVLNTDDAYASQEVNGPDGKPIGPPMGNPWIYMYPEGLKDLLMIMKNK
YGNPPIYITENGIGDVDTKETPLPMEAALNDYKRLDYIQRHIATLKESID
LGSNVQGYFAWSLLDNFEWFAGFTERYGIVYVDRNNNCTRYMKESAKWLK
EFNTAKKPSKKILTPA

Maize ZmBGAF (GRMZM2G172204)
MASLQVTPTSAFTEWNELKFEGLYLFHTPLGSGANQARVIDNKAPIGIGA
TVVNNWTVYDGPGPNAKLVARAQGLHIQAGNWVNSFSLVFVDQRFSGSTL
EVTGIVVESGEWAIVGGTGQFAMANGVIFKKFHEQRSEGNIIQLTIHAFC
PVLGPRKRSAAKVGPWGGSGGSPVDITAEPQRLKSITVATGIAVTSIAFS
YVDSAGQTQSAGRWGGSGGETEPVIQLGDSEVLTELSGTIGNVDGHTVIT
SIKFVTSLKTYGPFGAWGDGSDTPFAIPVQQGSAIVGFFARAGVYLDALG
VYVRSL

Sinapis alba myrosinase
DEEITCQENLPFTCGNTDALNSSSFSSDFIFGVASSAYQIEGTIGRGLNI
WDGFTHRYPNKSGPDHGNGDTTCDSFSYWQKDIDVLDELNATGYRFSIAW
SRIIPRGKRSRGVNEKGIDYYHGLISGLIKKGITPFVTLFHWDLPQTLQD
EYEGFLDPQIIDDFKDYADLCFEEFGDSVKYWLTINQLYSVPTRGYGSAL
DAPGRCSPTVDPSCYAGNSSTEPYIVAHHQLLAHAKVVDLYRKNYTHQGG
KIGPTMITRWFLPYNDTDRHSIAATERMKEFFLGWFMGPLTNGTYPQIMI
DTVGERLPSFSPEESNLVKGSYDFLGLNYYFTQYAQPSPNPVNSTNHTAM
MDAGAKLTYINASGHYIGPLFEKDKADSTDNIYYYPKGIYSVMDYFKNKY
YNPLIYVTENGISTPGDENRNQSMLDYTRIDYLCSHLCFLNKVIKEKDVN
VKGYLAWALGDNYEFNKGFTVRFGLSYIDWNNVTDRDLKKSGQWYQSFIS
P

FIGURE 6 | LC-MS^E-based proteomic identification of peptides (marked with an underline) derived from maize β-glucosidase ZmGlu1, maize β-glucosidase aggregating factor (BGAF), and *S. alba* myrosinase in frass samples.

frugiperda larvae (Chuang et al., 2013). Expression levels of the corresponding genes in maize leaves (Sekhon et al., 2011) are scattered widely (**Supplementary Table S1**) and do not correlate with numbers of detected peptides or sequence coverage, supporting a differential resistance to insect proteolysis among plant proteins. Many differences were observed in the general profiles of plant-derived polypeptides in total leaf extracts (**Supplementary Table S2**) relative to those isolated from frass (**Supplementary Table S1**). One of the major distinctions was in the large subunit (rbcL) of Rubisco (UniProt P00874) considered a marker for bulk leaf protein (Chen et al., 2005, 2007). In contrast to the good (48%) coverage of rbcL primary sequence in leaf extracts, our proteomic analyses did not detect any peptides derived from rbcL in frass extracts, supporting the degradation of bulk leaf protein during passage through the larval gut. In order to confirm this finding, we fed diet cubes containing bovine β-lactoglobulin in similar concentrations to those above for β-glucosidases, and analyzed non-ingested material and post-digestion samples. We could not detect a band corresponding to β-lactoglobulin with SDS–PAGE and Coomassie staining in insect-digested samples, and no peptides matching this protein could be found in frass proteomic data within the size range analyzed (∼7–35 kDa), again indicating that efficient proteolysis of bulk protein takes place during digestion by this herbivore.

DISCUSSION

Previous work had identified defensive plant proteins in insect frass following passage through the insect digestive system (Chen et al., 2005, 2007; Chuang et al., 2013; Ray et al., 2016), but their biological activities were assessed only in a few specific cases. Here, we fed three β-glucosidases presumed to be involved in activating plant defenses to *S. littoralis* caterpillars and found that they remained active after passage through the gut. A maize β-glucosidase involved in benzoxazinoid hydrolysis, a *S. alba* myrosinase involved in glucosinolate activation, and an almond β-glucosidase associated with cyanogenic glycoside accumulation were all detected in frass using both *in vitro* enzymatic assays and proteomic analyses. Our proteomic analyses identified several additional proteins in extracts from frass of maize-fed larvae that could participate in plant defense, such as oxidases, proteases, and other glycoside hydrolases. Interestingly, both arginine decarboxylase and agmatine deiminase were observed, suggesting that these may function sequentially to deplete the essential amino acid arginine from the food bolus as part of a defensive strategy. Other predicted glycosyl hydrolases were also detected, and their utilization of benzoxazinoids as substrates and roles in defense remain to be explored.

The persistence of these proteins and corresponding activities in the frass means they are at least partially resistant to the proteases, high pH (9.5–11) and detergents present in *S. littoralis* guts. These results are in agreement with previous reports showing that other β-glucosidases, such as cassava cyanogenic β-glucosidase isozymes (linamarases) and maize and almond β-glucosidases are stable against short treatments with trypsin, high temperature and chemical denaturants (Mkpong et al., 1990; Esen and Gungor, 1993). This unusual resistance may be a general trait of plant β-glucosidases and is an important aspect of their commercial value as industrial biocatalysts. In contrast to β-glucosidases which remained active in the resulting frass, bulk dietary proteins like Rubisco and artificially added bovine β-lactoglobulin were efficiently degraded in the *S. littoralis* gut.

The biophysical proprieties that contribute to the high stability of β-glucosidases remain to be fully understood, but some structural features common among them may be responsible. Alignment and structural superimposition have revealed that, despite relatively low primary-structure similarities (17–44%), the tertiary structures of family 1 β-glucosidases are remarkably similar. Several β-glucosidases, including those studied here, have a tight and stable folded core, presumably enabling activity over a wide range of conditions. Some amino acids present in

the binding and catalytic sites of these glucosidases, e.g., the peptide motifs TFNEP and ITENG, are also highly conserved and could help stabilize the protein core. In contrast to the tertiary structures, a diversity of β-glucosidase quaternary structures has been observed; oligomerization may lead to variable functions or allow specific regulation of bioactivation of defense compounds.

Enzyme stability might also be influenced by glycosylation. We detected two myrosinase-derived peptides (those containing N90 and N482) that contain residues proposed (Burmeister et al., 1997) to be modified by sugars. Their presence in our frass extracts coupled with the relatively broad migration of the parent protein in SDS–PAGE suggest that these proteins which are glycosylated may have been partially cleaved and deglycosylated during digestion. The actual contribution of these modifications to protein activity is still unclear, as previous studies have demonstrated that myrosinase glycosylation is not essential for activity (Zhou et al., 2012), while monocotyledonous glucosidases are not glycoproteins. However, glycosylation may contribute to stability with deglycosylated proteins being more susceptible to further attack by insect gut proteases, oxidants, electrophiles (including their enzymatic products), and to variation in temperature and pH.

Further features responsible for protein stability in insect guts have been revealed by investigations of maize ZmGlu1. The dimerization of this protein is stabilized by a disulfide bridge that shields a cluster of hydrophobic residues in the active site from the solvent (Rotrekl et al., 1999; Zouhar et al., 2001). ZmGlu1 also has a high number of proline residues as well as several intramolecular ion pairs and hydrogen and electrostatic bonds (Czjzek et al., 2001), which give the protein thermal stability and resistance to denaturing agents. The average number of hydrogen bonds per residue is rather high (>1) in ZmGlu1 and *S. alba* myrosinase MA1 (Czjzek et al., 2001).

Our proteomic analyses also support the assignment of more specific *in vivo* roles to individual β-glucosidase isoforms in spite of their overlapping substrate preferences *in vitro*. For example, ZmGlu1 but not ZmGlu2, and *S. alba* MA1 but not the other annotated *S. alba* myrosinases, were unambiguously identified in insect frass extracts. As most of the catalytic activity of the ingested foliage was still present in these frass extracts, these particular enzymes may therefore be the isoforms most relevant in activating their respective plant defensive substrates. However, this is in contrast to a previous proteomic analysis of frass from maize-fed *S. frugiperda* larvae, where ZmGlu2 was abundantly detected (Ray et al., 2016). Future studies are needed to clarify the comparative stabilities and substrate preferences of these close homologs and dissect their functions. The degraded isoforms may fulfill other roles, such as hydrolysis of cytokinins by ZmGlu enzymes (Brzobohatý et al., 1993). The almond enzyme, on the other hand, was found not to hydrolyze the cyanogenic diglucoside amygdalin *in vitro*, but may still act to hydrolyze prunasin (the corresponding monoglucoside), although this possibility was not examined and its activity was measured using the general glucosidase substrate pNPG.

The maize β-glucosidase aggregating factor (BGAF) whose aggregation of ZmGlu might protect the latter from insect proteases (Kittur et al., 2007) also resists the digestive system of *S. littoralis*. However, the roles of such aggregation factors have not been fully determined. Our recovery of maize BXD β-glucosidase activity did not differ between frass derived from feeding whole plant tissues (containing BGAF) and that from feeding semi-purified β-glucosidase preparations (presumably without BGAF), suggesting that BGAF did not protect against digestive inactivation. However, strongly interacting protein partners could have remained bound to ZmGlu during our simple purification procedure.

Insect gut pH can influence enzyme stability and also have a direct impact on catalytic activity. While larval Orthoptera, Hemiptera, and the larvae of most coleopteran families have slightly acidic to neutral midguts, many larvae of Lepidoptera, Diptera, and scarab beetles (Coleoptera) have highly alkaline midguts (Terra and Ferreira, 1994). In previous work, we observed that the midgut lumen of *S. frugiperda* fed on maize leaves is alkaline, but becomes neutral toward the hindgut (Wouters et al., 2014). While maize DIMBOA-Glc β-glucosidases were active under both neutral and basic conditions, their enzymatic activity was much higher at pH 7.0 (closer to their slightly acidic pH optima) than at pH 10.0 (Wouters et al., 2014). This resulted in slow hydrolysis of DIMBOA-Glc in the alkaline fore- and midgut, but extensive activation in the hindgut and neutral rectum where absorption of water and salts takes place. In the case of *Zygaena filipendula*, the cyanogenic β-glucosidases of *Lotus* spp. also had lower activity in the highly alkaline gut significantly reducing cyanogenesis (Pentzold et al., 2014). Therefore, by lowering the activity of these β-glucosidases, the high pH observed in the midgut (but not hindgut) lumen of these and other lepidopteran herbivores may help the insect to partially counteract the resistance of these defensive β-glucosidases to proteolytic inactivation in the gut.

CONCLUSION

Here, we have shown that selected plant β-glucosidases catalyzing the activation of chemical defenses survive digestion in a generalist lepidopteran herbivore, and are detectable in frass using *in vitro* activity assays and proteomic analyses. Although the structural features responsible for persistence in the digestive tract remain to be fully demonstrated, these enzymes seem particularly resistant because of their compact tertiary structure and the glycosylation of several residues, which have optimized them for resisting the protease-rich and high pH gut of lepidopteran herbivores. In parallel these features have allowed the use of such plant β-glucosidases in industrial applications even in the presence of detergents and organic solvents. The resistance to insect gut proteases even under high

pH is presumably a result of natural selection on these enzymes to catalyze the continuous release of toxic aglycones in the herbivore gut, especially in the hindgut and rectum where the lumen reverts to more neutral conditions. Further investigations are needed to establish this extended role of β-glucosidases in the herbivore gut, and to determine which features of the proteins or their binding partners are responsible.

AUTHOR CONTRIBUTIONS

DV and NW designed the experiments. DV, NW, AG, SG-J, and YH acquired and analyzed the data. DV, NW, AG, AS, and JG interpreted the data and drafted the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01389/full#supplementary-material

TABLE S1 | Maize proteins detected in *S. littoralis* frass extracts and their predicted functions, number of peptides observed by LC-MS and corresponding sequence coverage, and leaf expression levels (B73 maize).

TABLE S2 | Proteins detected during LC-MS proteomic analysis of maize leaf extracts.

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The Active Jasmonate JA-Ile Regulates a Specific Subset of Plant Jasmonate-Mediated Resistance to Herbivores in Nature

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The jasmonate hormones are essential regulators of plant defense against herbivores and include several dozen derivatives of the oxylipin jasmonic acid (JA). Among these, the conjugate jasmonoyl isoleucine (JA-Ile) has been shown to interact directly with the jasmonate co-receptor complex to regulate responses to jasmonate signaling. However, functional studies indicate that some aspects of jasmonate-mediated defense are not regulated by JA-Ile. Thus, it is not clear whether JA-Ile is best characterized as the master jasmonate regulator of defense, or if it regulates more specific aspects. We investigated possible functions of JA-Ile in anti-herbivore resistance of the wild tobacco *Nicotiana attenuata*, a model system for plant-herbivore interactions. We first analyzed the soluble and volatile secondary metabolomes of ir*JAR4*xir*JAR6*, as*LOX3*, and WT plants, as well as an RNAi line targeting the jasmonate co-receptor CORONATINE INSENSITIVE 1 (ir*COI1*), following a standardized herbivory treatment. ir*JAR4*xir*JAR6* were the most similar to WT plants, having a ca. 60% overlap in differentially regulated metabolites with either asLOX3 or ir*COI1*. In contrast, while at least 25 volatiles differed between ir*COI1* or as*LOX3* and WT plants, there were few or no differences in herbivore-induced volatile emission between ir*JAR4*xir*JAR6* and WT plants, in glasshouse- or field-collected samples. We then measured the susceptibility of jasmonate-deficient vs. JA-Ile-deficient plants in nature, in comparison to wild-type (WT) controls, and found that JA-Ile-deficient plants (ir*JAR4*xir*JAR6*) are much better defended even than a mildly jasmonate-deficient line (as*LOX3*). The differences among lines could be attributed to differences in damage from specific herbivores, which appeared to prefer either one or the other jasmonate-deficient phenotype. We further investigated the elicitation of one herbivore-induced volatile known to be jasmonate-regulated and to mediate resistance to herbivores: (*E*)-α-bergamotene. We found that JA was a more potent elicitor of (*E*)-α-bergamotene emission than was JA-Ile, and when treated with JA, ir*JAR4*xir*JAR6* plants

emitted 20- to 40-fold as much (*E*)-α-bergamotene than WT. We conclude that JA-Ile regulates specific aspects of herbivore resistance in *N. attenuata*. This specificity may allow plants flexibility in their responses to herbivores and in managing trade-offs between resistance, vs. growth and reproduction, over the course of ontogeny.

Keywords: jasmonate signaling, jasmonoyl isoleucine (JA-Ile), direct and indirect defense, plant metabolomics, plant-herbivore interactions, *Nicotiana attenuata*, *Manduca sexta*

INTRODUCTION

Plants employ sophisticated defense systems in response to herbivore attack. These include both direct defenses: traits which directly impair herbivore performance such as toxins, antifeedants, and repellents; and indirect defenses, which attract parasitoids or predators of attacking herbivores (Dicke and Baldwin, 2010; Mithöfer and Boland, 2012). Herbivory-induced direct and indirect defenses are regulated by complex signaling systems, activated when plants detect tissue damage and herbivore-derived chemical cues (Erb et al., 2012; Schuman and Baldwin, 2016). A central pathway regulating plant defense metabolites is the jasmonate signaling cascade comprising jasmonic acid (JA) and its derivatives, collectively referred to as jasmonates, and the molecular players in jasmonate biosynthesis, perception, and signal transduction (Erb et al., 2012; Wasternack and Strnad, 2016). JA biosynthesis begins with the hydrolysis of 9,12,15-octadecatrienoic acid (18:3) fatty acids from membrane lipids in the chloroplast. These fatty acids are then oxygenated by 13-lipoxygenases (LOX) to 13*S*-hydroperoxy-18:3 or−16:3 fatty acid peroxides, and then further oxidized and cyclized, leading to the formation of (9*S*,13*S*)-12-oxophytodeinoic acid (OPDA). OPDA is transported to the peroxisome, where several steps of beta-oxidation result in the formation of (3*R*,7*S*)-JA (Wasternack and Hause, 2013). The jasmonates comprise dozens of JA metabolites (Wasternack, 2007; Wasternack and Hause, 2013), including the isoleucine conjugate jasmonyl-isoleucine (JA-Ile) which has been identified as the active jasmonate hormone. The conjugation of JA to Ile is catalyzed by JASMONATE RESISTANT 1 (JAR1) in *Arabidopsis thaliana* and its homologs in other plant species, including JAR4 in *Solanum nigrum* and two enzymes, JARs 4 and 6, in *Nicotiana attenuata* (Staswick and Tiryaki, 2004; Wang et al., 2007; VanDoorn et al., 2011a,b). JA-Ile has been demonstrated in molecular interaction studies to interact more strongly than other jasmonates with their receptor complex (Chini et al., 2007; Thines et al., 2007). Specifically, the isomer (+)-7-iso-JA-L-Ile is perceived by a complex of one or more JAZ (JASMONATE ZIM DOMAIN protein) transcriptional repressor protein(s), inositol pentakisphosphate (InsP5), and the F-box protein COI1 (CORONATINE-INSENSITIVE 1), which is part of a Skp/Cullin/F-box complex (SCF^{COI1}) that functions as a ubiquitin ligase (Xu et al., 2002; Chini et al., 2007; Fonseca et al., 2009; Sheard et al., 2010). The binding of JA-Ile to the SCF^{COI1}-JAZ-InsP5 complex triggers the ubiquitination and degradation of the JAZ repressor(s) (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008). Intact JAZ proteins bind to transcription factors which regulate multiple jasmonate-inducible genes involved e.g. in secondary metabolite biosynthesis. JA-Ile-induced JAZ degradation releases these transcription factors, permitting the activation of defense metabolite biosynthesis (De Geyter et al., 2012).

A variety of jasmonates has been shown to exert biological activity in plants (Erb and Glauser, 2010). Furthermore, the JA precursor OPDA, which is an abundant molecule esterified to galactolipids in chloroplasts of *A. thaliana*, also induces COI1-dependent and -independent transcriptional regulation (Stintzi et al., 2001; Taki, 2005; Ribot et al., 2008), changes in intracellular calcium levels (Walter et al., 2007), and alterations of cellular redox status (Böttcher and Pollmann, 2009). OPDA is released by lipase activity present in oral secretions of feeding insects and regulates herbivory-induced transcriptional responses in *A. thaliana* (Schäfer et al., 2011). *cis*-Jasmone is a volatile jasmonate reported to activate defense responses in various plants, including *A. thaliana* and *Triticum aestivum* (Birkett et al., 2000; Bruce et al., 2008). Another volatile, methyl jasmonate (MeJA), elicits a constitutive defense response when over-produced in *A. thaliana* (Seo et al., 2001). However, in *N. attenuata*, herbivore resistance is reduced when MeJA production is upregulated, demonstrating the importance of analyzing biological functions of different jasmonates in diverse plant species (Stitz et al., 2011a). Other JA derivatives, which are thought to be inactive, may be involved in "switching off" jasmonate signaling: 12-O-β-D-glucopyranosyljasmonic acid (12-O-Glc-JA) and 12-OH-JA are abundant metabolites in many plant species, including *Solanum tuberosum*, *A. thaliana*, and *N. tabacum* (Yoshihara et al., 1989; Helder et al., 1993; Swiatek et al., 2004; Miersch et al., 2008). Interestingly, 12-O-Glc-JA, but not JA or JA-Ile, was shown to activate leaf closure in *Samanea saman* (Nakamura et al., 2011), supporting the idea that biological activities of jasmonates can be species- and tissue-specific.

Applying JA and JA-Ile to plants, as well as genetically manipulating jasmonate and JA-Ile biosynthesis and perception, has revealed that the responses elicited by these two jasmonates only partially overlap. In *Phaseolus lunatus*, for example, JA and JA-Ile treatments differentially regulate light-dependent extrafloral nectar production, an indirect defense which attracts predatory ants (Radhika et al., 2010). In *A. thaliana*, the emission of some herbivory-induced volatiles, such as terpenoids and methyl salicylate, but not green leaf volatiles (GLVs), is dependent on plants' ability to produce JA (Snoeren et al., 2009). Herbivore-attacked *jar1-1* mutants of *A. thaliana* are as attractive as WT plants for the parasitoid *Cotesia rubecula*, indicating that

JA-Ile-mediated signaling may not be involved in indirect defense in this plant (Van Poecke and Dicke, 2003), although an analysis of herbivory-induced volatiles in *jar1-1* mutants of *A. thaliana*, to our knowledge, has not yet been reported. Staswick and colleagues also found that JA-Ile is not required for full COI1-mediated resistance to pathogens and does not regulate all COI1-dependent transcriptional responses (Staswick et al., 1998; Suza and Staswick, 2008). Recently, Vandoorn and colleagues demonstrated that in *S. nigrum*, defense responses and resistance to herbivores in the field require jasmonate biosynthesis, and perception via COI1, but not JAR4, suggesting that JA-Ile plays minor roles in herbivore resistance in this plant (VanDoorn et al., 2011a). As of yet, there has not been an integrative analysis of the role of JA and JA-Ile in herbivory-induced direct and indirect resistance. Ideally, such an analysis would be performed in a model system in which herbivory-induced defense metabolites and their role in resistance to natural herbivores have been well characterized.

Nicotiana attenuata is an ecological model plant in which defensive roles of many metabolites and herbivore-induced plant volatiles have been demonstrated in nature. This plant specifically responds to attack from a variety of natural herbivores. For example, feeding by the specialist lepidopteran herbivore *Manduca sexta* is perceived via fatty acid-amino acid conjugates (FACs) present in the insect's oral secretions (Halitschke et al., 2003; Bonaventure et al., 2011) and more recently, the plant-derived elicitor 2-hydroxylinolenic acid (2-HOT) was shown to promote the production of resistance metabolites (Gaquerel et al., 2009, 2012). Applying *M. sexta* oral secretions (OS) to wounded leaves of *N. attenuata* dramatically amplifies transient wound-induced JA and JA-Ile production between 20 and 90 min post-elicitation, and the abundance of precursors within 5 min (Schittko et al., 2000; Kallenbach et al., 2010). Silencing JARs 4 and 6 in *N. attenuata* revealed that JA-Ile is not likely to be the only active oxylipin signal regulating direct defense metabolites: plants silenced in JAR4 and JAR6 by RNAi (inverted repeat, ir*JAR4*xir*JAR6*) were deficient in the resistance-related metabolites nicotine and trypsin protease inhibitors (TPI), but produced higher levels of both than did RNAi lines silenced in LIPOXYGENASE 3 (antisense, as*LOX3*), the lipoxygenase providing 18:3 fatty acid hydroperoxides for JA biosynthesis in this plant; ir*JAR4*xir*JAR6* plants were also intermediate between as*LOX3* and WT in their resistance to *M. sexta* (Halitschke et al., 2003; Wang et al., 2008). Microarray analysis of ir*JAR4*xir*JAR6* and as*LOX3* plants supported the conclusion that JA and JA-Ile have partially overlapping but distinct activities (Wang et al., 2008). Importantly, although JAR4 and JAR6 may also regulate conjugation of JA-Leu, which cannot be analytically distinguished from JA-Ile via standard mass spectrometry (MS) analysis, Wang and colleagues showed that JA-Ile application to ir*JAR4*xir*JAR6* plants was sufficient to restore gene expression (except for JAR4 and JAR6), nicotine and TPI production, and resistance to *M. sexta* larvae, to WT levels.

Meanwhile, much more is known about the herbivore-induced metabolome of *N. attenuata*, providing an appealing system in which to systematically investigated herbivore resistance-related metabolomic changes regulated by JA-Ile in comparison to total jasmonates, or jasmonate perception via COI1, and test the importance of JA-Ile vs. total jasmonate biosynthesis for resistance to plants' native herbivores in the field. We compared herbivore resistance of jasmonate-deficient (as*LOX3*) and JA-Ile-deficient (ir*JAR4*xir*JAR6*) plants in a field study, and our data reveal that JA-Ile has specific, and different, effects on herbivore resistance in comparison to jasmonate biosynthesis. Using targeted and untargeted metabolomics, and additional transformed lines manipulating jasmonate signaling (ir*COI1*) and accumulation (s*JMT*), we demonstrate that a large proportion of herbivory-induced changes to the soluble metabolome (known and putative metabolites of direct resistance) are regulated by JA-Ile, but that the biosynthesis of jasmonate-regulated herbivory-induced volatiles, known or putative indirect defenses, is JA-Ile independent. Thus, our study indicates that plants use different jasmonate metabolites to regulate jasmonate-dependent direct vs. indirect resistance.

MATERIALS AND METHODS

Plant Material

The genotype of *N. attenuata* used in this study was derived from the Desert Inn accession, UT (Baldwin et al., 1994) and wild-type (WT) plants were from the 30th inbred generation. The inverted repeat *JASMONATE RESISTANT 4* (ir*JAR4*) line A-05-355-6 and ir*JAR6* line A-05-380-6 and their hemizygous cross (third transformed generation, T3) were previously described by Wang and colleagues in comparison to independently transformed lines bearing the same construct (Wang et al., 2007, 2008). Two different hemizygous crosses generated from two different independently transformed lines per construct both accumulated only about 16% as much JA-Ile, but the same amount of JA as WT plants after herbivore elicitation; *JAR4* and *JAR6* transcripts were almost undetectable in Northern blots, in comparison to a strong signal for WT plants (Wang et al., 2008). Because possible maternal effects had not previously been tested for, reciprocal hand-pollinated crosses were generated from the ir*JAR4* and ir*JAR6* lines. In most cases a bulk collection of the reciprocal crosses was used for experiments, and where indicated in the Results and figure captions, collections from individual crossings were used. The JAR enzymes conjugate isoleucine to jasmonic acid to generate JA-Ile, but also form some other JA-AA conjugates including JA-Leu, which cannot be analytically separated from JA-Ile by standard LC-MS/MS/MS analysis (Wang et al., 2007). However, Wang and colleagues showed that differences in the transcript accumulation of defense- and growth-related genes between ir*JAR4*xir*JAR6* and WT plants could be restored by the application of pure JA-Ile (Wang et al., 2008). Thus, off-target effects are unlikely.

The antisense (as) *LOX3* line A-300-1 and the ir*COI1* line A-04-249-A-1 were used in the T3 generation, and s*JMT* lines ectopically expressing *Arabidopsis thaliana* jasmonate methyltransferase to channel jasmonate production to MeJA were used in the T2 generation (35S-*jmt*, lines A-07-287-3 and A-07-289-7); all lines were previously described in comparison to independently transformed lines bearing the same construct (Halitschke and Baldwin, 2003; Paschold et al., 2007; Stitz et al., 2011b).

We used WT plants as the control for all experiments. By the second (T2) or third transformed generation (T3) there are rarely measurable effects of the transformation process; screening to avoid off-target insertion effects is part of the normal screening process, and has been done for all lines used here. Consistently, empty vector control plants in only the second transformed generation (T2) have been shown to be indistinguishable from WT plants in their growth and herbivore-induced transcript, hormone and metabolite production (Schwachtje et al., 2008).

Glasshouse Growth Conditions

Seed germination and plant growth in the glasshouse were as previously described (Krügel et al., 2002; Adam et al., 2017). Briefly, seeds were germinated on Gamborg B5 medium and kept under 16 h light/8 h dark at 26 °C; 10 d later, seedlings were transferred to small pots (TEKU JJP 3050 104 pots, Poeppelmann GmbH & Co. KG, Lohne, Germany) in the glasshouse and then to 1 L pots 10 d later with soil, fertilization and watering regimes as previously described and grown under 19–35°C, 16 h light (supplemental lighting by Philips Sun-T Agro 400 W and 600 W sodium lights) and 55% humidity.

Field Growth Conditions

Importation and release of transgenic plants were carried out under Animal and Plant Health Inspection Service (APHIS) import permit numbers 07-341-101n and release permit number 11-350-101r. Seed germination and seedling growth was as previously described (McGale et al., 2018). Briefly, seedlings were germinated on Gamborg B5 media under illumination from fluorescent lights (GE Plant & Aquarium 40 W and GE Warm White 18 W) at ambient temperatures at the field station. Two to three weeks after germination, seedlings with four visible leaves were transferred into previously hydrated 50-mm peat pellets (Jiffy 703, www.jiffypot.com) treated with Borax to provide boron, an essential micronutrient (1:100 dilution of a 1.1 g/l stock solution) and adapted over 2 weeks to the field conditions of high light intensity and low relative humidity by keeping seedlings first in shaded, closed translucent plastic 34-quart boxes (Sterilite), then opening the boxes, and subsequently transferring open boxes to partial sunlight in mesh tents (Tatonka). Adapted size-matched seedlings were transplanted into an irrigated field plot at the Lytle Ranch Preserve, Santa Clara, Utah, in April 2012.

Leaf Treatments

For glasshouse experiments, the transition leaf, or the second fully expanded leaf (positions 0 or +2) on rosette-stage or elongated plants were used for treatments as described for specific analyses except for the analysis of s*JMT* lines and the accompanying WT, for which the second rosette leaf on elongated plants was used (older than +2, mature and non-senescent). Plants in both the rosette and the elongation stage show robust jasmonate-mediated responses in the glasshouse (Diezel et al., 2011) but it is easier to collect volatiles on-plant from leaves on elongated plants.

To mimic herbivory, leaves were treated by wounding with 3 rows of holes to the lamina on each side of the midvein (6 rows in total) using a tracing wheel, and the addition of 20 μL *M. sexta* oral secretions (W+OS) diluted 1:5 in distilled water, a procedure which has been shown to elicit most responses induced by *M. sexta* herbivory (Halitschke et al., 2001; Schittko et al., 2001). OS were collected from larvae from an in-house colony at the Max Planck Institute for Chemical Ecology fed on WT *N. attenuata* plants.

For elicitation with different jasmonates, purified substances synthesized in-house (JA, JA-Ile, JA-Leu) or obtained from Sigma-Aldrich (*cis*-jasmone) were first checked for purity by liquid chromatography-mass spectrometry analysis (UHPLC-ESI/TOFMS in negative mode, Bruker), and then 0.25 μmol of the pure compound was dissolved per 20 μL of 30% ethanol by first dissolving the corresponding mass in ethanol (Sigma-Aldrich) and then slowly adding distilled water to prevent precipitation; 30% ethanol in distilled water was used as a solvent control. Twenty microliter of jasmonate in 30% ethanol was added to 6 rows of wounds made with a tracing wheel, as described for W+OS treatment. For plants in the field, similar, fully expanded, non-senescent, and minimally damaged leaves were used for control and W+OS-treated samples.

Analysis of Jasmonates

The leaf at the +2 position on glasshouse-grown rosette-stage plants was treated with W+JA and treated leaves were harvested 1, 3, and 6 h later; harvests were conducted as described for the analysis of leaf secondary metabolites. For plants in the field, a similar, fully-expanded, minimally damaged leaf was treated with W+OS and harvested 1 h later and a similar, untreated leaf was harvested simultaneously from control plants into aluminum foil, then frozen immediately on dry ice and kept frozen on dry ice in a −20°C freezer until transport on dry ice to the Max Planck Institute for Chemical Ecology, Jena. Tissue was ground over liquid nitrogen to a find powder and kept at −80°C until extraction and analysis. Jasmonates were measured in ethyl acetate extracts of leaves, re-suspended in 70% methanol, on a Varian 1200L LC-MS/MS/MS system as previously described (Wang et al., 2007; Stitz et al., 2011b). For JA-treated leaves, JA was excluded from measurements. Results were calculated as concentrations in ng mg FM^{-1} using isotopically labeled internal standards except for OPDA and MeJA, for which no internal standard was available; the JA-Ile internal standard was used for all JA conjugates.

Analysis of Leaf Secondary Metabolites

The transition leaf (position 0) on rosette-stage plants was treated with W+OS and excised at the petiole 72 h later by which time treated leaves had grown to position +2 or +3. Midveins were excised, and leaf tissue was flash-frozen in liquid nitrogen. Tissue was ground over liquid nitrogen to a find powder and kept at −80°C until extraction and analysis. Extracts of leaves in acidified 40% methanol (soluble metabolome) were analyzed on a Bruker UHPLC-ESI/TOFMS in positive ionization mode as previously described (Gaquerel et al., 2010). Chromatograms were exported as netCDF files and peak detection, picking and integration was performed using the R package XCMS (Smith et al., 2006; Tautenhahn et al., 2008), and then ion traces were deconvoluted and putative in-source pseudo-spectra reconstructed with the R package CAMERA (Kuhl et al., 2012) as previously described (Gaquerel et al., 2010; Stitz et al., 2011a). The data matrix

was imported to Microsoft Excel and isotopic peaks and multi-charged m/z signals detected by CAMERA were excluded to reduce the redundancy within the data matrix. Consistent mass features—present (for a single plant genotype) in four out of the five biological replicates—with a retention time >50 s were considered for further statistical analysis.

Leaf Headspace Analysis From Glasshouse-Grown Plants

The leaf at the +2 position was used, which emits greater amounts of W+OS-induced volatiles than younger or older leaves (Halitschke et al., 2000) except for the analysis of sJMT lines and the accompanying WT, in which a slightly older rosette leaf on elongated plants was used (second rosette leaf, mature and non-senescent). Volatiles were sampled from elongated, pre-flowering plants unless otherwise noted. (Elongation provides easier access to the +2 leaf for volatile collection.) Several hours to 1 day after treatment according to peak emission times of different volatiles, as described in the Results and figure captions, treated leaves were enclosed in two 50 mL PET cups (Huhtamaki, Finland) lined on the edges with foam to protect leaves and with an activated charcoal filter attached to one side for incoming air, and secured with miniature claw-style hair clips as described previously (Schuman et al., 2009). Headspace volatiles were collected for several hours (see section Results and figure captions) on 20 mg of PoropakQ (Tholl et al., 2006) (Sigma-Aldrich) in self-packed filters (bodies and materials from ARS Inc.) by drawing ambient air through these clip cages at 300 mL min^{-1} using a manifold with screw-close valves set to provide equal outflow, via pushing air at 2–3 bar through a Venturi aspirator as previously described (Oh et al., 2012). Background VOCs present in ambient air were collected using empty foam-lined PET cups which were the same as those used for leaves, and background signals were later subtracted if necessary from raw intensities of plant samples prior to further processing. After trapping, Porapak Q filters were stored at −20°C until extraction, which was done by addition of 320 ng of tetralin as an internal standard (IS), and elution of volatiles with 250 μL of dichloromethane (Sigma–Aldrich). Filters were eluted into a GC vial containing a 250 μL glass insert.

For volatile metabolomic analysis, an Agilent 6890N gas chromatograph equipped with an Agilent 7683 autoinjector coupled with a LECO Pegasus III time-of-flight mass spectrometer with a 4D thermal modulator upgrade was used to collect three-dimensional GCxGC-TOFMS data and to generate a peak table as previously described (Gaquerel et al., 2009). The data matrix was imported to Microsoft Excel and consistent mass features—present (for a single plant genotype) in four out of the five biological replicates—with a retention time >150 s were considered for further statistical analysis.

For the targeted analysis of (*E*)-α-bergamotene, samples were analyzed on a on a Varian CP-3800 GC coupled to a Varian Saturn 4000 ion-trap mass spectrometer with a Varian CP-8400 autoinjector equipped with a Phenomenex ZB5 column from (Torrance, CA; 30 m × 0.25 mm i.d., 0.25 μm film thickness) and compounds were separated by a temperature ramp of 5°C min^{-1} between 40 and 180°C as previously described (Oh et al., 2012).

The identification of compounds was conducted by comparing GC retention times and mass spectra to those of standards and mass spectra databases: Wiley version 6 and NIST (National Institute of Standards and Technology) spectral libraries. The (*E*)-α-bergamotene peak was identified by retention index (RI) on two different columns and similarity to spectral libraries, and confirmed using an authentic standard as previously reported (Schuman et al., 2009).

Relative quantification of individual volatile compound peaks was done using the combined peak area of two specific and abundant ion traces per compound using MS Work Station Data Analysis software (Varian) or the ChromaToF software (LECO) and normalized by the 104 + 132 ion trace peak area from tetralin in each sample. The area of trapped leaves was quantified for comparison by scanning and calculating areas in pixels using SigmaScan (Systat Software Inc., San Jose, CA), and subsequently converting pixels to cm^2 using a size standard which was scanned with leaves. As leaf areas did not differ between lines (**Datasheet 1**), relative abundance of volatile compounds was expressed as a percentage of the IS peak area. The sesquiterpene β-caryophyllene was used as a standard to confirm that measured peak areas of (*E*)-α-bergamotene were within the linear range of detection.

Estimation of Damage From Native Herbivores in the Field

Observations of herbivore damage to the canopy of elongated plants were made on May 16th, 2012, by visual estimation after training for herbivore damage-type recognition. Damage was calculated as described in Schuman et al. (2012) by identifying damage from specific herbivores according to their characteristic feeding patterns, counting the number of leaves per plant (small leaves were counted as 1/5–1/2 of a leaf based on leaf area and large leaves were counted as 1 leaf), estimating the total percentage of leaf area damage due to each herbivore, and dividing the total leaf area damage from each herbivore by the total number of leaves, a protocol which has been used over 10 years of field studies in this system (e.g., Kessler et al., 2004; Steppuhn et al., 2004, 2008; Meldau et al., 2009; Kallenbach et al., 2012; Schuman et al., 2012). Damage estimates were made by C. D.

Leaf Headspace Analysis From Field-Grown Plants

Similar, fully expanded, non-senescent, and minimally damaged leaves on flowering plants were used for control or W+OS-treated samples on May 28th and May 29th, 2012. Flowering plants were used in the field, but not in the glasshouse, as this stage is more likely to receive oviposition from moths of *M. sexta*, which is a pollinator as well as an herbivore (Zhou et al., 2017); but in glasshouse-grown plants, jasmonate-mediated responses, although not the emission of (*E*)-α-bergamotene, are attenuated after flowering (Diezel et al., 2011; Schuman et al., 2014). Thus, we also measured jasmonates from WT plants in the field before and after flowering.

Silicone tubings (STs) were chosen as a more convenient method of field sampling (Kallenbach et al., 2014). ST preparation, volatile sampling from leaves, and TD-GC-QMS

analysis on a Shimadzu TD−20 thermal desorption unit connected to a quadrupole GC–MS-QP2010Ultra and equipped with a Phenomenex ZB5 column were conducted as previously described (Kallenbach et al., 2014, 2015). STs were exposed to leaf headspaces for 24 h immediately after W+OS treatment and simultaneously from control plants, with 3 technical replicates per leaf of which one was analyzed and the other two were kept as back-up.

ST samples were stored in tightly sealed amber glass screw-cap 1.5 ml vials at −20°C freezer prior to analysis, and were at room temperatures only during sample transport from Utah, USA to Jena, Germany (<1 day), which is known not to influence the analysis (Kallenbach et al., 2014, 2015). Identification of volatiles by spectral libraries, relative retention and comparison to standard compounds, and relative quantification of a single abundant m/z trace per peak using the Shimadzu software, as well as background correction based on samples of ambient air, were done as previously described (Kallenbach et al., 2014).

Statistical Analyses

Replicates were only excluded from statistical analyses when there was a valid biological reason to do so (death of plant, loss of leaf due to damage). We started with replicate numbers of 4-5 plants for jasmonate and soluble metabolite analysis in glasshouse experiments, 5–10 plants for volatile analyses in glasshouse experiments, and 10–21 plants for all analyses from the field experiment, and final replicate numbers are reported in figure captions and table legends.

Herbivore damage data and (*E*)-α-bergamotene emission from different plant genotypes were analyzed in SPSS 17.0, or in R 3.2.5 and RStudio 1.0.136 (*cis*-jasmone experiment). Datasets were evaluated for homogeneity of variance and normality using Levene's test and visual inspection (SPSS) or visual inspection of residual and Q-Q plots (R) and when these requirements could not be met after log transformation, nonparametric Kruskal-Wallis tests were used for multiple comparisons and Mann-Whitney *U*-Tests for binary comparisons. Holm-Bonferroni corrections were used to maintain Type I error below $\alpha = 0.05$ when multiple tests were conducted on the same data, and the adjusted *P*-values are reported.

For soluble and volatile metabolome analysis, statistical evaluation was conducted using the Multiple Experiment Viewer (MEV). Analytes were considered to differ significantly between wild-type and transgenic lines which had a fold-change of 1.5 (up-regulation) or 0.67 (down-regulation) at a significance level of $\alpha = 0.05$, as determined by Student's *t*-tests corrected for the testing of multiple analytes using the Benjamini and Hochberg false discovery rate (FDR) method.

Corrected integrated peak areas from field-collected headspace samples (Kallenbach et al., 2014), and corrected (visual check of integration) normalized peak areas from phytohormone analysis after W+OS, W+EtOH or W+JA treatment, were analyzed using Metaboanalyst (www.metaboanalyst.ca) (Xia et al., 2009, 2015; Xia and Wishart, 2016). Analytes having zero values for all samples in a treatment group were removed and the remaining peak areas were log-transformed and mean-centered to meet assumptions of normality and homoscedasticity. For headspace samples, after an initial exploratory analysis to visualize differences, data were evaluated by one-way analysis of variance (ANOVA) for each treatment group and Tukey's Honestly Significant Difference (HSD) *post-hoc* tests were performed for analytes with FDR<0.05, to control for the testing of multiple analytes. Pearson's correlation tables were also calculated and are available as part of the source data files for this manuscript along with the input data and R script (**Datasheet 1**). For phytohormones, data were evaluated by one- or two-way analysis of variance (ANOVA) and *P*-values were adjusted to an FDR<0.05, to control for the testing of multiple analytes.

RESULTS

JA-Ile Regulates ca. 60% of Jasmonate-Responsive Secondary Metabolites, but Not Volatile Metabolites

We first asked what portion of the jasmonate-regulated secondary metabolome was likely to be regulated by JA-Ile, as opposed to total jasmonate products of LOX3 activity, or jasmonate signaling mediated by COI1. We investigated both the soluble and volatile secondary metabolome, which include many small molecule metabolites that have been shown to confer direct resistance to herbivores as a result of toxic, antifeedant, or deterrent effects, as well as indirect resistance as a result of increased predation rates on herbivores (Kessler and Baldwin, 2001; Kallenbach et al., 2012; Schuman and Baldwin, 2016). We employed a controlled simulated herbivory treatment comprising wounding and the addition of *M. sexta* oral secretions (W+OS) (Halitschke et al., 2001; Schittko et al., 2001) in order to elicit standardized responses in as*LOX3*, ir*JAR4*xir*JAR6*, ir*COI1*, and WT plants. We then conducted an untargeted analysis of metabolite extracts from tissue samples, and headspace samples: without identifying specific metabolites, we analyzed the patterns in relative abundance of ions (m/z features) after filtering raw mass spectral data to identify ions which likely represented different metabolites (i.e., one ion per metabolite).

We found that JA-Ile synthesis controlled by JAR4 and JAR6 was required for ca. 60% of the response of the soluble secondary metabolome to W+OS treatment, as estimated by the difference between WT and ir*COI1* or as*LOX3* plants, in comparison to the difference between WT and ir*JAR4*xir*JAR6* plants (**Figure 1**, **Supplementary Table 2**). In contrast, the abrogation of JA-Ile synthesis in ir*JAR4*xir*JAR6* plants had no significant effect at all on the jasmonate-dependent emission of at least 25 herbivore-induced volatiles (**Figure 1**).

Measurements of peak herbivore-induced JA and JA-Ile accumulation from the same plants used for metabolite sampling showed that asLOX3 plants were deficient in JA (15% of WT values) and JA-Ile (29% of WT), while ir*JAR4*xir*JAR6* were deficient in JA-Ile (20% of WT) but not JA (99% of WT), and ir*COI1* produced less JA (21% of WT) but similar JA-Ile levels (106% of WT) (**Table 1**, **Supplementary Figure 1**). In addition, we analyzed known JA-Ile metabolites and abscisic acid (ABA) in these samples. Overall, in addition to the significant differences in JA and JA-Ile, we also found differences in OH-JA-Ile and COOH-JA-Ile, although the levels of these JA-Ile metabolites

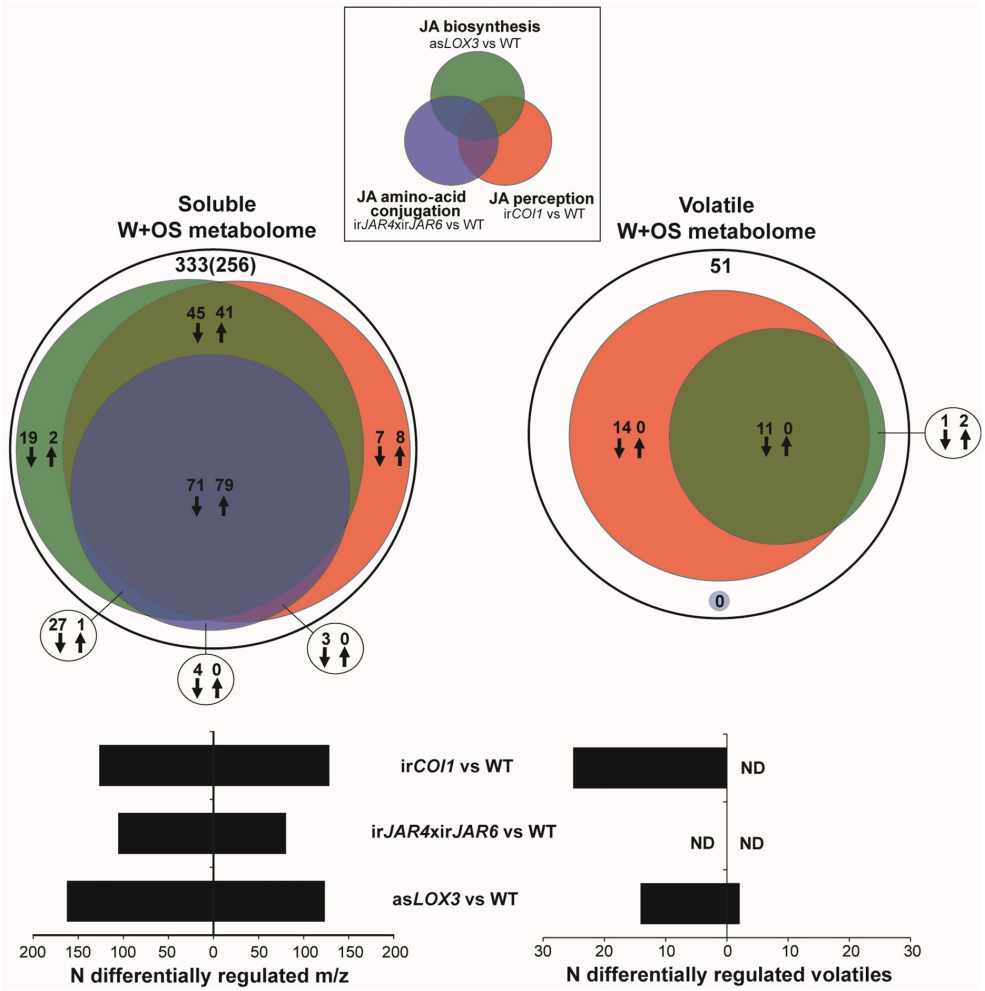

FIGURE 1 | JA-Ile formation regulates a subset of the jasmonate-dependent soluble metabolome, but not volatile emissions induced after simulated herbivore attack. Venn diagram representations of the relative contributions of total jasmonate biosynthesis (as*LOX3* vs. WT), of JA-Ile signaling (ir*JAR4*xir*JAR6* vs. WT), and jasmonate perception (ir*COI1* vs. WT) to metabolic changes activated in *Nicotiana attenuata* rosette-stage leaves during simulated herbivore attack (W+OS) underscore the minor role of JA-Ile formation for induced volatile production. Methanolic extracts (soluble metabolome) of leaves collected 72 h after W+OS ($n = 5$ plants) were analyzed using UHPLC-ESI/TOFMS in positive ionization and processed mass-to-charge ratio (m/z) peak matrices analyzed for differential expression between transgenic lines and WT (Gaquerel et al., 2010). Plant volatiles (volatile metabolome) were collected 0–6 and 6–24 h after elicitation by W+OS ($n = 5$ plants) and analyzed using GCxGC-TOFMS (Gaquerel et al., 2009). Processed volatile matrices obtained for the two collection times were combined and analyzed for differential expression between transgenic lines and WT. Deconvoluted volatiles and m/z features were called deregulated in transgenic lines compared to in WT when their fold change (ratio of averages: transgenic line vs. WT) was higher than 1.5 (up-regulation: up arrows) or lower than 0.67 (down-regulation: down arrows) at a significance level of 0.05 (*t*-test, $P < 0.05$ corrected for multiple testing using the Benjamini and Hochberg false discovery rate method). Black circles depict the total number of W+OS-regulated features in WT leaves. For the soluble metabolome, the number of predicted metabolites after informatic-deconvolution (see section Materials and Methods) and clustered metabolite-derived m/z in-source fragments is presented in brackets.

were low at 1 h after elicitation; and only weak differences in ABA (**Table 1**).

Jasmonoyl Isoleucine (JA-Ile) Deficiency Accounted for ca. 30% of Jasmonate-Mediated Resistance to Herbivores in Nature

We then askedwhether the active jasmonate hormone JA-Ile is responsible for jasmonate-mediated resistance to herbivores for *N. attenuata* plants in nature. We evaluated the herbivore resistance of ir*JAR4*xir*JAR6* plants, deficient in JA-Ile biosynthesis, in comparison to as*LOX3* plants deficient in the synthesis of all jasmonates, or wild-type (WT) controls, by estimating the total canopy damage to field-grown plants by native herbivores. We chose to use asLOX3 and not irCOI1 because asLOX3 plants had a similar level of JA as JA-Ile deficiency, and the reduction in both hormones was similar to the reduction of JA-Ile in ir*JAR4*xir*JAR6* compared to WT plants, in contrast to the more complex changes in jasmonate biosynthesis in ir*COI1* plants (**Table 1**, Paschold et al., 2008). In this experiment, we did not aim to compare resistance due to

TABLE 1 | ABA, JA, and JA-Ile levels and JA-Ile metabolites in lines deficient in JA-Ile synthesis (ir*JAR4*xir*JAR6*), total jasmonate biosynthesis (as*LOX3*), or jasmonate perception (ir*COI1*) vs. wild-type (WT) plants during peak JA and JA-Ile accumulation, 1 h after W+OS treatment (n = 3–5 plants).

Analyte	$F_{(3, 17)}$[a]	Adj. *P*	Tukey[b]	Genotype	Mean ± SE (ng g^{-1} FM)[c]
JA	67.28	**<0.0001**	**a**	WT	3,833 ± 354
			a	ir*JAR4*xir*JAR6*	3,799 ± 413
			b	as*LOX3*	585.4 ± 91.3
			b	ir*COI1*	820.1 ± 92.0
JA-Ile/Leu	38.35	**<0.0001**	**a**	WT	244.6 ± 28.0
			b	ir*JAR4*xir*JAR6*	49.10 ± 3.82
			b	as*LOX3*	71.13 ± 12.03
			a	ir*COI1*	259.5 ± 26.0
OH-JA-Ile	10.07	**0.0009**	**a**	WT	124.2 ± 14.8
			ab	ir*JAR4*xir*JAR6*	30.29 ± 6.19
			a	as*LOX3*	40.40 ± 12.91
			b	ir*COI1*	10.26 ± 3.78
ABA	3.652	**0.0391**	ns[d]	WT	260.2 ± 9.3
			ns	ir*JAR4*xir*JAR6*	283.0 ± 22.5
			ns	as*LOX3*	203.9 ± 33.7
			ns	ir*COI1*	105.7 ± 9.7
COOH-JA-Ile	ns	ns	ns	WT	8.265 ± 1.280
			ns	ir*JAR4*xir*JAR6*	7.332 ± 2.417
			ns	as*LOX3*	5.277 ± 2.138
			ns	ir*COI1*	4.722 ± 0.9662

One-way ANOVAs were conducted to identify significant differences (false discovery rate-adjusted P-value, Adj. $P < 0.05$) after log transformation and mean-centering to achieve normality and homogeneity of variance. Significant differences are indicated in bold. See also ***Supplementary Figure 1*** *and* ***Figure 3B****.*

[a] *Degrees of freedom.*

[b] *Significantly different contrasts ($P < 0.05$) in Tukey post-hoc tests.*

[c] *Calculated based on closest internal standard: JA, ABA, or JA-Ile (used for JA-Ile/Leu, OH-JA-Ile, COOH-JA-Ile).*

[d] *ns, not significant.*

jasmonate perception by COI1, but only relative resistance due to JA-Ile production vs. the production of all jasmonates. Because jasmonate-inducible defense in leaves is reported to be strongest prior to flowering (Diezel et al., 2011), we monitored plant canopy damage inflicted by herbivores before plants flowered.

We found that plants deficient only in JA-Ile suffered 20% more herbivore damage than WT, while as*LOX3* plants, deficient in total jasmonate production, suffered 66% more herbivore damage than WT plants (**Figure 2**). When comparing damage from specific herbivores, ir*JAR4*xir*JAR6* plants, deficient in JA-Ile, were more susceptible only to *Trimerotropis* spp. grasshoppers, which did not damage WT plants, but caused at most 1% of the total canopy damage in this year. The as*LOX3* plants, deficient in total jasmonates, were also attacked by *Trimerotropis* spp., and were furthermore significantly more damaged by *Empoasca* sp. leafhoppers than were either WT or ir*JAR4*xir*JAR6* plants (**Figure 2** and figure caption).

These data indicate that JA-Ile regulates specific aspects of jasmonate-mediated herbivore resistance in *N. attenuata*.

JA-Ile Does Not Regulate Herbivore-Induced Volatile Emissions in Field-Grown, Flowering Plants

The metabolomic profiling experiment shown in **Figure 1** was conducted before plants flowered because in the glasshouse, flowering plants have abrogated jasmonate responses, which may affect the induction of soluble secondary metabolites more than volatiles (Diezel et al., 2011; Schuman et al., 2014); and we aimed for a rigorous comparison of these two groups of secondary metabolites.

However, flowering plants are more likely to experience oviposition by the herbivore/pollinator *M. sexta*, and this is the stage for which the importance of volatile-mediated defense is best understood (Kessler and Baldwin, 2001; Schuman et al., 2012; Zhou et al., 2017; Joo et al., 2018). We therefore sampled volatiles from flowering plants as part of the field experiment, in order to determine whether JA-Ile might be important for herbivore-induced volatile emissions at this critical stage. The analysis of these samples was consistent with the results of the glasshouse experiment: as*LOX3* plants had abrogated emission of 9 herbivore-induced volatiles quantifiable in these samples, while ir*JAR4*xir*JAR6* plants had reduced emission of only 1 volatile and emitted even greater amounts of the herbivore-induced sesquiterpene 5-epi-aristolochene in comparison to WT plants (**Table 2**, **Supplementary Figures 2**, **3**, **Supplementary Tables 2**, **3**).

Jasmonate Accumulation Is Reduced, but Herbivore Inducibility of Jasmonates Is Increased After Flowering in Field-Grown Plants

In order to determine whether jasmonate responses were abrogated by the transition to flowering in field-grown plants, we measured jasmonates in response to simulated herbivory both prior to flowering, when herbivore damage rates were assessed (**Figure 2** and above), and after flowering, when volatiles were sampled (**Table 2**, **Supplementary Figures 2**, **3**, **Supplementary Tables 2**, **3**). Induced levels of JA were attenuated after flowering by ca. 70%, but the fold change between control and induced levels was only 4-fold before flowering and 60-fold after flowering; similarly, induced levels of JA-Ile were reduced by 30% after flowering, while the fold change between control and induced plants was 7-fold before flowering and 140-fold after flowering (**Table 3**). Timepoint, treatment, and the timepoint*treatment interaction were all highly significant in a general linear model on log-transformed data (df: 1, 37; all P-values < 0.001; treatment, JA: $F = 110.5$, JA-Ile: $F = 181.9$; timepoint, JA: $F = 87.78$, JA-Ile: $F = 34.88$; interaction, JA: $F = 32.04$, JA-Ile: $F = 17.60$).

These data indicate that JA-Ile is required to elicit a subset of jasmonate-regulated herbivore-induced secondary metabolites, but not herbivore-induced volatiles in *N. attenuata*. Furthermore, although JA and JA-Ile levels may be reduced in the leaves of flowering plants, we found that both remained strongly inducible, and thus we infer that induced jasmonates could maintain the

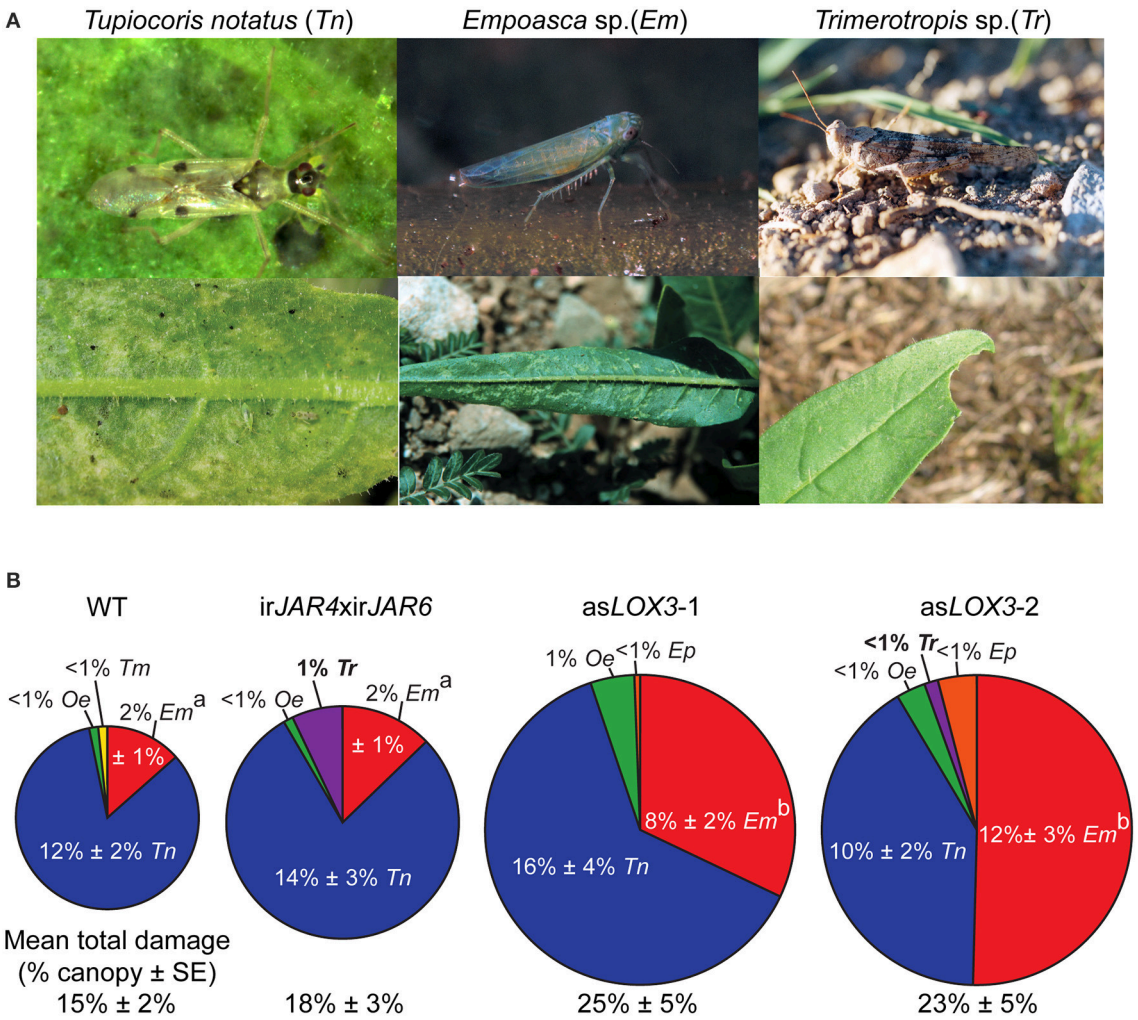

FIGURE 2 | Plants deficient in the active jasmonate JA-Ile (ir*JAR4*xir*JAR6*) were only marginally more susceptible to native herbivores compared to as*LOX3* plants deficient in all jasmonate hormones. **(A)** Upper row: pictures of herbivores which caused more than 1% canopy damage on at least one of the three *Nicotiana attenuata* genotypes investigated (*Tupiocoris notatus*, *Tn*; *Empoasca* spp., *Em*) or which caused significantly different levels of damage across genotypes (*Empoasca* spp., *Em*; *Trimerotropis* sp., *Tr*). Lower row: damage typical of each herbivore. Photographs © C. Bruetting (*Tn* adult), © A. Kessler (*Em* and *Em* damage, reproduced with permission from Kessler et al., 2004), © A. Steppuhn (*Tr* adult), and © D. Kessler (*Tn* damage, *Tr* damage). **(B)** Plants deficient in JA-Ile (ir*JAR4*xir*JAR6*) suffered only 20% more damage from native herbivores than wild-type (WT) plants (mean percent canopy damage ± SE), while plants deficient in total jasmonate biosynthesis (as*LOX3*) suffered 65% more damage. *Trimerotropis* sp. (***Tr***) caused at most 1% canopy damage, but only to ir*JAR4*xir*JAR6* or as*LOX3* plants. In contrast, *Empoasca* spp. (*Em*), opportunistic herbivores sensitive to plant jasmonate signaling capacity, caused more damage on as*LOX3* plants than on either WT or ir*JAR4*xir*JAR6*. The labels as*LOX3*-1 and as*LOX3*-2 refer to a pair of plants of the same transformed line placed at two different positions within each experimental quadruplet ($n = 20$–21); similarities between these two groups indicate the reproducibility of the data set. [a,b]Different letters indicate significantly different levels of *Empoasca* spp. damage ($P < 0.05$ after a Holm-Bonferroni *post-hoc* correction) in Mann-Whitney *U*-tests following a significant Kruskal-Wallis test across all genotypes.

induction of herbivore-induced volatile emission in flowering plants (e.g., Schuman et al., 2014).

JA-Ile Does Not Elicit the Volatile (*E*)-α-Bergamotene, a Highly Effective Component of Plant Jasmonate-Mediated Resistance to Herbivores

Because there were very few changes in the herbivore-induced volatile emission of ir*JAR4*xir*JAR6* plants in comparison to as*LOX3* and ir*COI1* plants in both glasshouse and field experiments and over two growth stages (**Figure 1**, **Table 2**), we investigated whether JA-Ile or other known jasmonates elicit volatile emission to mediate herbivore resistance. We used the volatile (*E*)-α-bergamotene (initially reported as (*Z*)-α-bergamotene; Halitschke et al., 2000; Schuman et al., 2009) for which the jasmonate-mediated and herbivore-induced elicitation of emission is well characterized. (*E*)-α-Bergamotene emission from leaves has been demonstrated to attract native predators and reduce herbivore populations on plants (Halitschke et al., 2000, 2008; Kessler and Baldwin, 2001; Schuman et al., 2009, 2014, 2015).

TABLE 2 | Herbivore-induced volatile emission was abrogated in field-grown plants with impaired jasmonate biosynthesis (as*LOX3*), but not JA-Ile biosynthesis specifically (ir*JAR4*xir*JAR6*) ($n = 5$–10 plants).

Treatment	df[a]	Analyte	*F*	Adj. *P*	Tukey[b]	Genotype
Control	2, 27	α-Terpineol[c]	25.52	**<0.0001**	**a**	WT
					a	ir*JAR4*xir*JAR6*
					b	as*LOX3*
		(*Z*)-3-Hexenol[d]	12.84	**0.0022**	**a**	WT
					a	ir*JAR4*xir*JAR6*
					b	as*LOX3*
		(*Z*)-3-Hexenyl-2-methylbutanoate[d]	11.00	**0.0039**	**a**	WT
					a	ir*JAR4*xir*JAR6*
					b	as*LOX3*
		(*Z*)-3-Hexenyl isobutanoate[d]	9.606	**0.0064**	**a**	WT
					b	ir*JAR4*xir*JAR6*
					b	as*LOX3*
		Unidentified green leaf volatile	6.563	**0.0342**	**a**	WT
					ab	ir*JAR4*xir*JAR6*
					b	as*LOX3*
W+OS	2, 20	(*E*)-α-Bergamotene[d]	24.66	**0.0002**	**a**	WT
					a	ir*JAR4*xir*JAR6*
					b	as*LOX3*
		(*Z*)-3-Hexenol[d]	22.42	**0.0002**	**a**	WT
					a	ir*JAR4*xir*JAR6*
					b	as*LOX3*
		Unidentified sesquiterpene (RT27.547)	11.63	**0.0064**	ab	WT
					a	ir*JAR4*xir*JAR6*
					b	as*LOX3*
		α-Terpineol[c]	10.50	**0.0081**	**a**	WT
					a	ir*JAR4*xir*JAR6*
					b	as*LOX3*
		(*Z*)-3-Hexenyl isobutanoate[d]	9.065	**0.0112**	**a**	WT
					a	ir*JAR4*xir*JAR6*
					b	as*LOX3*
		(*Z*)-3-Hexenyl-2-methylbutanoate[d]	9.010	**0.0112**	**a**	WT
					a	ir*JAR4*xir*JAR6*
					b	as*LOX3*
		(*Z*)-3-Hexenyl butanoate[d]	6.396	**0.0341**	**a**	WT
					a	ir*JAR4*xir*JAR6*
					b	as*LOX3*
		5-epi-Aristolochene[d]	6.345	**0.0341**	**a**	WT
					b	ir*JAR4*xir*JAR6*
					ab	as*LOX3*
		(*Z*)-3-Hexenyl acetate[d]	6.278	**0.0341**	**a**	WT
					a	ir*JAR4*xir*JAR6*
					b	as*LOX3*

One-way ANOVAs were conducted to identify significant differences (false discovery rate-adjusted P-value, Adj. $P < 0.05$) in the leaf headspace abundance of 49 peaks, of which 36 were above the limit of quantification in the headspace of untreated leaves (control), vs. 38 in headspace samples from herbivore-elicited leaves (wounding plus Manduca sexta oral secretions, W+OS). In total, 5 analytes differed significantly by genotype in control samples (box plots in ***Supplementary Figure 2****) vs. 9 after W+OS treatment (box plots in* ***Supplementary Figure 3****). In 12 of 14 cases, the analyte is reduced in asLOX3 plants compared to both WT and irJAR4xirJAR6 plants. In only 2 cases do irJAR4xirJAR6 and WT plants differ (in bold): 1 case in which irJAR4xirJAR6 plants, like asLOX3 plants, emit less [the GLV (Z)-3-hexenyl isobutanoate], and 1 case in which they emit more (the sesquiterpene 5-epi-aristolochene). Indicators of significant differences (P-values and letters) are also in bold.*

[a] *Degrees of freedom.*

[b] *Significantly different contrasts ($P < 0.05$) in Tukey post-hoc tests.*

[c] *Tentative identification based on relative retention and comparison to spectral libraries.*

[d] *Identity confirmed using a standard.*

TABLE 3 | Herbivore-induced jasmonic acid (JA) and jasmonoyl-isoleucine (JA-Ile) levels in WT leaves 1 h after W+OS treatment vs. concurrently harvested, untreated leaves was measured in field-grown plants before and after flowering ($n = 9$–10 plants).

Timepoint[a]	Treatment[a]	Analyte[a]	Concentration[a] (ng/g FM)	FC[b]
Pre-flowering	Control	JA	279.1 ± 52.6	4.45
		JA-Ile	25.86 ± 7.40	6.61
	W+OS	JA	1323 ± 230	
		JA-Ile	170.9 ± 32.0	
Post-flowering	Control	JA	6.934 ± 4.632	60.8
		JA-Ile	0.8573 ± 0.3559	139
	W+OS	JA	421.6 ± 77.6	
		JA-Ile	119.2 ± 20.5	

*Timepoint, treatment, and the timepoint*treatment interaction were all highly significant in a general linear model (see section Results).*

[a] *Mean ± SE.*

[b] *Fold change W+OS/control.*

We first analyzed (*E*)-α-bergamotene emission from leaves of WT and ir*JAR4*xir*JAR6* plants after W+OS treatment and found no difference (**Figure 3A**) although JA-Ile accumulation was reduced by 80% in comparison to WT plants during peak accumulation, 1 h after W+OS treatment (**Figure 3B**, **Supplementary Figure 1**, Wang et al., 2007). We then tested the effect of pure jasmonates on (*E*)-α-bergamotene emission from ir*JAR4*xir*JAR6* and WT plants and found that JA was a more potent elicitor than either JA-Ile or JA-Leu, both of which conjugates are synthesized by JAR4 and JAR6; interestingly, the application of JA greatly amplified (*E*)-α-bergamotene emission from ir*JAR4*xir*JAR6* plants, to ca. 40-fold WT emission (**Figure 3C**) while JA-Ile/Leu accumulation in JA-treated ir*JAR4*xir*JAR6* plants was reduced by 89% compared with

WT (**Figure 3D**, **Table 4**). A similar effect on (*E*)-α-bergamotene emission, 20-fold amplification, was reproduced in the reciprocal crosses of ir*JAR4* with ir*JAR6*, while emission from ir*COI1* plants remained near or below the detection limit (**Figure 3E**).

We asked whether other known jasmonates might be more active than JA-Ile in eliciting (*E*)-α-bergamotene emission. Interestingly, the jasmonate elicitor cis-jasmone showed the same (*E*)-α-bergamotene-inducing activity as JA in WT plants, but was less active than JA in eliciting emission from ir*JAR4*xir*JAR6* plants (ca. 20% of JA-elicited emission), and was also not active in ir*COI1* (emission not detected) (**Supplementary Figure 4**). Two lines which convert jasmonates to methyl jasmonate due to the ectopic expression of JASMONATE METHYL TRANSFERASE from *Arabidopsis thaliana* (s*JMT*) (Stitz et al., 2011b) had significantly lower (*E*)-α-bergamotene emission after W+OS elicitation, compared to WT plants (ca. 30% of WT levels; **Supplementary Figure 5**).

We also analyzed volatile emission from systemic leaves, but although these were lower and more variable, the pattern of relative emission did not vary substantially from elicited leaves (**Datasheet 1**).

To ensure that JA-Ile deficiency of ir*JAR4*xir*JAR6* plants was not restored by JA treatment, and to determine whether JA treatment enhanced production of another jasmonate in ir*JAR4*xir*JAR6* plants, we used LC-MS/MS to measure all jasmonates so far identified in *N. attenuata* as well as OPDA, ABA, and SA from W+JA-treated leaves (because JA was used as a treatment, it was not measured). JA-Ile and its metabolites OH-JA-Ile and COOH-JA-Ile, as well 6 other JA-AA conjugates were significantly reduced in ir*JAR4*xir*JAR6* plants compared to WT, while JA-glucose was elevated in ir*JAR4*xir*JAR6*, and levels of the other measured hormones did not differ by plant genotype (**Table 4**, **Figure 3D**, **Datasheet 1**).

These data indicate that an unidentified jasmonate—perhaps JA-glucose—or JA itself, is responsible for the jasmonate-mediated elicitation of (*E*)-α-bergamotene emission, and that JA-Ile may be a negative regulator of jasmonate-induced (*E*)-α-bergamotene emission.

DISCUSSION

Here, we used a JA-Ile-deficient cross of lines silenced in two jasmonate-isoleucine conjugating enzymes, ir*JAR4*xir*JAR6*, in comparison to lines with abrogated jasmonate biosynthesis (as*LOX3*) and perception (ir*COI1*)—all of which have been previously characterized in comparison to independently transformed lines bearing the same construct (Halitschke and Baldwin, 2003; Paschold et al., 2007; Wang et al., 2007, 2008; Stitz et al., 2011b)—to test hypotheses about the role of JA-Ile in the jasmonate-mediated defense response of the wild tobacco *N. attenuata* to herbivory. The ir*JAR4*xir*JAR6* cross produces only about 20% as much JA-Ile but the same amount of JA as WT plants after herbivore elicitation (**Table 1**, Wang et al., 2008); JAR4 and JAR6 transcripts were almost undetectable in Northern blots, in comparison to a strong signal for WT plants (Wang et al., 2008). We conducted reciprocal crossing between the ir*JAR4* and ir*JAR6* lines because possible maternal effects had not previously been tested for, and results from both reciprocal crosses were consistent with each other, and with the bulk collection combining both reciprocal crosses which was used for most analyses (**Figure 3**). All transgenic lines used in this study were in the second (T2) or third (T3) transformed generation; empty vector control plants in only the second transformed generation (T2) have been shown to be indistinguishable from WT plants in their growth and herbivore-induced transcript, hormone and metabolite production (Schwachtje et al., 2008), and thus we used WT plants as controls. Importantly, although JAR4 and JAR6 may also regulate conjugation of JA-Leu, which cannot be analytically distinguished from JA-Ile via standard mass spectrometry (MS) analysis, Wang and colleagues showed that JA-Ile application to ir*JAR4*xir*JAR6* plants was sufficient to restore gene expression (except for JAR4 and JAR6), nicotine and TPI production, and resistance to *M. sexta* larvae, to WT levels. Thus, off-target effects are unlikely.

Individual silencing of *JAR4* or *JAR6* in *N. attenuata* results in a weaker effect than the silencing of both JAR homologs, but similarly to an *S. nigrum* study, independent silencing of the *NaJAR* genes does significantly affect the accumulation of certain jasmonate-regulated secondary metabolites (Wang et al., 2007; VanDoorn et al., 2011a). Wang and colleagues showed that several defense- and growth-related genes were differentially expressed in as*LOX3* vs. ir*JAR4*xir*JAR6* lines of *N. attenuata*, and that differences in gene expression between ir*JAR4*xir*JAR6* lines and WT could be restored by JA-Ile application (except for the downregulation of the *JAR4* and *JAR6* target transcripts), indicating that JA-Ile may regulate a subset of jasmonate-mediated defense responses in *N. attenuata* (Wang et al., 2008). Together with our data, these studies indicate that JA-Ile regulates a subset of jasmonate-mediated defense in solanaceous plants. A study in *Solanum lycopersicum* using RNAi lines deficient in OPDA REDUCTASE 3 (OPR3) or JASMONATE INSENSITIVE 1 (JAI1, the homolog of COI1) showed that both the jasmonate precursor 12-oxophytodienoic acid (OPDA) and JA-Ile can mediate local defense responses, whereas JA-Ile appears to be required for systemic defense activation (Bosch et al., 2014). Thus, the specific role of JA-Ile signaling in herbivore-induced resistance may in part be explained by differences in local vs. systemic regulation of jasmonate-mediated responses. We also analyzed volatile emission from systemic leaves, but although these were lower and more variable, the pattern of relative emission was similar to elicited leaves (**Datasheet 1**). This likely cannot be dissected without accounting for tissue-specific expression and functions of the several JASMONATE ZIM DOMAIN (JAZ) protein repressors of jasmonate signaling, which are variable components of the SCF^{COI1}-jasmonate co-receptor complex (Chini et al., 2007; Thines et al., 2007; Oh et al., 2012; Li et al., 2017), and future work along these lines may help to clarify the mechanisms of specificity in jasmonate signaling and interactions between jasmonates and other hormones.

Consistently with previous studies which quantified a few metabolites as markers of resistance, our investigation of plants' soluble metabolome revealed that JA-Ile deficiency had a relatively large effect on *N. attenuata*'s herbivore-induced

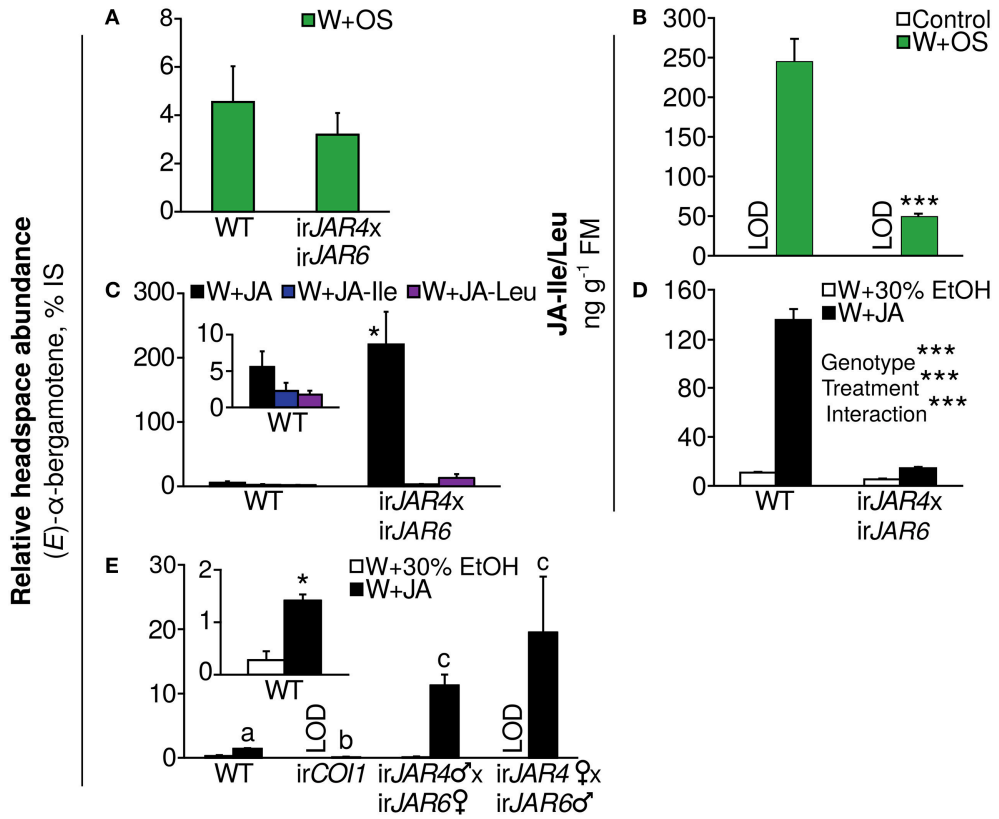

FIGURE 3 | Induction of the volatile (*E*)-α-bergamotene, an effective mediator of resistance to herbivores, depends on jasmonate perception by COI1 and not on the synthesis of JA-Ile by JAR4 and JAR6. Emission of (*E*)-α-bergamotene is shown as a percentage of the internal standard peak area in the same sample (mean + SE) and was measured during peak emission 24–32 h after elicitation. **(A)** The (*E*)-α-bergamotene emission of WT and ir*JAR4*xir*JAR6* plants does not differ when leaves are subjected to simulated herbivory (W+OS, $n = 10$ plants). **(B)** The ir*JAR4*xir*JAR6* cross produces significantly less JA-Ile/JA-Leu than WT plants at peak accumulation, 1 h after plants are subjected to simulated herbivory (W+OS, $n = 3$–5 plants) (Wang et al., 2007); levels in leaves of undamaged plants were below the limit of detection (control: LOD). ***$P < 0.001$, WT vs. ir*JAR4*xir*JAR6*, Tukey's HSD *post-hoc* test following an ANOVA by genotype for a dataset including asLOX3 and irCOI1 (**Table 1**) after log10 transformation to achieve homogeneity of variance. **(C)** ir*JAR4*xir*JAR6* plants emit significantly more (*E*)-α-bergamotene than WT when treated with JA, but not with equimolar amounts of either JA-Ile or JA-Leu, both conjugates synthesized by JAR4 and JAR6; JA-Ile and JA-Leu are also weaker elicitors of (*E*)-α-bergamotene emission in WT plants compared to JA ($n = 4$–6 plants). *Emission from ir*JAR4*xir*JAR6* differs significantly from WT plants ($P < 0.05$) in a Mann-Whitney *U*-test. **(D)** The ir*JAR4*xir*JAR6* cross produces significantly less JA-Ile/JA-Leu than WT plants at peak accumulation 3 h after plants are wounded and supplemented with JA (black bars), as well as in the wounding plus 30% ethanol solvent control (white bars); and also responds significantly less to the JA treatment ($n = 4$ plants). (Peak JA-Ile accumulation occurs later after W+JA treatment than after W+OS treatment; 1, 3, and 6 h were tested). ***$P < 0.001$ in a two-way ANOVA on data after log-transformation and mean-centering to achieve homogeneity of variance and normality; corrected for multiple testing using the false discovery rate method as part of multivariate analysis with the full measured jasmonate profile (**Table 4**). **(E)** Wounding of leaves and the addition of JA significantly enhances (*E*)-α-bergamotene emission from WT but not from ir*COI1* plants compared to a solvent control (30% ethanol), and this enhancement is dramatically increased in ir*JAR4*xir*JAR6* plants ($n = 4$–5 plants); results are shown separately for reciprocal crosses of the same ir*JAR4* and ir*JAR6* lines [data in **(A–C)** are from a bulk collection of these reciprocal crosses]. ^{a,b}Different letters indicate significantly different emission of (*E*)-α-bergamotene ($P < 0.05$ after a Holm-Bonferroni *post-hoc* correction) in Mann-Whitney *U*-tests following a significant Kruskal-Wallis test across all genotypes; *WT plants treated with W+JA emit significantly more (*E*)-α-bergamotene ($P < 0.05$ in a *t*-test followed by the Holm-Bonferroni *post-hoc* correction). LOD, below the limit of detection.

soluble secondary metabolites, although less than did deficiency in jasmonate biosynthesis via *LOX3* or perception via *COI1*. In contrast, JA-Ile deficiency did not affect the volatile metabolome after herbivore elicitation. Van Poecke and Dicke (2003) also showed that *jar1-1* mutants of the brassicaceous plant *Arabidopsis thaliana*, which have reduced JA-Ile production, are able to attract parasitoids as well as WT plants, indicating that JA-Ile may not be a key jasmonate regulator of herbivore-induced plant volatile emission.

We then planted ir*JAR4*xir*JAR6* plants together with as*LOX3* and WT plants out into a field plot in the plant's native habitat in order to monitor their resistance to naturally occurring herbivores. Over more than 10 years of research at this field site and in nearby wild populations, we have found that herbivore populations on the plot reflect those in wild populations (e.g., Steppuhn et al., 2008; Kallenbach et al., 2012; Schuman et al., 2013). We found that JA-Ile deficiency had relatively small effects on susceptibility to native herbivores when compared with total jasmonate deficiency in a field experiment. The small difference in susceptibility we observed between ir*JAR4*xir*JAR6*, and WT plants, mostly resulted from a change in preference by *Trimerotropis* spp. grasshoppers: one of two generalist

TABLE 4 | Results of a 2-way ANOVA on hormone concentrations (mean ± SE) in leaves of the WT or ir*JAR4*xir*JAR6* genotype 3 h after wounding and treatment with 0.25 μmol JA in 20 μL 30% ethanol (W+JA), or only 30% ethanol as a control ($n = 4$ plants).

Analyte	Treatment	Genotype	Mean ± SE (peak value g^{-1} FM)	Genotype Adj. *P*	Treatment Adj. *P*	Interaction Adj. *P*
JA-Ile/Leu	W+EtOH	WT	11.18 ± 0.59[a]	**<0.0001**	**<0.0001**	**<0.0001**
		ir*JAR4*xir*JAR6*	5.676 ± 0.320[a]			
	W+JA	WT	136.1 ± 8.1[a]			
		ir*JAR4*xir*JAR6*	14.77 ± 1.06[a]			
OH-JA-Ile	W+EtOH	WT	13.88 ± 3.41[a]	**<0.0001**	**<0.0001**	**0.0116**
		ir*JAR4*xir*JAR6*	4.122 ± 0.735[a]			
	W+JA	WT	205.9 ± 32.3[a]			
		ir*JAR4*xir*JAR6*	14.03 ± 2.49[a]			
JA-Met	W+EtOH	WT	0.1409 ± 0.1409[a]	**0.0015**	**0.0003**	**0.0116**
		ir*JAR4*xir*JAR6*	LOD[a]			
	W+JA	WT	13.30 ± 1.85[a]			
		ir*JAR4*xir*JAR6*	0.2123 ± 0.2123[a]			
COOH-JA-Ile	W+EtOH	WT	7.794 ± 0.604[a]	**0.0015**	0.0558	0.0778
		ir*JAR4*xir*JAR6*	5.897 ± 0.632[a]			
	W+JA	WT	14.19 ± 2.254[a]			
		ir*JAR4*xir*JAR6*	5.666 ± 0.573[a]			
JA-Phe	W+EtOH	WT	LOD[a]	**0.0030**	**<0.0001**	**0.0076**
		ir*JAR4*xir*JAR6*	LOD[a]			
	W+JA	WT	37.47 ± 1.66[a]			
		ir*JAR4*xir*JAR6*	0.7768 ± 0.5262[a]			
JA-Asn	W+EtOH	WT	0.4039 ± 0.0611[a]	**0.0151**	**0.0101**	0.8998
		ir*JAR4*xir*JAR6*	0.2162 ± 0.1905[a]			
	W+JA	WT	2.171 ± 0.381[a]			
		ir*JAR4*xir*JAR6*	0.4799 ± 0.2237[a]			
JA-glucose	W+EtOH	WT	LOD[a]	**0.0321**	**<0.0001**	**0.0473**
		ir*JAR4*xir*JAR6*	19.85 ± 6.80[a]			
	W+JA	WT	1010 ± 133[a]			
		ir*JAR4*xir*JAR6*	1047 ± 173[a]			
JA-Gly	W+EtOH	WT	0.2631 ± 0.1623[a]	**0.0361**	**0.0001**	0.1305
		ir*JAR4*xir*JAR6*	1.701 ± 0.480[a]			
	W+JA	WT	9.205 ± 1.01[a]			
		ir*JAR4*xir*JAR6*	12.80 ± 2.46[a]			
JA-Val	W+EtOH	WT	0.6380 ± 0.4895[a]	**0.0361**	**<0.0001**	0.4755
		ir*JAR4*xir*JAR6*	0.2151 ± 0.2151[a]			
	W+JA	WT	94.51 ± 5.64[a]			
		ir*JAR4*xir*JAR6*	7.522 ± 0.218[a]			
MeJA	W+EtOH	WT	11460 ± 514.6[b]	0.2067	**<0.0001**	0.8310
		ir*JAR4*xir*JAR6*	13440 ± 696.5[b]			
	W+JA	WT	159100 ± 10550[b]			
		ir*JAR4*xir*JAR6*	180200 ± 24440[b]			
JA-Tyr	W+EtOH	WT	LOD[a]	0.2707	**0.0001**	0.3790
		ir*JAR4*xir*JAR6*	LOD[a]			
	W+JA	WT	1.155 ± 0.386[a]			
		ir*JAR4*xir*JAR6*	0.4841 ± 0.2167[a]			
JA-Glu	W+EtOH	WT	LOD[a]	0.2707	**0.0183**	0.3790
		ir*JAR4*xir*JAR6*	LOD[a]			
	W+JA	WT	0.1886 ± 0.1077[a]			
		ir*JAR4*xir*JAR6*	0.0587 ± 0.0587[a]			

(Continued)

TABLE 4 | Continued

Analyte	Treatment	Genotype	Mean ± SE (peak value g^{-1} FM)	Genotype Adj. *P*	Treatment Adj. *P*	Interaction Adj. *P*
JA-Ala	W+EtOH	WT	LOD[a]	0.3556	**<0.0001**	0.4377
		ir*JAR4*xir*JAR6*	LOD[a]			
	W+JA	WT	6.836 ± 0.822[a]			
		ir*JAR4*xir*JAR6*	9.852 ± 2.513[a]			
JA-glucose formiate	W+EtOH	WT	6.487 ± 5.431[a]	0.4366	**<0.0001**	0.5237
		ir*JAR4*xir*JAR6*	19.25 ± 7.79[a]			
	W+JA	WT	21710 ± 2575[a]			
		ir*JAR4*xir*JAR6*	23370 ± 4120[a]			
JA-Gln	W+EtOH	WT	0.8359 ± 0.6437[a]	0.5901	**0.0056**	0.4377
		ir*JAR4*xir*JAR6*	1.994 ± 0.781[a]			
	W+JA	WT	10.32 ± 1.64[a]			
		ir*JAR4*xir*JAR6*	6.662 ± 1.281[a]			
12-OH-JA	W+EtOH	WT	402.8 ± 53.7[a]	0.5901	**<0.0001**	0.5782
		ir*JAR4*xir*JAR6*	401.0 ± 18.3[a]			
	W+JA	WT	8809 ± 840[a]			
		ir*JAR4*xir*JAR6*	7741 ± 1249[a]			

*Of 20 analytes, these 16 showed significant variation (false discovery rate-adjusted P-value, Adj. P < 0.05) by genotype, treatment, or the interaction of genotype and treatment: JA-glucose (in bold) was elevated while 8 other jasmonates were reduced in irJAR4xirJAR6 compared to WT. The remaining 4 analytes, salicylic acid, abscisic acid, JA-Arg, and OPDA, did not show any significant patterns, and JA-Trp and JA-His were analyzed but not detected in samples. Analytes are organized from smallest to largest adjusted P-value by genotype. The 3 h timepoint was chosen as this was the time that JA treatment-induced jasmonates were in greatest abundance (**Datasheet 1**). JA was not measured since it was used as a treatment. Significant adjusted P-values are in bold. JA-Ile/Leu data are also shown in Figure 3D.*

[a] *ng g^{-1} FM calculated using the JA-Ile internal standard.*

[b] *Raw peak area mg^{-1} FM, LOD: below the limit of detection.*

herbivores present in this season which we found to be affected by *N. attenuata*'s jasmonate-mediated defense. *Trimerotropis* spp. are sensitive to nicotine, which is regulated by JA-Ile (Steppuhn et al., 2004; Wang et al., 2008). It should be noted that grasshoppers are devastating herbivores in some years and so although *Trimerotropis* spp. preferences did not contribute strongly to plant damage in this year, over multiple seasons they are likely to act as a selective pressure. In contrast, another generalist, *Empoasca* sp., appear to respond to plants' jasmonate signaling capacity, and particularly to jasmonate biosynthesis, rather than to jasmonate-mediated defense (Kallenbach et al., 2012). Our data indicate that *Empoasca* sp. sense jasmonates other than JA-Ile, since this herbivore caused more damage to as*LOX3*plants, which have lower levels of all jasmonates, but not to ir*JAR4*xir*JAR6* plants, which are deficient in JA-Ile. Consistent with this inference, Kallenbach and colleagues showed that *Empoasca* sp. prefer to attack as*LOX3* and ir*COI1* vs. WT plants; while both genotypes are strongly deficient in JA, ir*COI1* is not deficient in JA-Ile biosynthesis (**Table 1**; Paschold et al., 2008; Kallenbach et al., 2012).

Interestingly, Vandoorn and colleagues showed that a JA-Ile-deficient ir*JAR4* line of another solanaceous plant, *Solanum nigrum*, was not more susceptible to herbivores in a study at the same field site, although a line silenced in *SnCOI1* was more susceptible (VanDoorn et al., 2011a). Vandoorn and colleagues also showed that gene regulation after herbivory changed very little in ir*JAR4 S. nigrum* plants compared to WT, and that the ir*JAR4*-regulated metabolome overlapped by about 50% with the *COI1*-regulated metabolome (VanDoorn et al., 2011a). Interestingly, *S. nigrum* ir*JAR4* plants accumulated more JA-glucose after wounding than did WT plants, but in this species the ir*JAR4* plants also accumulated more JA after wounding; these differences were eradicated after mock herbivory (W+OS) treatment which elicited larger amounts of JA and JA-glucose in both WT and ir*JAR4* (VanDoorn et al., 2011b). Vandoorn and colleagues also noted that sensitivity for JA-glucose was poor using standard LC-MS/MS analysis and instead developed a method using atmospheric pressure chemical ionization (APCI). Thus, our measurement of elevated JA-glucose in ir*JAR4*xir*JAR6 N. attenuata* plants is consistent, and intriguing, but should be interpreted cautiously.

In the field experiment, we measured herbivore-induced volatile emission from flowering plants. In the glasshouse, flowering plants have abrogated jasmonate responses, which may affect the induction of soluble secondary metabolites more than volatiles (Diezel et al., 2011; Schuman et al., 2014); and thus we used plants before flowering in glasshouse studies. However, flowering plants are more likely to experience oviposition by the herbivore/pollinator *M. sexta*, and this is the stage for which the importance of volatile-mediated defense is best understood (Kessler and Baldwin, 2001; Schuman et al., 2012; Zhou et al., 2017; Joo et al., 2018). Our volatile measurements from flowering plants in the field were consistent with the glasshouse data:

while as*LOX3* plants were deficient in the emission of several leaf volatiles which were induced by herbivory in WT plants, volatile emission from ir*JAR4*xir*JAR6* plants was similar to WT. Interestingly, we found that while herbivore-induced JA and JA-Ile levels were generally lower in field-grown plants after flowering, as has been shown for plants in the glasshouse (Diezel et al., 2011), the herbivore inducibility of both jasmonates—in terms of the fold-change between basal and induced levels—was higher after flowering in field-grown plants, indicating that both may still be important regulators of induced leaf defenses after flowering.

We then used ir*JAR4*xir*JAR6* plants in further glasshouse studies employing different jasmonate elicitors, and other transgenic lines, to investigate the contribution of jasmonate signaling to herbivore-induced volatile emission more closely, focusing on the resistance compound (*E*)-α-bergamotene (initially reported as (*Z*)- α-bergamotene; Halitschke et al., 2000; Schuman et al., 2009). (*E*)-α-Bergamotene is an herbivore-induced sesquiterpene common to, but variably emitted among wild *N. attenuata* plants (Halitschke et al., 2000; Kessler and Baldwin, 2001; Schuman et al., 2009; Zhou et al., 2017), which has been shown to be regulated by jasmonate signaling (Halitschke et al., 2000, 2008; Schuman et al., 2009, 2015) and to attract native predators, resulting in the removal of up to 90% of herbivores from *N. attenuata* plants in nature (Kessler and Baldwin, 2001; Halitschke et al., 2008; Schuman et al., 2015). We found that JA-Ile deficiency as a result of *JAR4* and *JAR6* silencing had little to no effect on (*E*)-α-bergamotene emission after simulated herbivory. It has been suggested that the remaining levels (ca. 10% of WT) of JA-Ile in *A. thaliana jar1-1* mutants and *N. attenuata* ir*JAR4*xir*JAR6* plants may be sufficient for the activation of defense responses (Suza and Staswick, 2008), and other members of the *JAR* gene family could potentially be responsible for the residual 10% of JA-Ile (Staswick and Tiryaki, 2004; Wang et al., 2008). However, we show that in WT plants, JA elicited more abundant (*E*)-α-bergamotene emission than an equimolar amount of JA-Ile; and interestingly, the application of JA to ir*JAR4*xir*JAR6* plants resulted in greatly increased emission of (*E*)-α-bergamotene. Thus, JA-Ile does not appear to be a strong elicitor of (*E*)-α-bergamotene emission in *N. attenuata*. It is tempting to speculate that it may even be a negative regulator. However, Woldmariam and colleagues identified a JA-Ile hydrolase (JIH) in *N. attenuata* and surprisingly, an RNAi line deficient in JIH had greater emission of sesquiterpenes, including (*E*)-α-bergamotene. These ir*JIH* lines had ca. 5-fold the induced levels of JA-Ile as WT plants, as well as elevated JA-Ile metabolites, while other jasmonates were not affected. Together with our study, this indicates that the regulation of indirect defense responses by jasmonates cannot be fully attributed to a single compound. A study by Dinh and colleagues showed that plants deficient in a regulator of abscisic acid (ABA) metabolism, HERBIVORE-ELICITED RESPONSE 1 (HER1), had reduced levels of ABA as well as reduced accumulation of several herbivore-induced metabolites, and reduced emission of several volatiles including (*E*)-α-bergamotene (Dinh et al., 2013). ABA and JA-Ile production are correlated after simulated herbivore treatment in wild genotypes of *N. attenuata* (Schuman et al., 2009) and in *A. thaliana*, ABA has been shown to prime systemic jasmonate-mediated defense (Vos et al., 2013). This indicates that JA-Ile signaling may be involved in the separate regulation of systemic vs. local responses (Bosch et al., 2014). It should be noted that we did not identify differences in ABA accumulation between ir*JAR4*xir*JAR6* and WT plants, but the ratio of ABA:JA-Ile would differ in the two lines due to JA-Ile deficiency in ir*JAR4*xir*JAR6*. However, measurements of volatile emission from systemic leaves, though more variable and having lower signal, reflect the patterns from elicited leaves (**Datasheet 1**).

As an alternative to the local-systemic hypothesis, it is possible that different jasmonate signaling modules allow for the coordinated function of specific metabolites, which may not be explained only by their local vs. systemic elicitation patterns, or their volatility, and that these modules may be regulated in different ways by specific jasmonates and their interactions with other hormone signaling systems (reviewed e.g., by Robert-Seilaniantz et al., 2011). For example, RNAi-mediated silencing of JAZh de-represses the jasmonate-induced accumulation of trypsin protease inhibitors, hydroxygeranyllinalool diterpene glycosides (HGL-DTGs), and the emission of several herbivore-induced volatiles (Oh et al., 2012); all of these responses show strong local and weaker systemic induction in response to herbivory, with a distribution that meets the predictions of optimal defense theory: induced in proportion to herbivory, but also constitutively enriched in younger and reproductive tissues (Halitschke et al., 2000; van Dam et al., 2001; Heiling et al., 2010; Brütting et al., 2016; Li et al., 2017; Schäfer et al., 2017). In addition, HGL-DTGs effectively reduce the growth of *M. sexta* larvae within the background of a wild-type plant defense profile (Heiling et al., 2010), and TPIs may increase susceptibility of larvae to predation by *Geocoris* spp. predators attracted to the plant volatiles the larvae induce (Schuman et al., 2012). Interestingly, RNAi-mediated silencing of *JAZh* also suppressed nicotine accumulation (Oh et al., 2012), and the induction of nicotine is also attenuated upon plant recognition of OS from nicotine-tolerant *M. sexta* larvae (von Dahl et al., 2007). Thus, one hypothesis is that JAZh represses a functional defense module responding to attack by *M. sexta* and perhaps other nicotine-tolerant specialist herbivores (Kessler and Baldwin, 2004), and by downregulating repression by JAZh, plants might emphasize indirect over direct defense responses. It remains an open question as to what extent the regulation of JAZh and other JAZs may occur by JA-Ile-independent mechanisms.

Furthermore, it is well known that plant defense responses change with ontogeny, and optimal defense theory predicts that plants should invest more in defending first young leaves, and then reproductive tissue such as buds and flowers (Stamp, 2003; Boege and Marquis, 2005; Barton and Koricheva, 2010; Brütting et al., 2016). In *N. attenuata*, leaf jasmonate- and ethylene-mediated defense responses change drastically once plants start to flower, at least under glasshouse conditions (Diezel et al., 2011), yet jasmonate-mediated volatile emission is not abrogated in flowering plants (Schuman et al., 2014), and in fact the flowering stage is when volatile-mediated defenses, which can be highly

effective against the extremely damaging specialist herbivore *M. sexta*, may be most important (Kessler and Baldwin, 2001; Kessler et al., 2010, 2015; Zhou et al., 2017). Interestingly, we show here that although JA and JA-Ile production appear to be abrogated in leaves after plants flower, their inducibility was maintained in leaves, with an even greater fold-change after induction due to lower basal levels. Because these hormone data come from field-grown plants, we cannot exclude influence of naturally occurring herbivore damage on our measurements of basal or induced levels at either growth stage. However, basal jasmonate levels in floral buds are high in comparison to leaves, and recent work on the tissue specificity of JAZ function in *N. attenuata* has provided more insight on how plants independently regulate jasmonate-mediated floral defense (Li et al., 2017). Our understanding of the complexities that determine jasmonate regulation of plant defense is likely to benefit from combining mechanistic advances with an integrative functional understanding of plant defense responses (Li et al., 2016).

DATA AVAILABILITY STATEMENT

All source data for this study are included in the manuscript and the supplementary files. The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MS, SM, EG, and IB: conceptualization; MS, SM, and EG: data curation; MS, SM, EG, EM, and SG: formal analysis; MS and IB: funding acquisition; MS, SM, EG, CD, SG, and IB: investigation; MS, SM, EG, and IB: methodology; MS, SM, EG, and IB: project administration; IB: resources; MS and IB: supervision; MS, SM, EG, and EM: validation; , MS and EG: visualization; MS, SM, and EG: writing—original draft; MS, SM, EG, EM, and IB: writing—review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00787/full#supplementary-material

Datasheet 1 | Source data files for the article.

Supplementary Figure 1 | JA and JA-Ile accumulation after mock herbivory treatment (W+OS) in WT, as*LOX3*, ir*COI1*, and ir*JAR4*xir*JAR6* plants ($n = 3$–5 plants); JA-Ile data for WT and ir*JAR4*xir*JAR6* plants is also shown in **Figure 3B**. ^{a,b}Different letters represent statistically significant differences ($P < 0.0001$) in Tukey's HSD *post-hoc* tests following one-way ANOVAs with a false discovery rate correction for multiple analytes, after log transformation and mean-centering to achieve normality and homogeneity of variance. See also **Table 1** and **Figure 3B**.

Supplementary Figure 2 | Boxplots showing normalized peak areas of analytes which differed significantly by plant genotype (left to right: WT, red; ir*JAR4*xir*JAR6*, green; or as*LOX3*, blue) in headspace measurements of leaves on field-grown plants before W+OS treatment (control), to accompany **Table 2** ($n = 10$ plants). The lowest boxplot shows data from an unidentified green leaf volatile. ^{a,b}Different letters represent statistically significant differences in Tukey *post-hoc* tests following a one-way ANOVA and corrected for multiple testing using the false discovery rate method, after log transformation and mean-centering to achieve normality and homogeneity of variance.

Supplementary Figure 3 | Boxplots showing normalized peak areas of analytes which differed significantly by plant genotype (left to right: WT, red; ir*JAR4*xir*JAR6*, green; or as*LOX3*, blue) in headspace measurements of W+OS-treated leaves on field-grown plants, to accompany **Table 2** ($n = 5$–8 plants). ^{a,b}Different letters represent statistically significant differences in Tukey *post-hoc* tests following a one-way ANOVA and corrected for multiple testing using the false discovery rate method, after log transformation and mean-centering to achieve normality and homogeneity of variance.

Supplementary Figure 4 | A known jasmonate elicitor of volatiles, *cis*-jasmone, is not more potent than JA in eliciting (*E*)-α-bergamotene emission ($n = 4$–5 plants). Addition of either JA or an equimolar amount of *cis*-jasmone (CJ) affects (*E*)-α-bergamotene emission similarly in WT plants, but only JA and not *cis*-jasmone enhances emission in ir*JAR4*xir*JAR6* plants; results are shown separately for reciprocal crosses of the same ir*JAR4* and ir*JAR6* lines. ^{a,b}Different letters indicate significantly different emission of (*E*)-α-bergamotene ($P < 0.05$ after a Holmes-Bonferroni *post-hoc* correction) in Mann-Whitney U-tests following a significant Kruskal-Wallis test across all genotypes.

Supplementary Figure 5 | Methyl jasmonate is not the elicitor of (*E*)-α-bergamotene. Two independently transformed transgenic lines ectopically expressing the *Arabidopsis thaliana* jasmonate methyltransferase (s*JMT*), which converts jasmonates to methyl jasmonate, have reduced emission of (*E*)-α-bergamotene from leaves following W+OS treatment ($n = 9$–10 plants). *s*JMT* lines differ significantly from WT ($P < 0.05$ after a Holmes-Bonferroni *post-hoc* correction) in Mann-Whitney *U*-tests following a significant Kruskal-Wallis test across all genotypes.

Supplementary Table 1 | Deduplicated feature table from the untargeted analysis of WT, ir*JAR4*xir*JAR6*, as*LOX3*, and ir*COI1* plants in the glasshouse experiment.

Supplementary Table 2 | Peak table for control leaf headspace samples from flowering plants in the field experiment.

Supplementary Table 3 | Peak table for W+OS-treated leaf headspace samples from flowering plants in the field experiment.

Image 1 | Contains all supplementary figures.

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Whole-Plant Metabolic Allocation Under Water Stress

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Trade-offs between plant growth and defense depend on environmental resource availability. Plants are predicted to prioritize growth when environmental resources are abundant and defense when environmental resources are scarce. Nevertheless, such predictions lack a whole-plant perspective—they do not account for potential differences in plant allocation above- and belowground. Such accounting is important because leaves and roots, though both critical to plant survival and fitness, differ in their resource-uptake roles and, often, in their vulnerability to herbivores. Here we aimed to determine how water availability affects plant allocation to multiple metabolic components of growth and defense in both leaves and roots. To do this, we conducted a meta-analysis of data from experimental studies in the literature. We assessed plant metabolic responses to experimentally reduced water availability, including changes in growth, nutrients, physical defenses, primary metabolites, hormones, and other secondary metabolites. Both above- and belowground, reduced water availability reduced plant biomass but increased the concentrations of primary metabolites and hormones. Importantly, however, reduced water had opposite effects in different organs on the concentrations of other secondary metabolites: reduced water increased carbon-based secondary metabolites in leaves but reduced them in roots. In addition, plants suffering from co-occurring drought and herbivory stresses exhibited dampened metabolic responses, suggesting a metabolic cost of multiple stresses. Our study highlights the needs for additional empirical studies of whole-plant metabolic responses under multiple stresses and for refinement of existing plant growth-defense theory in the context of whole plants.

Keywords: abiotic stress, biotic stress, drought, growth-defense trade-off, herbivory, multiple stresses, resource allocation, roots

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INTRODUCTION

Plants experience many forms of stress, from both the abiotic and the biotic environment. As sessile organisms, plants have evolved various physiologic and metabolic responses to individual stresses, but the nature of such responses strongly depends on whether and how stresses co-occur in the plant's environment (Atkinson and Urwin, 2012; Suzuki et al., 2014; Nguyen et al., 2016). In particular, the co-occurrence of resource limitation and herbivory can steepen the trade-off between growth and defense by altering both the availability of chemical precursors and the strategic value of defense (Herms and Mattson, 1992; Mole, 1994; Donaldson et al., 2006). The strategic value of defense (*i.e.*, optimal defense) should depend on the cost of defense traits,

the value of the tissue, and the risk of attack from herbivores (McKey, 1974; Rhoades, 1979). Although roots are the first responders to many kinds of stress (Brunner et al., 2015; Weemstra et al., 2016), work to date on growth-defense trade-offs and optimal defense has focused mostly aboveground, on shoots and leaves, rather than on the whole plant (van Dam, 2009). There are still few predictions about how simultaneous abiotic and biotic stresses should drive whole-plant allocation strategies.

Water availability is a central resource affecting plant fitness. Predicted increases in the frequency of extreme precipitation events under ongoing global climate change (Bates et al., 2008; Donat et al., 2016) threaten reliable sources of water for terrestrial ecosystems (Easterling et al., 2000; Weltzin et al., 2003). Plants experiencing drought or flooding can adjust their morphology to optimize water uptake by the roots while decreasing the rate of photosynthesis by the leaves, thereby changing the production of growth and defense metabolites (Koricheva et al., 1998; Grant et al., 2005; Nicotra et al., 2007; Kleine and Mueller, 2014). Changes in biochemistry under water stress can determine plant physiology and performance, including the fitness-defining production of flowers and seeds (Taiz and Zeiger, 1998). Despite the potentially vital role of water availability in driving trade-offs between growth and defense, few studies to date have evaluated whole-plant metabolic responses to water stress in combination with herbivory stress, either experimentally induced or inferred from the plant's production of defensive secondary metabolites. Because plant chemistry links multi-species trophic interactions with biogeochemical cycles, determining how plant chemistry responds to changes in precipitation may be critical to determining the response of entire ecosystems (Hunter, 2016).

Plant responses to water stress can affect the concentration, composition, and distribution of both primary and secondary metabolites. Plant primary metabolites, such as amino acids, enzymes, and carbohydrates, maintain life processes and facilitate growth (Díaz et al., 2004). Secondary metabolites allow plants to adapt to their environments by defending them against abiotic stresses, pathogens, and herbivores (Agrawal, 2007; Ramakrishna and Ravishankar, 2011). A plant's response to stress typically begins with an elaborate signaling network, with frequent crosstalk between primary and secondary metabolic pathways (Robert-Seilaniantz et al., 2011; Atkinson and Urwin, 2012; Bonaventure, 2014; Suzuki et al., 2014; Jacobo-Velázquez et al., 2015). Signaling pathways can also be shared between responses to different forms of stress, including between biotic and abiotic stresses (Santner and Estelle, 2009; Robert-Seilaniantz et al., 2011; Atkinson and Urwin, 2012; Denancé et al., 2013; Nguyen et al., 2016). Changes in the quantity and composition of signal molecules induced by simultaneous stresses may in fact allow plants to alter their physiologies and metabolic mechanisms to cope with multiple stresses at once (Krasensky and Jonak, 2012). For example, abscisic acid (ABA) and jasmonic acid (JA) hormone signaling regulate plant responses to both drought and foliar insect herbivores (Pieterse et al., 2012; Berens et al., 2017). The cost of plant responses to both stresses may be reduced by this overlap (Mittler, 2006; Nguyen et al., 2016). In other cases, different stresses elicit opposing reactions. For example, salicylic acid (SA) and ABA/JA signaling pathways are commonly antagonistic to one another (Pieterse et al., 2012; Berens et al., 2017). Elevated SA signaling in response to biotrophic pathogens is often correlated with reduced ABA/JA signaling and decreased resistance to drought and insect herbivores (Zarate et al., 2007; Suzuki et al., 2014).

Changes in plant chemistry in response to water stress, although measured less frequently in ecological studies than changes in biomass and reproduction (but see Koricheva et al., 1998), will strongly affect the surrounding ecological community via direct and indirect trophic interactions (Hunter, 2016). These interactions can then feed back to affect plant chemistry and nutrient cycles. Such complex interactions initiate not only in leaves, but also in roots. Plant metabolic responses to water stress are also likely to differ between leaves and roots (Parker et al., 2012). Because water is sensed by the roots, root metabolic allocation under water stress is probably critical to defining whole-plant responses (Wilkinson and Davies, 2010; Basu et al., 2016). Although water stress often appears correlated with differences in herbivore pressure aboveground (White, 1969; Mattson and Haack, 1987; Huberty and Denno, 2004), very little is known about how water stress affects the susceptibility of roots to attack.

In this study, we assemble a meta-analytic database to synthesize our knowledge so far of: (1) how whole plants respond metabolically to reduced water; and (2) if and how these responses differ when plants suffer from co-occurring herbivory stress. To obtain sufficient studies for our analysis, we examined any study that reduced water compared to controls (see Results for details). For convenience, we use "drought" interchangeably with reduced water, a convention that is consistent conceptually, although not in operational detail, with the formal hydrological definition of drought (Mishra and Singh, 2010). Specifically, we test the hypotheses that: (1) leaves and roots produce different metabolic responses under reduced water; (2) reduced water negatively affects nutrient concentrations in both leaves and roots (He and Dijkstra, 2014); (3) reduced water increases the concentration of primary metabolites in both leaves and roots (Chaves et al., 2003), but the effects on composition vary between above- and belowground organs; (4) changes in secondary metabolites under reduced water are related to distinct growth-defense trade-offs in roots and leaves because the higher relative growth of roots alone can mitigate the effects of drought; and (5) the cost of co-occurring drought and herbivory stresses is mitigated by the overlap in ABA/JA defense signaling pathways.

MATERIALS AND METHODS

Study Search and Data Collection

We compiled the database by conducting a key-word search in the Web of Science (ISI) in September 2017. We considered all resulting peer-reviewed studies with no date restrictions from searches using the terms "secondary metabolites or compounds", "chemical compounds or defens*", "plant or leav* or root*" and "herbivor* or insect or parasitoid" as a topic in all possible factorial combinations, but always with "water or precipitation or

drought" as the title category. This initial search resulted in 1,475 studies. Our analysis did not include book chapters, graduate theses, or unpublished data. We attempted to analyze the effects of increased water, or flooding, stress on plant metabolism as well, but found too few studies that had addressed this alternative water stress to be confident in our results (data not shown).

To be included in the analysis, each study had to meet three criteria. First, water or precipitation had to have been manipulated experimentally, *i.e.*, the study had to have both control and treatment levels of water. Observational studies comparing plants growing in variable natural conditions were thus excluded from the analysis. Second, at least one secondary chemical metabolite (including hormones) had to have been reported. Third, studies had to provide each of the following variables (directly or indirectly): means, measure of variance (SD or SE), and the sample sizes of the control and the treatments. When this statistical information was not reported in the text, we extracted these values from the data using "GetData Graph Digitizer" (v2.24; Fedorov, 2002). Unspecific error bars were assumed to show standard error.

We also followed two rules when collecting the data from each study: (1) When a single study presented results for several plant species and response variables, we included them all. (2) When plant species were subjected to a gradient of water treatments or several treatments in a factorial design, we chose the control, and the lowest value of water as "drought." Although an imperfect proxy for real-world drought, this definition allows us to begin to address how plants may respond metabolically to changes in precipitation. Results from factorial designs in which water treatment was in combination with another treatment (*e.g.*, low nutrient, shade) were excluded.

We investigated plant metabolic responses to drought alone and to drought combined with herbivory. We grouped the response variables into six overarching groups: (1) growth, including root and/or shoot weight (dry or fresh); (2) nutrients, including nitrogen (N), phosphorus (P), and potassium (K) concentrations from whole plants, roots, and/or shoots; (3) physical traits, including lignin, specific leaf area, and root length; (4) primary metabolites, including leaf soluble sugars (mono-, di-, and trisaccharides), complex carbohydrates (starch and/or total non-structural carbohydrates), vitamins, amino acids, and enzymes; (5) hormones, including abscisic acid (ABA) and jasmonic acid (JA), which are important for plant drought and herbivory responses; and (6) other secondary metabolites, including both carbon- and nitrogen-based secondary metabolites (*i.e.*, flavonoids, phenolics, tannins, terpenoids, volatiles, alkaloids, stilbenes, and glucosinolates) from roots and shoots. Although the 'volatiles' mainly comprised monoterpenes and sesquiterpenes, we separated them from "terpenoids" due to the method of extraction: volatiles were extracted from head-space collections, whereas terpenoids were extracted from ground leaves. We also collected the following additional information from each study: plant species, study location (greenhouse, growth chamber, or field), type of reduced water treatment, herbivore species, plant part attacked by herbivores (leaf, roots, or both), herbivore feeding type (*i.e.*, chewing or sap-sucking), and the indirect effect on parasitoids.

Statistical Analyses

For each study and response variable, we estimated the mean effect size of a plant subjected to water treatments using Hedges' *d* statistic (Koricheva et al., 2013). We estimated the magnitude of the treatment effect (effect size, *d*) by calculating the difference between the treatment and control estimated means, adjusted by their sample sizes and standard deviation, and weighted by a correction term (Gurevitch et al., 2001; Koricheva et al., 2013). We use Hedges' *d* because it is not affected by unequal sampling variances, and it includes a correction factor for small sample sizes (Gurevitch et al., 2001). We calculated the mean effect size and confidence interval (CI) for each class of response variable. The water treatment was considered to have a statistically significant effect when the 95% CI of the variable did not overlap zero. A positive effect indicates that the drought treatment increased the amount of a given plant trait, whereas a negative effect indicates that the water treatment decreased the amount of a given plant trait.

To test if there was variation among studies beyond that due to sampling error, we used the model heterogeneity statistic (Q_M, also known as heterogeneity between groups Q_B). Q_M describes the amount of heterogeneity that can be explained by the model (Gurevitch et al., 2001; Koricheva et al., 2013). Here this means that if Q_M for a given plant trait is significant ($P < 0.05$), some of the variance can be explained by the water treatment. We calculated Q_M using the Q statistic and then compared against the Q_E (unexplained heterogeneity, also known as Q_{Error}) using a chi-squared distribution.

We conducted all analyses using the R statistical programming language (v3.2.4; R Core Team, 2016) with the package *metafor* (v2.0-0; Viechtbauer, 2010). We used the standardized mean difference (SMD) and the rma() function as the meta-analytic random-effect model with the Hedges estimator (Viechtbauer, 2010). We also performed some additional analyses to test for publication bias. Publication bias occurs when the mean effect size in the overall dataset generates different conclusions from those obtained when the mean effect size comes from a representative sample with reliable results (Koricheva et al., 2013). We used both funnel plots (*i.e.*, scatterplots of effect sizes against their variance) and Spearman rank correlations between the mean effect size and sample sizes to test for publication bias (Koricheva et al., 2013). In the absence of bias, the funnel plots should show symmetry around the mean effect size, and effect sizes should not correlate with sample sizes (Koricheva et al., 2013).

RESULTS

The literature search resulted in 1,475 publications, of which 61 papers published between January 1992 and August 2017 met our criteria. The full data set is deposited in the Dryad Digital Repository. The publications were reported in 41 different

journals (see Supplementary Materials). Considering only the studies that experimentally manipulated water availability we found three types of experiments: (1) studies that reduced the percentage of moisture in the soil compared to the control (44%), (2) studies that reduced the amount of water given to the plant (28%), and (3) studies that deprived the plants of water for the length of the study (28%). Eleven studies manipulated drought and aboveground herbivory simultaneously, and only one study also investigated the effects of root herbivory. Herbivory studies included nine species of herbivores who engaged in chewing (seven studies), sap-sucking (five studies), and artificial mechanical damage (two studies). Three studies measured the indirect effect of drought, by means of volatiles, on parasitoids. Forty-one studies measured leaf traits, 18 measured traits of both leaves and roots, and two measured only root traits. In total, the studies we reviewed investigated the responses of 92 plant species or genotypes in 48 genera. Crop species were used in 42 studies, native species in 16 studies, an invasive species in two studies, and a medicinal plant in one study. Sixty-seven percent of the studies were conducted in greenhouses or growth chambers, and 33% were field experiments.

Water treatment explained a significant proportion of trait variation for almost all plant traits measured (**Table 1**). For most traits, funnel plots of effect sizes versus sample sizes indicated that, overall, few studies reported these traits and that those studies were biased towards smaller sample sizes (Supplementary Figure A1). Nevertheless, Spearman's rank correlations did not show significant relationships between the mean effect size and the sample size for 17 of the 19 traits we quantified (Supplementary Table A1), which indicates that bias in the meta-analysis is mostly non-significant, and our results are reliable estimates.

Growth and Physical Traits

Drought treatments reduced shoot and root biomass (**Figure 1**; $P < 0.001$ for both). Drought treatments also negatively affected physical traits (*i.e.*, lignin) in leaves (**Figure 1A**; $P < 0.01$). In contrast, drought did not change pooled physical traits in roots (*i.e.*, lignin, and root length; **Figure 1B**; $P = 0.3$), but it did significantly reduce root length (Hedges' $d = -0.548$, CI = -0.939 to -0.157, $P < 0.01$).

Nutrients and Primary Metabolites

Drought treatments did not change overall nutrient content in leaves (**Figure 1A**; $P = 0.8$) or in roots (**Figure 1B**; $P = 0.7$). In leaves, this lack of effect emerged from the opposing effect of drought on nitrogen (N) compared with its effect on phosphorus (P) and potassium (K). Reduced water increased leaf N (**Figure 2**; $P = 0.03$) but reduced both P (Hedges' $d = -0.874$, CI = -1.45 to -0.298, $P < 0.01$) and K (Hedges' $d = -2.798$, CI = -4.111 to -1.485, $P < 0.001$). In contrast, drought did not affect root N (**Figure 2**; $P = 0.6$), and no data were available on changes in root P or K.

Drought treatments increased the concentrations of pooled primary metabolites in both leaves and roots, but this effect was significant only in leaves (**Figure 1**; $P < 0.05$ for leaves and $P = 0.09$ for roots). When primary metabolites were subdivided, drought treatments tended to increase sugars but reduce complex carbohydrates in leaves, but these effects were not significant (**Figure 2**). Very few studies examined sugars or complex carbohydrates in roots (**Figure 2**), and no studies examined

TABLE 1 | The model heterogeneity (Q_M) for changes in plant traits under drought treatment alone, and under drought and herbivory treatments combined.

Treatment per plant part	Plant trait measured	Q_M	df	*P*
Drought				
Leaf	Primary metabolites	2400	240	*<0.001*
	Secondary metabolites	966	222	*<0.001*
	N-Based compounds	55.2	20	*<0.001*
	C-Based compounds	911	201	*<0.001*
	Physical traits	10.6	7	0.2
	Hormone	107	14	*<0.001*
	Biomass	329	33	*<0.001*
	Nutrients	263	35	*<0.001*
Root	Primary metabolites	158	17	*<0.001*
	Secondary metabolites	143	13	*<0.001*
	Physical traits	22.1	9	*<0.01*
	Hormone	7.18	3	0.07
	Biomass	154	20	*<0.001*
	Nutrients	86.6	9	*<0.001*
Drought x Herbivory				
Leaf	Primary metabolites	1.84	2	0.4
	Secondary metabolites	242	93	*<0.001*
	Hormone	30.6	14	*<0.01*

A significant Q_M ($P < 0.05$) indicates that the treatment explains a significant proportion of the variance among observations.

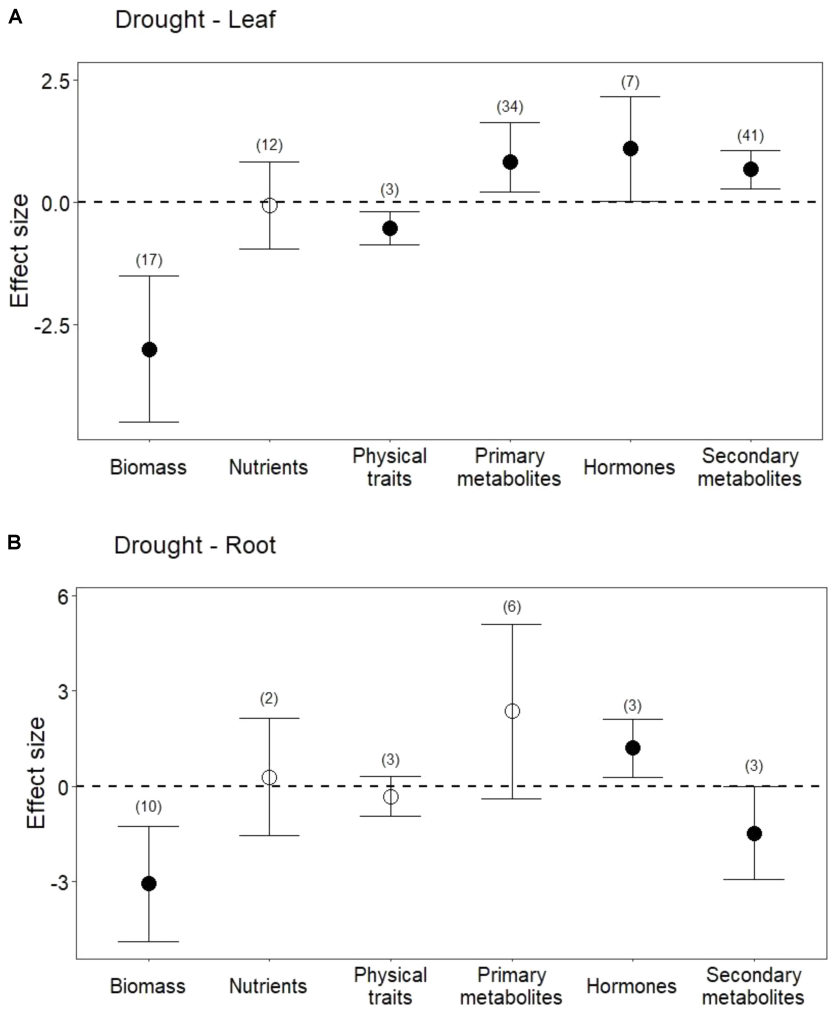

FIGURE 1 | Influence of drought treatments on leaf and root traits. **(A)** Effect of drought on leaves. **(B)** Effect of drought on roots. Numbers in parentheses represent the number of studies considered. Mean effect sizes are shown with 95% confidence intervals (CIs). Effects are considered significant if their associated CIs do not overlap zero (dashed line) and are illustrated with solid circles.

amino acids or enzymes. In leaves, drought treatments had no effect on enzymes ($P = 0.2$) but did increase pooled amino acids (**Figure 3**; $P < 0.03$). Nevertheless, this increase in leaf amino acids depended almost entirely on proline (**Figure 3**).

HORMONES AND SECONDARY METABOLITES

Drought increased the concentrations of hormones (ABA and JA) in both leaves and roots (**Figure 1**; $P < 0.05$ and $P < 0.01$, respectively). On the other hand, salicylic acid (SA) was unchanged or marginally lower in leaves subjected to drought (Hedges' $d = -0.1015$, CI = −1.007 to 0.803, $P = 0.8$).

Drought treatments also significantly increased secondary metabolites in leaves when all compounds were pooled (**Figures 1A**, **4**; $P < 0.01$). Interestingly, and in contrast to leaves, drought treatments significantly reduced secondary metabolites in roots (**Figures 1B**, **4**; $P < 0.05$). This difference between leaves and roots depended on carbon (C)-based secondary compounds, which were higher in leaves under drought but lower in roots (**Figure 4A.1**; $P < 0.01$ and $P < 0.05$, respectively). Drought did not affect nitrogen (N)-based secondary compounds in leaves (**Figure 4A.2**; $P = 0.8$), and these compounds were not measured in roots. Among C-based secondary metabolites, concentrations of flavonoids, phenolics, and terpenoids all increased in leaves under drought, but only the increase in flavonoids was significant (**Figure 4B**; $P < 0.01$). In contrast, phenolics in roots were significantly reduced by drought (**Figure 4B**; $P < 0.04$). Interestingly, although phenolics in leaves were not significantly higher under reduced water when all data were pooled, drought treatments significantly increased phenolics in non-trees (Hedges' $d = 1.953$, CI = 0.191 to 3.715, $P = 0.03$), and marginally decreased phenolics in trees (Hedges' $d = -0.242$, CI = −0.564 to 0.08, $P = 0.1$). Volatiles, mainly comprising monoterpenes and sesquiterpenes did not change in leaves under drought treatments

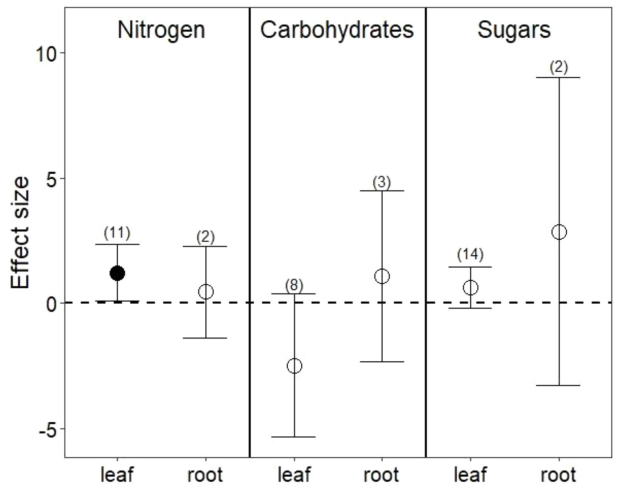

FIGURE 2 | Drought effects on nitrogen content, complex carbohydrates, and sugars for leaves and roots. Numbers in parentheses represent the number of studies considered. Mean effect sizes are shown with 95% confidence intervals (CIs). Effects are considered significant if their associated CIs do not overlap zero (dashed line) and are illustrated with solid circles.

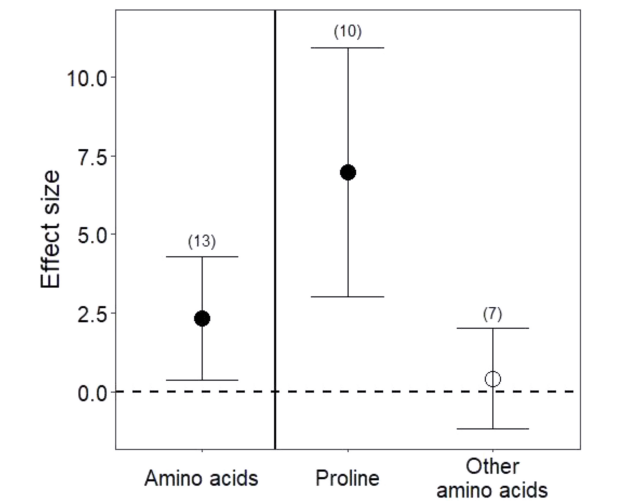

FIGURE 3 | Drought effects on leaf amino acid content. "Amino acids" include all pooled amino acids; "Other amino acids" includes all amino acids except for proline. Numbers in parentheses represent the number of studies considered. Mean effect sizes are shown with 95% confidence intervals (CIs). Effects are considered significant if their associated CIs do not overlap zero (dashed line) and are illustrated with solid circles.

(**Figure 4B**; $P = 0.6$), resulting from the opposing effects of two studies that found increases in volatiles and one that found a decrease. Volatiles under drought stress were not measured in roots.

Herbivory and Tritrophic Interactions

Among the 1,475 studies that were returned by our search criteria, only 11 studies manipulated water and herbivory simultaneously and contained sufficient observations for us to calculate an effect size. Overall, the co-occurrence of herbivory with reduced water appeared to dampen plant metabolic responses relative to those under reduced water alone (**Figure 5**). Hormones were slightly higher in leaves (**Figure 5**) and lower in roots in co-occurring drought and herbivory treatments relative to controls (*i.e.*, plants sustaining neither drought nor herbivory), but these effects were not significant ($P = 0.5$ in leaves and $P = 0.2$ in roots). Among studies that manipulated both drought and herbivory and also assessed root metabolic traits (Tariq et al., 2013a; Ederli et al., 2017), hormones were the only metabolite measured. In leaves, sugars were the only primary metabolites measured under co-occurring drought and herbivory treatments, and the slight increase in sugars was not significant (**Figure 5**; $P = 0.1$). Finally, co-occurring drought and herbivory treatments increased secondary metabolites in leaves when all compounds were pooled (**Figure 5**; $P < 0.03$), due to increases in C-based secondary metabolites ($P < 0.04$) and flavonoids in particular ($P < 0.02$). Still, the effect size of this increase in secondary metabolites under co-occurring drought and herbivory treatments was smaller than the increase observed under drought treatment alone (**Figure 5**).

The three studies that measured the effect of drought on volatiles also looked at parasitoid responses. These three studies illustrated a wide variety of possible outcomes: drought increased volatiles, which increased parasitoid attraction (Salerno et al., 2017); drought decreased volatiles, which decreased parasitoid attraction (Tariq et al., 2013b), and drought increased volatiles, but this had no effect on parasitoid attraction (Weldegergis et al., 2015).

DISCUSSION

Our results highlight the importance of considering whole-plant metabolic responses under water stress. Despite the key role of roots in sustaining and mitigating abiotic and biotic stress, early theories of trade-offs between plant growth and defense focused on aboveground tissues (Rhoades, 1979; Coley et al., 1985; Herms and Mattson, 1992). Although attention to roots has increased recently (*e.g.*, in our meta-analysis, a third of papers with root measurements were conducted since 2010), some metabolic comparisons between leaves and roots under water stress will be possible only with further studies. Additional studies will also be necessary to differentiate among levels of water stress, which could be associated with different plant metabolic responses. In the comparisons that were possible, leaves and roots often produced different metabolic responses, consistent with our first hypothesis. The leaf economics spectrum now provides simple predictions of how leaf traits should vary with resources, and such traits also correlate with vulnerability to herbivores (Wright et al., 2004; Agrawal and Fishbein, 2006). In contrast, an analogous root economics spectrum remains elusive (Valverde-Barrantes et al., 2015; Weemstra et al., 2016; also see Roumet et al., 2016), perhaps because roots respond to a wider variety of environmental constraints (Weemstra et al., 2016). Consistent with the existence of differential constraints operating above- and

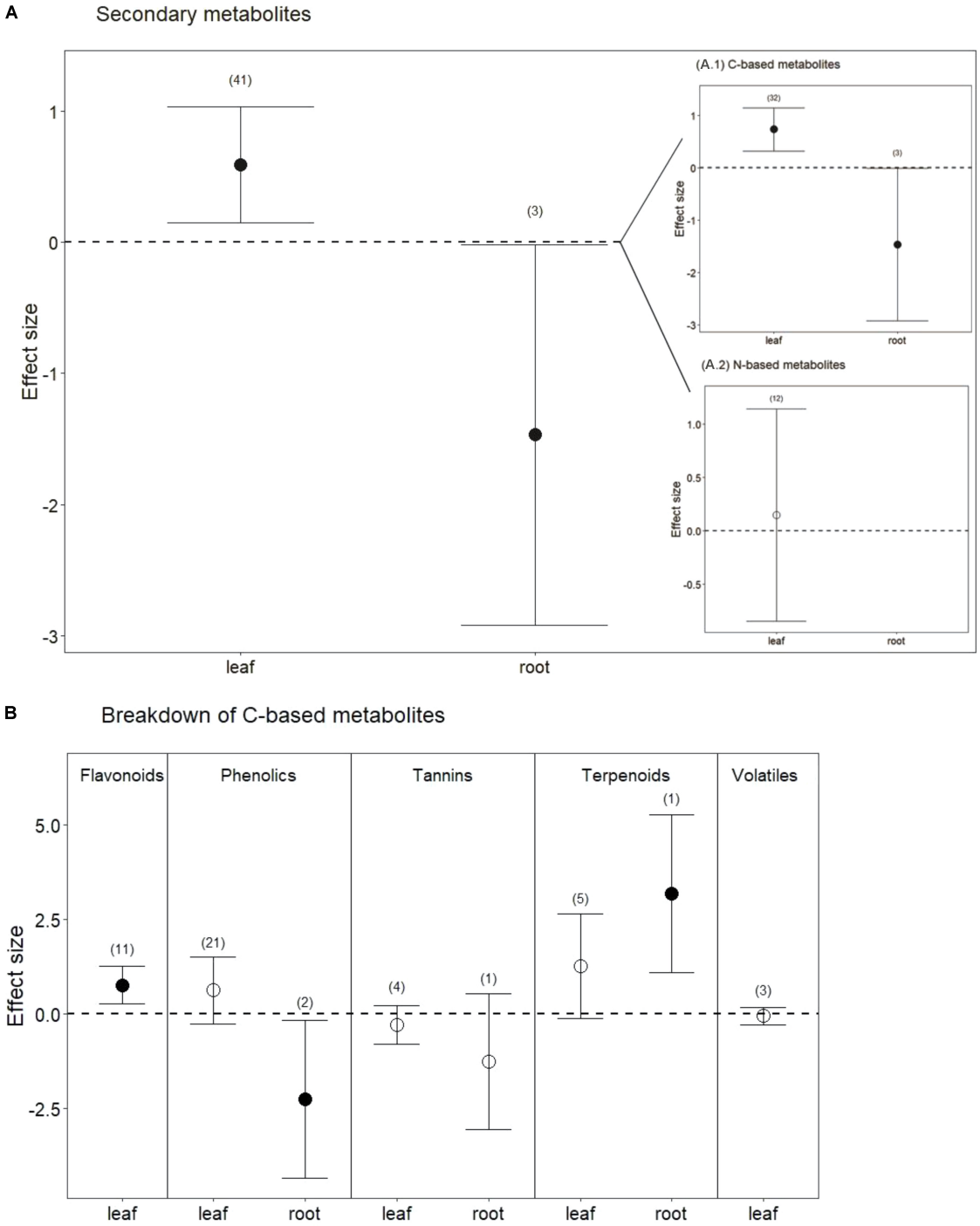

FIGURE 4 | Secondary metabolite response to drought for both leaves and roots. **(A)** Secondary metabolites, including all measured metabolites (C-based and N-based) for each tissue (from **Figure 1**). **(A.1)** Carbon-based metabolites, including flavonoids, phenolics, tannins, terpenoids, and volatiles. **(A.2)** Nitrogen-based metabolites, including glucosinolates, alkaloids and glycoalkaloids. **(B)** Breakdown of C-based secondary metabolites for each tissue. Numbers in parentheses represent the number of studies considered. Mean effect sizes are shown with 95% confidence intervals (CIs). Effects are considered significant if their associated CIs do not overlap zero (dashed line) and are illustrated with solid circles.

belowground, our results suggest that leaves and roots respond to water stress via partially decoupled growth-defense trade-offs.

Contrary to our second hypothesis, drought did not affect overall nutrient concentrations in either leaves or roots. However, this result obscured some interesting differences among

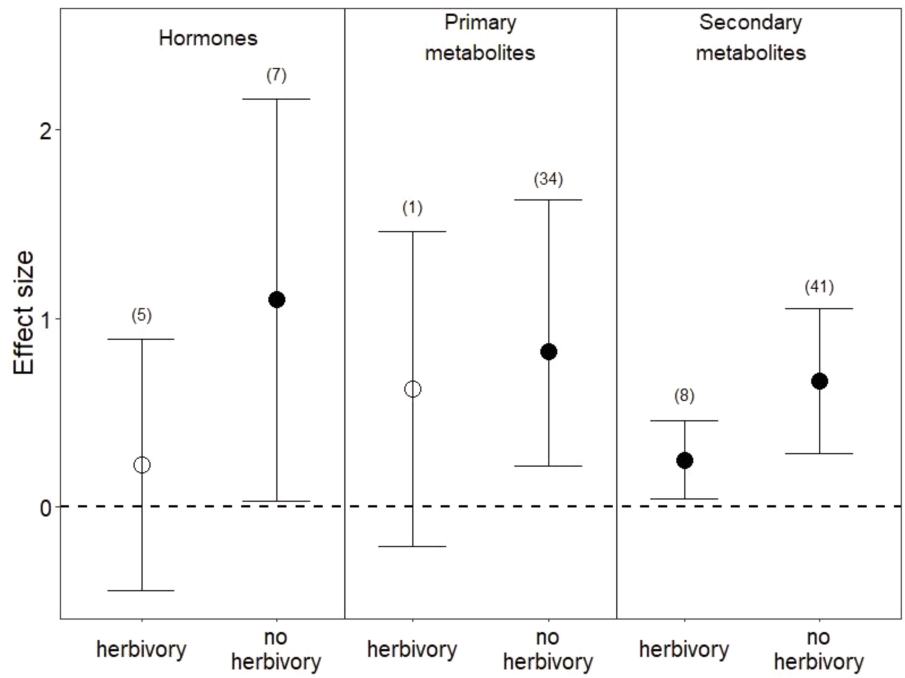

FIGURE 5 | Effects of drought with and without herbivory on leaf metabolic responses. "No herbivory" effects are the same as those shown in **Figure 1** and are reproduced here for comparison. Numbers in parentheses represent the number of studies considered. Mean effect sizes are shown with 95% confidence intervals (CIs). Effects are considered significant if their associated CIs do not overlap zero (dashed line) and are illustrated with solid circles.

nutrients, as well as between leaves and roots. Whereas P and K concentrations decreased in leaves under drought, N concentrations increased. This latter finding is in line with early work tying drought to increased N and higher herbivore pressure on aboveground tissues (*e.g.*, White, 1969), and it may result from studies where either the duration of drying or the frequency of rewetting was relatively high (He and Dijkstra, 2014). Surprisingly, a recent meta-analysis of N and P responses to drought found that soil extractable N, unlike P, can actually increase under drought treatment (He and Dijkstra, 2014; but see Delgado-Baquerizo et al., 2013). Roots, in contrast to leaves, showed no discernible changes in N concentration under drought, but only two studies were available for analysis.

Consistent with our third hypothesis, we found that some primary metabolites increased in both leaves and roots under drought. Plant growth is particularly sensitive to drought stress (Shao et al., 2008; Ings et al., 2013), and slowed growth may immediately redirect primary metabolism to the production of stress metabolites, such as sugars and free amino acids (Chaves et al., 2003). Reduced photosynthesis under drought also profoundly alters primary metabolism (Bradford and Hsiao, 1982; Lawlor, 2002). We found higher amino acids in leaves under drought stress, although this result was driven largely by increases in proline, an amino acid well known to adjust osmotic pressure, scavenge free radicals, and increase expression of stress-related genes under drought (Mali and Mehta, 1977; Chaves et al., 2003; Hayat et al., 2012). Among the studies we analyzed (Appendix 1), only one measured proline in the roots, but it too found that concentrations of proline nearly doubled under drought (El Sayed, 1992). These increases in free amino acids could be advantageous to nitrogen-limited herbivores (White, 1969), but proline metabolism has also been implicated in the production of plant phenolic secondary metabolites (Lattanzio et al., 2009), such that gains in accessible nitrogen to herbivores under drought may commonly be offset by higher plant toxicity (Gershenzon, 1984 and see below). Although sugars also marginally increased under drought in both leaves and roots (**Figure 2**), roots and leaves may metabolize complex carbohydrates at different rates under drought stress (**Figure 2**; Chaves, 1991). Because root:shoot biomass ratios increase under water stress (Poorter et al., 2012; Eziz et al., 2017), implying differential primary metabolism in below- and aboveground organs, future work quantifying primary metabolites in whole plants will be useful for determining whether and how primary metabolic responses reflect the source of stress and its relationship to organ function (*e.g.*, Merewitz et al., 2011; Tardieu, 2012; Gargallo-Garriga et al., 2014).

One of the main criteria of our meta-analysis was to find studies that measured secondary metabolites under water stress. Consistent with our fourth hypothesis, our results showed that concentrations of C-based secondary metabolites increased in leaves but decreased in roots under drought treatments. The effects of drought were also associated with plant type (tree or non-tree) and compound class. Phenolics were the most

commonly measured secondary metabolites in drought studies. In all plants but trees, leaf phenolics increased whereas root phenolics decreased in response to drought. Although leaf phenolics in trees marginally decreased in response to drought, we did not find any studies that had measured root phenolics in trees for comparison. Increases in phenolics under water stress may be explained partly by their function as antioxidants (Nakabayashi et al., 2014), a function that may in fact be less necessary in roots than in leaves, because roots lack the spikes in reactive oxygen species under stress that are associated with chloroplasts (*e.g.*, Lodeyro et al., 2016). Many important secondary metabolites (e.g., alkaloids and flavonoids) known to be produced in roots (van Dam, 2009) were not measured in studies considering the effects of water stress. It will be important to consider a broader spectrum of root secondary metabolites and their responses to water stress in future studies. For example, flavonoids might be expected to increase in both roots and leaves under drought because these compounds can be enhanced by ABA signaling and modulate plant growth (Brown et al., 2001; Besseau et al., 2007).

Although water and herbivory stress frequently co-occur belowground (Bardgett and Wardle, 2003; Ryalls et al., 2016) and roots play a vital role in plant metabolism and fitness (Zangerl and Bazzaz, 1992; Lambers et al., 2002), growth-defense trade-offs and patterns of optimal defense are rarely determined for roots (van Dam, 2009). If the plant is integrating its responses above- and belowground under water stress, and if water stress increases the relative value of roots because they determine whole-plant water availability (Eziz et al., 2017), the plant might be expected to reduce its chemical defense of leaves and increase its chemical defense of roots. Our results, however, revealed the opposite pattern (**Figure 1**; see also Gargallo-Garriga et al., 2014). Notably, our results are consistent with the model of Zangerl and Bazzaz (1992), who argued that a higher root:shoot ratio under resource stress makes the roots comparatively *less* valuable per unit of structural investment. We propose that, at the level of the whole plant, there could be organ-specific variation in the optimal growth-defense ratio that is determined by the cost of the stress for the tissue and the tissue's function for the plant (see also Freschet et al., 2013). Plants may invest in tolerance and regrowth of roots under drought stress, perhaps partly to explore a larger area of soil for water (*e.g.*, Hodge, 2004). In contrast, producing new leaves under drought conditions may be particularly expensive, leading to higher investment in aboveground chemical defenses. More information is needed to evaluate whole-plant growth-defense trade-offs under water stress, particularly because some important metabolic comparisons require additional root measurements. Addressing the trade-off only aboveground is problematic because, in addition to the stress responses of the roots themselves, root responses will influence leaf responses, as well as play a significant role in determining plant fitness and yield (*e.g.*, Bardgett and Wardle, 2003; War et al., 2012; Tariq et al., 2013a; Gargallo-Garriga et al., 2014).

Plants are known for their remarkable ability to respond to multiple stress conditions, sometimes using the same signaling pathway (Rizhsky et al., 2004; Fujita et al., 2006; Ramakrishna and Ravishankar, 2011). Our results are consistent with evidence that drought and herbivory may be regulated cooperatively by ABA/JA signaling. However, contrary to our fifth hypothesis, our results also suggest that the responses of secondary metabolites under co-occurring herbivory and drought were smaller than those under drought alone. Although this could be due to the smaller sample size of studies addressing the two stresses simultaneously, it is also possible that the combined energetic costs of drought and herbivory reduce the availability of energy and chemical precursors for an effective stress response (*e.g.,* Rennenberg et al., 2006; Zhu, 2016). Determining whether such costs are additive or synergistic should be a key goal for future research (*e.g.*, Bansal et al., 2013; Ben Rejeb et al., 2014).

Herbivores are strongly influenced not only from the bottom up by plant metabolites, but also from the top down by interactions with predators. Studies of tritrophic interactions in the context of drought stress typically examine only the population dynamics of herbivores and their natural enemies (Tariq et al., 2013b; Weldegergis et al., 2015; Salerno et al., 2017; see, *e.g.,* Hoover and Newman, 2004; Ahmed et al., 2017). Studies examining plant chemistry will be necessary to add predictive power to these, frequently context-dependent, observations. For example, plant metabolites can influence the effectiveness of the herbivore immune system, physical barriers against entomopathogens, and sequestration of secondary metabolites (War et al., 2012; Biere and Bennett, 2013; Duisembecov et al., 2017). Plant metabolites can also have differential effects on specialist and generalist herbivores (Ali and Agrawal, 2012), which could further modify community-wide food webs. We found only three studies of the indirect effects of drought on parasitoid predators via plant chemistry, and the results were inconsistent. Moreover, combining drought stress with herbivory stress may have additional important, non-additive effects on plant chemistry, and thus on the multitrophic interactions mediated by the plant (Shikano, 2017; Tariq et al., 2013b). Because plants are powerful mediators of interactions between otherwise loosely connected food webs, understanding phytochemical responses to co-occurring stresses will be crucial to predicting how terrestrial ecosystems will respond to global change.

CONCLUSION

Drought and herbivory are common and important stresses in terrestrial ecosystems that can cause whole-plant changes in growth, physiology, and biochemistry. Whereas specific primary metabolites and changes in biomass are often measured in studies of drought and secondary metabolites are often measured in studies of herbivory, assessment of changes in a variety of primary and secondary metabolites, as well as physical traits, would lend insight into the complex metabolic and structural demands required for plants to acclimate and maintain function when faced with multiple stresses. Recent studies of plant-

herbivore interactions clearly demonstrate that metabolic profiles of shoots can be altered by root herbivory and vice versa (Erb et al., 2008; Kaplan et al., 2008; Wondafrash et al., 2013), but it remains surprisingly rare to examine whole-plant responses to herbivory under co-occurring abiotic stress. Allocation to metabolic processes to tolerate and/or protect tissues from damage under stress can impact plant fitness and competitive ability, as well as plant mediation of multi-species trophic interactions. Prediction of the effects of multiple stresses on plant metabolic allocation and its ecological ramifications will ultimately require a theoretical framework for the whole plant.

AUTHOR CONTRIBUTIONS

FM and EP contributed to the design and implementation of the study, to the analysis of the results, and to the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00852/full#supplementary-material

All of the data and R code for the figures and supplementary material are deposited in the Dryad Digital Repository.

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MINI REVIEW
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The Galling Truth: Limited Knowledge of Gall-Associated Volatiles in Multitrophic Interactions

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Galls are the product of enclosed internal herbivory where the gall maker induces a plant structure within which the herbivores complete their development. For successful sustained herbivory, gall makers must (1) suppress the induction of plant defenses in response to herbivory that is usually mediated through the jasmonic acid pathway and involves volatile organic compound (VOC) production, or (2) have mechanisms to cope with herbivory-induced VOCs, or (3) manipulate production of VOCs to their own advantage. Similarly, plants may have mechanisms (1) to avoid VOC suppression or (2) to attract galler enemies such as parasitoids. While research on VOCs involved in plant–herbivore–parasitoid/predator interactions is extensive, this has largely focussed on the impact of piercing, sucking, and chewing external herbivores or their eggs on VOC emissions. Despite the importance of gallers, owing to their damage to many economically valuable plants, the role of volatiles in gall-associated herbivory has been neglected; exceptions include studies on beneficial gallers and their enemies such as those that occur in brood-site pollination mutualisms. This is possibly the consequence of the difficulties inherent with studying internally occurring herbivory. This review examines the evidence for VOCs in galler attraction to host plants, potential VOC suppression by gallers, increased emission from galls and neighboring tissues, attraction of galler enemies, and the role of galler symbionts in VOC production. It suggests a research focus and ways in which studies on galler-associated VOCs can progress from a philatelic approach involving VOC listing toward a more predictive and evolutionary perspective.

Keywords: galler, herbivory, parasitoid, multitrophic interactions, plant–galler interactions, plant–insect interactions, volatile organic compounds, VOCs

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INTRODUCTION

Galls are a classic example of niche construction (Gilbert, 2009) and partly of the extended phenotype of the galling organism (Stone and Cook, 1998). Galls are constructed by gallers in concert with plant tissue that is coerced into gall formation (Favery et al., 2016; Borges, 2017). These hypertrophied tissues provide protection and nutrition for one or more galler generations (Wool and Burstein, 1991). Diverse organisms including viruses, bacteria, fungi, and invertebrates induce galls on plants (Mani, 1964; Raman, 2011; Fernandes and Santos, 2014). Of invertebrates, galling insects are possibly the most diverse and most studied and include gall midges (Diptera: Cecidomyiidae), gall wasps (Hymenoptera: Cynipidae), and aphids (Hemiptera:

Aphididae); nematodes and mites are also important. Most galls are an infestation; to be sustained, gallers must suppress or cope with plant defenses such as herbivore-induced plant volatiles (HIPVs) or manipulate them to their own advantage (**Figure 1**). This review is restricted to invertebrate-induced galls, and focuses on the less-examined role of volatiles in galler–plant–galler enemy interactions (**Figure 1**). Beneficial galls occur in some brood-site pollination mutualisms when gallers themselves are pollinators, e.g., fig wasps (Borges, 2016; **Figure 2**). Here the interests of the host-plant and the gallers are aligned, and plants actively signal to their galler pollinators.

PLANT VOLATILE ORGANIC COMPOUNDS (VOCs) THAT ATTRACT GALLERS

Plant tissues rich in meristems are likely most suitable for gall initiation (Carneiro et al., 2017; Silvia and Connor, 2017) and should attract gallers.

Floral Volatiles

In the fig pollination mutualism, where gallers are pollinators and gall individual flowers at the expense of seeds, a diverse volatile organic compound (VOC) blend attracts agaonid wasp pollinators (Hossaert-McKey et al., 2010; Borges, 2016). These are likely produced by glandular cells in the outer wall of fig syconia (enclosed globular inflorescences) or in bracts surrounding the syconium opening at the pollen-receptive stage (Souza et al., 2015). These blends comprise mostly terpenoids, with some benzenoids and aliphatic compounds (Borges, 2016). In one study, 4-methylanisole was proposed as the major pollinator attractant (Chen et al., 2009). Another study determined that enantiomeric mixtures of some dominant monoterpenes were more attractive to pollinators than others (Chen and Song, 2008). Besides pollinating gallers, most fig syconia also harbor non-pollinating, parasitic galler wasp species (Herre et al., 2008); these arrive for oviposition either very early or much later in the development of the syconium (Segar et al., 2013) attracted by stage-specific VOCs; some species are attracted to the same blends that attract pollinating gallers (Borges et al., 2013; **Figure 2C**), and therefore exploit signals meant for mutualistic gallers.

Sometimes floral VOCs serves as cues for leaf gallers. Floral volatiles in *Salix* are long-distance attractants for leaf-galling sawflies *Pontania proxima* (Kehl et al., 2010). Although the target galling sites are leaves, flowering twigs produce 90 times more VOC quantities than non-flowering twigs suggesting that using floral volatiles as a proxy for leaves may be an efficient host-finding strategy; more flowering than non-flowering plants were galled. In electroantennogram detection (EAD) studies on VOCs from male flowering twigs, compounds absent from vegetative VOC blends, e.g., 1,4-dimethoxybenzene, were strongly detected by sawfly antennae, confirming that such floral compounds may constitute key attractants for leaf gallers. Interestingly, the antennae also responded to green leaf volatiles (GLVs).

Stem and Leaf Volatiles

Considering the voluminous research on cecidomyiid and cynipid galls, very little work exists on host volatiles as attractants. Volatiles of flowering stems of the herbaceous perennial *Silphium* (Asteraceae) attracted the cynipid gall wasp *Antistrophus rufus* (Tooker et al., 2005). A monoterpene blend consisting of a racemic mixture of α-pinene and β-pinene (+ for both), (+)-limonene, and (−) camphene served as principal attractants for ovipositing females (Tooker et al., 2005). The compound ratios in the blend must be crucial since these monoterpenes are present in sympatric *Silphium* species to which the cynipids are not attracted. Male cynipid wasps use parts of this same blend to locate females within galled stems indicating that host volatiles are employed as mate location cues (Tooker et al., 2002; Tooker and Hanks, 2004) as in several other non-galling phytophagous insects (Xu and Turlings, 2018). The cynipid chestnut gall wasp *Dryocosmus kuriphilus* was attracted to a GLV blend from *Castanea* stems 60–120 min after damage, and failed to be attracted to intact stems (Germinara et al., 2011). All these compounds were detected by wasp antennae. C6 volatiles from young apple leaves were major attractants, eliciting EAD responses in the apple cecidomyiid midge *Dasineura mali* (Anfora et al., 2005). Female orange wheat blossom cecidomyiid midges *Sitodiplosis mosellana* were attracted by key compounds, e.g., (*Z*)-3-hexenyl acetate, acetophenone, and 3-carene, present in minor proportions in the overall volatile profile of wheat panicles (Birkett et al., 2004). The African rice gall cecidomyiid midge *Orseolia oryzivora* preferred volatiles from uninfested plants while those from infested plants served as repellents; the major difference between these volatile profiles was a considerable increase in the HIPV (*E*)-β-caryophyllene (Ogah et al., 2017).

Root Volatiles

For root-knot nematodes, CO_2 seems to be the most important attractant released by actively respiring roots (Rasmann et al., 2012); there is scant information on other root volatiles that serve as galler/plant parasitic nematode attractants in the absence of plant damage (Rasmann et al., 2012; Johnson and Rasmann, 2015). Low concentrations of lauric or dodecanoic acid attract root-knot nematodes while this VOC is repellent at high levels (Dong et al., 2014).

IMPACT OF GALL MAKERS ON PLANT VOLATILES

Suppression of Volatile Production

A successful galling strategy may require that gall makers suppress the induction of plant defenses (Kant et al., 2015), since a galler must have prolonged residence within the plant. Since many plant-induced defenses involve activation of the jasmonic acid (JA) pathway, which also often results in volatile release, it is therefore not surprising that VOC production is often suppressed during galling. A meta-analysis of secondary metabolites that are up-regulated on gall induction found that, unlike other

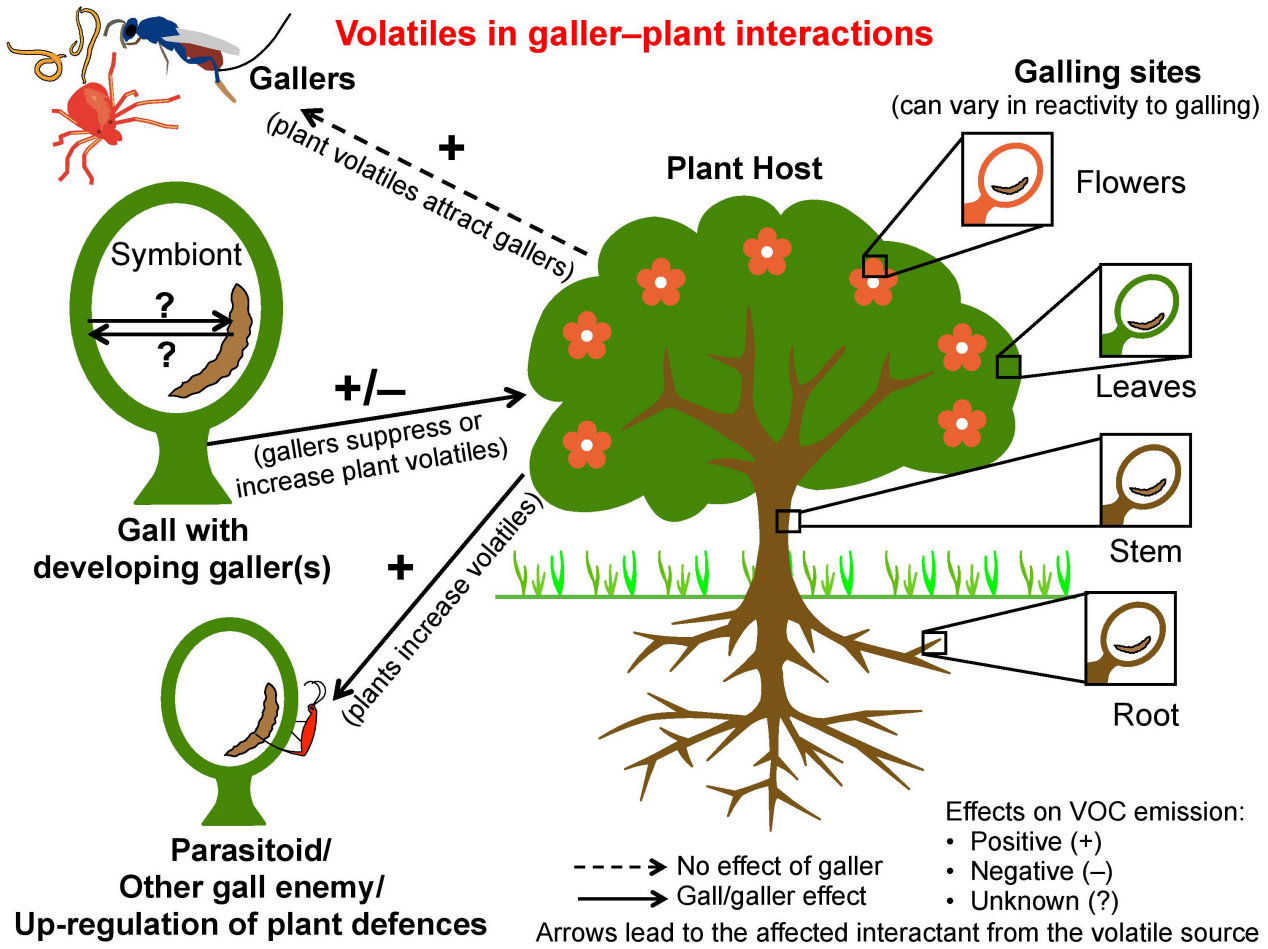

FIGURE 1 | Interactions between gallers and plants by means of volatiles. Arrows point toward the affected interactant from the volatile source. Volatiles may attract gallers to plants; the galler may suppress plant volatile production, or plants may increase volatile production to attract galler enemies or to up-regulate defenses. Symbionts within galls or gallers may affect volatile production.

metabolites, volatiles were usually unaffected (Hall et al., 2017). For example, goldenrod plants *Solidago altissima* showed no increase in VOC emission after attack by galling flies *Eurosta solidagnis* or galling moths *Gnorimoschema gallaesolidaginis* (Tooker et al., 2008), as also in Japanese elms attacked by galling aphids (Takei et al., 2015). Furthermore, infestation by *E. solidagnis* suppressed HIPVs in response to subsequent herbivory by generalist caterpillars (Tooker et al., 2008). Consistent with JA suppression, galls accumulated salicylic acid (SA) instead (Tooker et al., 2008). Tooker and De Moraes (2008) speculate that gallers are adapted to suppress JA, since JA inhibits plant growth hormones such as auxin and also cytokinins, both of which must be locally up-regulated in gall formation (Tooker and Helms, 2014). Whether gallers can suppress ethylene production which could impact VOC production (Broekgaarden et al., 2015) is unknown. Some insect gallers may synthesize phytohormones, e.g., auxin (Bailey et al., 2015); this may impact JA synthesis as suggested by cross-talk between auxin and JA observed in many plant-associated bacteria and fungi (Berens et al., 2017). Gallers may succeed in suppressing plant defenses by deploying effector molecules (Zhao et al., 2015) among which are ATP-hydrolysing enzymes, calcium-binding proteins, and ubiquitin ligases (Giron et al., 2016; Guiguet et al., 2016; Nabity, 2016).

Five non-mutually exclusive mechanisms have been suggested for the absence of increased VOC emission after galling (Tooker et al., 2008). Besides the SA up-regulation mentioned above, they include (a) avoidance of galler detection, (b) targetting relatively non-reactive tissues, e.g., stems, (c) depletion by galler larvae of plant resources needed for VOC production, and (d) active suppression of host-plant defense (e.g., Nyman and Julkunen-Tiitto, 2000).

Increase in Volatiles After Galling

While gallers often suppress VOCs, increased VOC production in and around galled tissues may occur. In flower galls produced by the dipteran *Myopites stylatus* on the woody fleabane *Dittrichia viscosa* (Asteraceae), emission of the phenylpropene compound estragole, an isomer of anethole, increased six times compared to ungalled flowers (Santos et al., 2016). A moderate increase in anethole was also evident. The terpene eucalyptol (1,8-cineole)

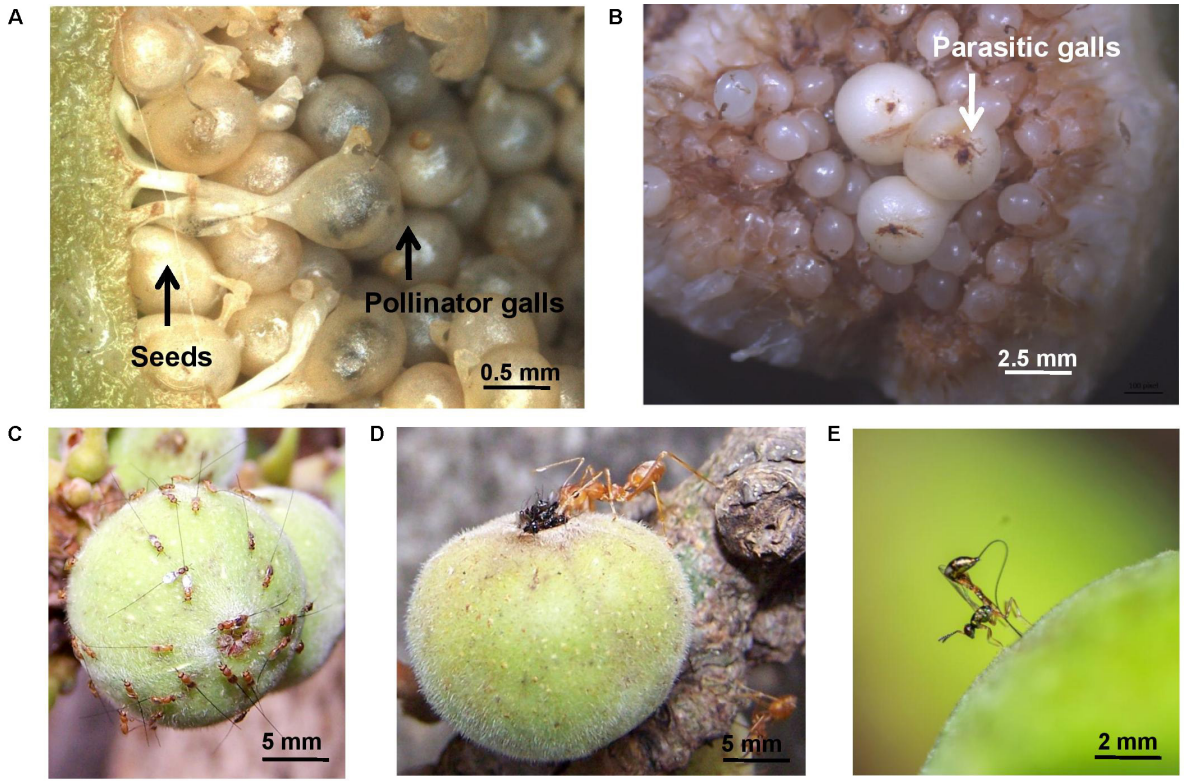

FIGURE 2 | Interactions of plants with beneficial and parasitic gallers. Examples are from the cluster fig *Ficus racemosa* **(A)** seeds and pollinator galls in a syconium. Note remnants of stigma and single developing pollinator in each galled uniovulate flower. **(B)** Large galls of an early-arriving parasitic galler *Sycophaga stratheni* in a syconium; these gallers target tissues of the syconium lumen. **(C)** Aggregations of parasitic gallers *Sycophaga fusca* on a syconium; these gallers are attracted by the syconium volatile blend emitted at pollen-receptive stage. **(D)** The weaver ant *Oecophylla smaragdina* preying upon pollinator gallers *Ceratosolen fusciceps* entering a syconium through the ostiole; ants are attracted by syconial volatiles at pollen-receptive phase. **(E)** Oviposition by parasitoid *Apocrypta* sp. 2 into galls hidden within the syconium; oviposition decision are made using chemosensory features of the ovipositor. Photo credits: **(A,D)** Mahua Ghara and Yuvaraj Ranganathan; **(B,C)** Pratibha Yadav; image in **C** is adapted from Yadav et al. (2018) and is reproduced with permission from Springer Nature; **(E)** Nikhil More.

was emitted in large quantities only from galls, and was absent from floral scents. However, the gall emission of compounds such as α-pinene, β-pinene, limonene, and linalool was significantly lowered. Terpenes such as α-pinene, limonene, and linalool may have a toxic but hormetic effect on dipterans (Papanastasiou et al., 2017), while estragole and anethole are generally toxic to dipterans (Chang et al., 2009). Eucalyptol emitted by another Asteraceae plant was a repellent and oviposition-deterrent to mosquitoes (Klocke et al., 1987). Many interesting questions arise from these observations on the fleabane–fly interaction. First, the concentrations of the VOCs within the gall are unknown; therefore, whether high concentrations of estragole and anethole are also present within gall tissues is not known. If the gall also contains high amounts of these phenylpropanoids, that are usually toxic to many insects, one may speculate that the galler larvae/pupae are resistant to these toxins. If so, are they being produced by the galler by hijacking plant biochemical machinery to their advantage, so that non-resistant galler enemies such as parasitoids are also deterred? Additionally, is it possible that concentrations of α-pinene, limonene, and linalool are lowered within the gall to non-toxic but hormetic levels under the action of the galler? Is eucalyptol being produced in very high quantities to deter galler enemies such as parasitoids or for its antifungal/antibacterial activities? The parasitoids of this tephritid fly species include eurytomid, eupelmid, pteromalid and torymid wasps, and appear to be attracted by gall and/or host cues (Santos et al., 2016). It is also possible that gallers are unable to manipulate VOC release and that VOC emission is under multifactorial control resulting in unexpected VOC emission patterns.

In another example, the aphid *Baizongia pistaciae* induces galls on the terminal buds of the pistachio *Pistacia palaestina* (Anacardiaceae). Gall tissue extracts contained much higher levels of terpenes, especially α-pinene and limonene, than surrounding ungalled leaves, while leaves accumulated more sesquiterpenes (Rand et al., 2014). The high terpene levels resulted from increased biosynthetic activity within the galls rather than accumulation from surrounding tissue (Rand et al., 2017). High terpene levels within the gall could result from a need for antibacterial/antifungal activity, or to deter parasitoids. Stored and emitted terpenes were also in higher concentrations in galls formed by the aphid *Slavum wertheimae* on the lateral buds of *Pistacia atlantica* (Rostás et al., 2013). Concentrations of three terpenes, i.e., α-pinene, limonene, and sabinene, were

much higher compared to others. The principal galler enemies in this study were mammalian herbivores, i.e., goats, that were reluctant to consume galls or food pellets to which these three terpenes were added in biologically relevant concentrations. While the authors speculate that terpenes were largely responsible for the feeding deterrence, they admit that the high gall tannin concentrations may also have deterrent effects.

In the oak *Quercus robur*, cynipid galls do not have altered VOC emissions but affect emissions of neighboring portions of gall-bearing leaves (Jiang et al., 2018). The change in leaf emission depends on whether the galler attacks major veins or intercostal areas. Major vein galls resulted in more GLV production and less terpenes from neighboring tissues, while galls in undifferentiated parenchyma of intercostal areas resulted in much more terpene and benzenoid production. Notably both types of galls caused increased α-pinene and limonene emission, while the intercostal tissue galls also induced the emission of other monoterpenes such as linalool, camphene, β-myrcene, and the sesquiterpene β-bergamotene that were not produced by ungalled leaves. Galls also produced far less isoprene than ungalled leaves. Therefore, in this example also, monoterpene production was most affected. Similarly, an increase in α-pinene and limonene emission occurred in galls induced by psyllids on *Schinus polygamus* (Anacardiaceae) (Damasceno et al., 2010); C6 volatiles increased in neighboring leaf portions bearing the galls.

In *S. altissima* attacked by the rosette gall-midge *Rhopalomyia solidaginis*, emissions of terpenes such as copaene and β-pinene increased post-galling (Uesugi et al., 2016); these attracted herbivorous beetles whose presence facilitated galler performance, suggesting that VOC emission patterns must be viewed in an integrated manner.

PLANT OR GALL VOLATILES THAT ATTRACT GALLER ENEMIES

Herbivore-induced plant volatiles used by parasitoids in host location have been largely investigated for externally feeding chewing, piercing, and sucking herbivores (Aartsma et al., 2017), where herbivore feeding mode appears an accurate predictor of the HIPV blend (Danner et al., 2017). Surprisingly, there is almost no work on VOCs attracting parasitoids to galls. While Borges et al. (2013) examined changes in the volatilome during fig syconial development, including stages when a multiplicity of gallers and parasitoids of these gallers are also attracted, here too, the study did not specifically test a set of volatiles on parasitoids. Using an adaptation of weighted gene coexpression network analysis (WGCNA), co-emitted modules of VOCs were detected. Early-arriving gallers triggered the release of GLVs such as (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenol. Later-arriving gallers triggered the release of compounds such as (*E*)-β-ocimene, (*Z*)-β-ocimene, and methyl salicylate in response to galler feeding; these are well known HIPVs and parasitoid attractants in other plant–herbivore systems (Turlings and Erb, 2018). In Y-tube olfactometer experiments, parasitoid wasps were attracted by VOCs of fig syconia containing their galler hosts (Proffit et al., 2007). Such attracted parasitoids make decisions about which galls to parasitise by sampling syconial odors with their probing ovipositor. For the first time, Yadav and Borges (2017) showed that the ovipositor of fig wasp gallers and parasitoids is a volatile sensor, and that it responds both behaviourally as well as with neuronal firing to cues such as CO_2 and syconial stage-specific volatiles. For parasitoids that need to seek out hidden hosts within galls (**Figure 2E**), the use of cues such as CO_2 that signal locations of actively respiring galler larvae/pupae are potentially extremely important in successful parasitism. Parasitoids in the fig system may be considered apparent mutualists if they target non-beneficial gallers or control the population of pollinating gallers within fig syconia (Krishnan et al., 2015); therefore, the role of VOCs in maintaining such tritrophic interactions in order to stabilize the core mutualism is likely profound.

Volatiles emitted by fig syconia in pollen-receptive phase to attract galling pollinators are also attractive to predatory ants that eavesdrop on this pollinator signal (Ranganathan and Borges, 2009; **Figure 2D**). These ants are also attracted to syconial volatiles at a later stage when F1 galler wasps and parasitoids exit the syconia; ant attraction toward stage-specific volatiles in Y-tube olfactometer tests is a learnt association based on prior exposure to syconial volatiles (Ranganathan and Borges, 2009). Since ants are important predators of galler and parasitoid fig wasps (Ranganathan et al., 2010; Bain et al., 2014), and are also important in other galling systems (Fernandes et al., 1999), their response to gall-associated volatiles deserves more attention.

Inquilines of gallers are also attracted by volatiles. Goldenrod *Solidago* stems are infested by galls induced by *E. solidaginis* tephritid flies. These flies can infest *S. altissima* and *Solidago gigantea* plants, and in turn their galls are attacked by gall-boring inquiline beetles *Mordellistena convicta*. These beetles were attracted to volatiles associated with galls of their natal host plants, and avoided those of alternate host plants occupied by their galler hosts suggesting that inquiline speciation and subsequent radiation is driven by olfaction (Rhodes et al., 2012). The inquiline wasps *Diaziella yangi* and *Lipothymus* sp. that parasitise the pollinating galler *Eupristina* sp. of *Ficus curtipes* are attracted by 6-methyl-5-hepten-2-one (Gu and Yang, 2013) which is an important volatile in the pollen-receptive scent of the fig syconium (Gu et al., 2012); they showed no attraction to 6-methyl-5-hepten-2-ol which is another important scent constituent.

ROLE OF SYMBIONTS IN GALLER–PLANT INTERACTIONS

Plant scents may be influenced by symbionts. For example, bacteria have been recently implicated in VOC production in floral tissues (Helletsgruber et al., 2017). Bacteria in oral secretions of caterpillars can also suppress JA responses (Wang et al., 2016). Fungal root symbionts can influence above-ground production of HIPVs that attract parasitoids (Rasmann et al., 2017; Simon et al., 2017). Whether microbes are involved in VOC production that is beneficial to the galler or to the plant is as yet unknown (**Figure 1**). However, symbiosis has benefitted many

gall-inducing insect lineages such as ambrosia gall midges whose diversification has been aided by fungal symbionts (Joy, 2013). The role of endophytic fungi present in many insect-induced galls is also unknown (Lawson et al., 2014).

CONCLUSION

Moving From Philately to Understanding the Volatilome

While information is still scarce, gall induction may affect the volatilome of plants. Making sense of this effect may be tackled at several levels. (1) Classes of VOCs may have particular relevance at different stages of galler–plant interactions. For example, that many gallers are attracted to non-specific GLVs suggests that GLVs are used as habitat cues rather than host-specific cues (Webster and Cardé, 2017) and enable navigation toward suitable vegetation patches from long distances. (2) Mirroring research on externally feeding herbivores, where HIPVs have been investigated from the egg-laying stage (Hilker and Fatouros, 2015), comparable information is needed in galler–plant–parasitoid/inquiline interactions including stages such as tissue damage by ovipositor probing, egg laying, damage by larval mandibles, and finally gall induction. (3) Metabolic plasticity can occur in host-plant–galler interactions vis-a-vis different gallers and plant genotypes (Uesugi et al., 2013, 2016), resulting in varied responses that must, therefore, be interpreted within the context of the specific interactants. (4) We need to understand correlation networks of VOCs produced by plants under a variety of circumstances. Junker et al. (2017) and Junker (2017) have examined enzymatic and volatile hubs; such efforts may lead to predictions and an understanding of why certain VOCs tend to co-occur, e.g., α-pinene, limonene, and linalool in many of the above-cited examples. (5) Chemists and behavioral ecologists must appreciate the importance of VOCs produced in small quantities; small peaks are often ignored at analytical and testing stages, but these may often contain the real signaling messages (Clavijo McCormick et al., 2014). (6) Since VOC emission is likely an active process controlled by transporters across plant cell membranes (Adebesin et al., 2017), and is not merely controlled by physical processes such as volatility (Borges et al., 2013), the field of plant signaling using volatiles must move toward making predictions about the costs and benefits of VOC production, types of VOCs expected, and their consequences. Only then will the study of volatiles progress from philately to viewing gall-associated volatiles within an ecological and evolutionary framework.

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The author confirms being the sole contributor of this work and approved it for publication.

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Evidence for the Involvement of Fatty Acid Biosynthesis and Degradation in the Formation of Insect Sex Pheromone-Mimicking Chiloglottones in Sexually Deceptive *Chiloglottis* Orchids

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Hundreds of orchid species secure pollination by sexually luring specific male insects as pollinators by chemical and morphological mimicry. Yet, the biochemical pathways involved in the synthesis of the insect sex pheromone-mimicking volatiles in these sexually deceptive plants remain poorly understood. Here, we explore the biochemical pathways linked to the chemical mimicry of female sex pheromones (chiloglottones) employed by the Australian sexually deceptive *Chiloglottis* orchids to lure their male pollinator. By strategically exploiting the transcriptomes of chiloglottone 1-producing *Chiloglottis trapeziformis* at distinct floral tissues and at key floral developmental stages, we identified two key transcriptional trends linked to the stage- and tissue-dependent distribution profiles of chiloglottone in the flower: (i) developmental upregulation of fatty acid biosynthesis and β-oxidation genes such as *KETOACYL-ACP SYNTHASE, FATTY ACYL-ACP THIOESTERASE,* and *ACYL-COA OXIDASE* during the transition from young to mature buds and flowers and (ii) the tissue-specific induction of fatty acid pathway genes in the callus (the insectiform odor-producing structure on the labellum of the flower) compared to the labellum remains (non-odor-producing) regardless of development stage of the flower. Enzyme inhibition experiments targeting KETOACYL-ACP SYNTHASE activity alone in three chiloglottone-producing species (*C. trapeziformis*, *C. valida*, and *C.* aff. *valida*) significantly inhibited chiloglottone biosynthesis up to 88.4% compared to the controls. These findings highlight the role of coordinated (developmental stage- and tissue-dependent) fatty acid gene expression and enzyme activities for chiloglottone production in *Chiloglottis* orchids.

Keywords: *Chiloglottis*, chiloglottone, sexual deception, pollination, transcriptome, fatty acid

INTRODUCTION

Flowers have evolved diverse strategies to attract potential pollinators by conveying visual and olfactory signals (Raguso, 2004; Borghi et al., 2017). Some plants even use specific "semiochemical" signals that mimic the sex pheromone of female insects to sexually lure their highly specific male insect pollinators. This fascinating pollination strategy, commonly known as sexual

deception (SD), has evolved multiple times on several continents (Gaskett, 2011). Amongst the Orchidaceace, hundreds of species spanning more than 20 genera across different subtribes are involved. Other cases are known in two other plant families (Bohman et al., 2016). In orchids, pollination by SD is often achieved when the male insect pollinator attempts to copulate with the flower, with volatiles holding the key to this interaction (Bohman et al., 2016; Wong et al., 2017b).

The Australian SD orchids of the genus *Chiloglottis* employ chiloglottones, a class of 2,5-dialkylcyclohexan-1,3-dione natural products (Peakall et al., 2010), to lure their specific male thynnine wasp pollinator (See Supplementary Figures 1A,B). For example, chiloglottone 1 is employed by *Chiloglottis trapeziformis* to attract its primary male pollinator, *Neozeleboria cryptoides* while the mostly allopatric *C. valida* employs chiloglottone 1 but attracts *N. monticola* for pollination. Conversely, a morphologically cryptic species with taxonomic affinity to *C. valida* (here after *C.* aff. *valida* secures pollination using a specific blend of chiloglottones 1 and 2 (Peakall et al., 2010; Peakall and Whitehead, 2014). In *C. trapeziformis*, chiloglottone 1 production has been shown to be specific to the densely clustered "insectiform" calli structure (callus) on the labellum, and its production is unexpectedly UV-B dependent (Falara et al., 2013). Flowers that open in the field become depleted of chiloglottone within 3–5 days when they are grown under light lacking in the UV-B range. Re-exposure to UV-B light, or natural sunlight, rapidly initiates chiloglottone production. Chiloglottone 1 is also dependent on the flowers' developmental stage (Amarasinghe et al., 2015). In all but the very young buds, chiloglottone 1 can be detected when flowers are exposed to UV-B or sunlight, with levels increasing from trace amounts from manually opened young buds to appreciable amounts in very mature buds and in fully open flowers.

The formation of chiloglottones has been predicted to involve the condensation of intermediates of varying chain lengths in the fatty acid (FA) biosynthetic and degradative (β-oxidation) pathways, followed by decarbonylation (Franke et al., 2009; Bohman et al., 2016). For example, the condensation of activated 3-oxohexanoic acid (CoA or ACP) and activated 2-hexenoic acid precursors and subsequent decarboxylation may give rise to chiloglottone 1 (See Supplementary Figure 1C). As such, formation of chiloglottone 2 may involve activated 3-oxohexanoic acid (CoA or ACP) and activated 2-octenoic acid precursors. In plants, such intermediates are ubiquitous, existing in the plastids as ACP derivatives or the peroxisomes as CoA derivatives. However, just how these precursors are made available for chiloglottone formation in an otherwise iterative pathway is unknown. One hypothesis for this may involve the mid-cycle termination (i.e., after a specified number of cycles) of FA biosynthesis and/or degradation (β-oxidation) pathway enzymes (Bohman et al., 2016).

Previously, we reported that tissue-specific floral transcriptome analysis, comparing the chiloglottone-emitting callus with the non-active labellum of *C. trapeziformis*, revealed that FA biosynthesis and β-oxidation pathways were highly coordinated in a tissue-specific manner whereby pathway transcripts are often highly or exclusively expressed in the callus (Wong et al., 2017a). This finding matched with the known tissue-specific distribution of chiloglottone 1 (Falara et al., 2013; Amarasinghe et al., 2015) and supported the current hypothesis of chiloglottones formation (Franke et al., 2009; Bohman et al., 2016). Interestingly, no induction of FA pathways was observed under UV-B treatment in the callus of mature buds and flowers despite the activation of UVR8-mediated signaling pathways, suggesting an unknown mechanism operating on chiloglottone 1 production at the transcriptional and metabolic level.

Building upon these previous biochemical, chemical, and transcriptional observations linked to chiloglottone formation, here we address three outstanding questions using targeted gene expression analysis and molecular inhibitor experiments. We ask the following three specific questions: (1) Are FA metabolic pathway genes developmentally regulated during the transition from young buds to mature flowers in *C. trapeziformis*? (2) Are FA metabolic pathway genes regulated in a tissue-specific manner in *C. trapeziformis*? (3) Does the inhibition of FA biosynthesis impair chiloglottone production in a cross section of *Chiloglottis* orchids?

MATERIALS AND METHODS

Study Species

Whole *Chiloglottis trapeziformis* plants with single flowers at two developmental stages, namely very young buds (*vyb*) and naturally opened flowers (*flw*) (Amarasinghe et al., 2015), were sampled from a colony growing naturally within the Australian National Botanic Gardens (Canberra, ACT, Australia) in September 2014. Whole *C. valida* and *C.* aff. *valida* plants with single naturally opened flowers were also sourced from wild populations within the Kosciuszko National Park in NSW, Australia, in November 2014. See supplementary materials for plant growth conditions.

RNA Extraction, Library Construction, RNA Sequencing, and Transcriptome Analysis

Fifteen plants (three biological replicates with each replicate containing five individual plants) were used for each treatment. Floral tissues of *vyb* were carefully dissected to separate the stalked callus from the remaining labellum then immediately snap-frozen in liquid nitrogen. RNA extractions, library construction, and RNA sequencing was performed as previously described (Wong et al., 2017a; Xu et al., 2017). All raw sequence reads obtained in this study have been added to the existing BioProject accession PRJNA390683 and SRA study accession SRP109328[1]. Paired-end reads from (Wong et al., 2017a), and from non UV-B treated callus and labellum at very mature buds (*vmb*) and *flw* stages, were used in conjunction with sequenced tissue-specific reads at *vyb* stage obtained in this study and analyzed according to previously pipelines (Wong et al., 2017a).

[1]http://www.ncbi.nlm.nih.gov/sra

The classifications of bud and flower stages used in this study follows Amarasinghe et al. (2015). The *vyb* stage is characterized by a small and very tightly closed buds with both green stalk and bud. The *vmb* stage is characterized by larger buds that are about to open with sepals and petals beginning to separate. Fully open flowers define the *flw* stage. See supplementary materials for detailed methods.

Pharmacological Inhibition Experiments and Chemical Analysis

All inhibition experiments were conducted on chiloglottone-depleted cut flowers from growth-chamber acclimatized whole plants (*C. trapeziformis*, *C. valida*, and *C.* aff. *valida*). Stock solutions (40 mM) of Cerulenin (Cayman Chemical, United States) were dissolved in Dimethyl Sulfoximine (Sigma, United States) and diluted to a final concentration of 100 μM with water (assay buffer). An assay buffer without the inhibitor served as the solvent control. The stalks of cut flowers were immersed into either 100 μM of Cerulenin (assay buffer) or solvent control. Next, the tip of each test tube was sealed with parafilm to ensure no direct contact between the solution and the flowers (3 flowers/tube) and then held in a test tube for 3 days. To induce chiloglottone production, UV-B treatments were conducted over a 2-h period on inhibitor-treated and control plants using a custom made light-box following Amarasinghe et al. (2015). The calli of all three species were immediately excised and assayed for chiloglottones as previously described (Falara et al., 2013). See supplementary materials for detailed chemical analysis methods.

Statistical Analysis

Differential expression analysis was performed using DESeq2 (Love et al., 2014). Transcripts differentially expressed between any given contrasts are defined as having an absolute log2 fold change (log2FC) >0.5 with a false discovery rate (FDR) <0.05. For all inhibition experiments, at least six flowers were used in each treatment or control group. The outcomes of inhibition treatments were analyzed with Student's *t*-test in R[2].

NEW INSIGHTS INTO THE FORMATION OF INSECT SEX PHEROMONE-MIMICKING CHILOGLOTTONES

Spatio-Temporal Gene Expression of Fatty Acid Pathways Coincides With Chiloglottone 1 Production in *C. trapeziformis*

In this study, we specifically addressed the first two objectives by interrogating the callus and labellum transcriptomes of *vyb* (obtained in this study; Supplementary Table 1) in conjunction with *vmb* and *flw* transcriptomes collected from the same colony in the same year obtained from our previous study (Wong et al., 2017a). Principal component analysis revealed that major differences among treatments was mainly driven by developmental stage followed by tissue specificity. (Supplementary Figure 2A). Enrichment (FDR <0.05) of gene ontology categories such as carbohydrate and lipid metabolic process were often observed in both developmental stage and tissue-specific contrasts (Supplementary Figures 2A–C). Our findings revealed that many genes of the fatty acid (FA) biosynthesis and β-oxidation pathways were only upregulated in the callus during the transition from *vyb* to *vmb*. This coincides with a strong enrichment of lipid metabolism processes (FDR $< 6.43 \times 10^{-11}$). Meanwhile, tissue-specific pairwise contrasts (callus vs. labellum) revealed that lipid metabolism process were also enriched in the callus regardless of developmental stage ($FDR_{vmb} < 3.50 \times 10^{-6}$, $FDR_{vyb} < 1.81 \times 10^{-3}$, $FDR_{flw} < 2.02 \times 10^{-2}$).

Three Fatty Acid Biosynthesis and Two β-Oxidation Pathway Genes Exhibit Callus-Specific Developmental Upregulation

Most of the FA biosynthesis such as one *KETOACYL-ACP SYNTHASE III* (*CtKASIII*), four *KETOACYL-ACP REDUCTASE* (*CtKAR-L1 – 3,* mitochondrial *CtKAR*), two *KETOACYL-ACP SYNTHASE I* (*CtKASI-1* and *CtKASI-2*), and one *FATTY ACYL-ACP THIOESTERASE* (*CtFATB2*) and FA β-oxidation pathway genes such as three *ACYL-COA OXIDASE* (*CtACX2/3*, *CtACX4*, and *CtACX1/5*) and one *MULTIFUNCTIONAL PROTEIN* (*CtMFP3*) were consistently upregulated in the callus compared to the labellum **(Figures 1A,B)**. In addition, the transition from *vyb* to *vmb* stages generally involved coordinated upregulation of pathway genes, with transcripts remaining high in *flw* (no significant change from *vmb* to *flw*). Interestingly, four genes exhibited striking tissue (callus)-specific developmental upregulation (e.g., *CtKASI*-2, *CtFATB*, *CtACX2/3*, and *CtACX4*). These genes showed consistently (i) higher expression in the callus compared to the labellum and (ii) exhibited callus-specific developmental upregulation during the transition from *vyb* to *vmb*. We hypothesized that they may have direct involvement in chiloglottone 1 formation compared to those that are expressed at higher levels in the callus but are non-specifically upregulated in both callus and labellum tissues during the transitions from *vyb* to *vmb* (i.e., *CtKASI-1*, CtACX1/5, *CtMFP1*, *CtMFP2*, and *CtMFP3*) and *vmb* to *flw* (i.e., *CtKAR-L1*, *CtMFP3,* and *CtKAT2L*).

Inhibition of Fatty Acid Biosynthesis Significantly Block Chiloglottone Production in *Chiloglottis* Flowers

In light of the striking developmental stage- and tissues-specific upregulation of several key FA pathway genes (**Figures 1A,B**), we hypothesized that inhibition of their activities may affect chiloglottone 1 biosynthesis in *C. trapeziformis*. Therefore, we tested the effect of KAS inhibition using Cerulenin. Studies in several plant species have shown that Cerulenin specifically

[2]https://www.r-project.org/

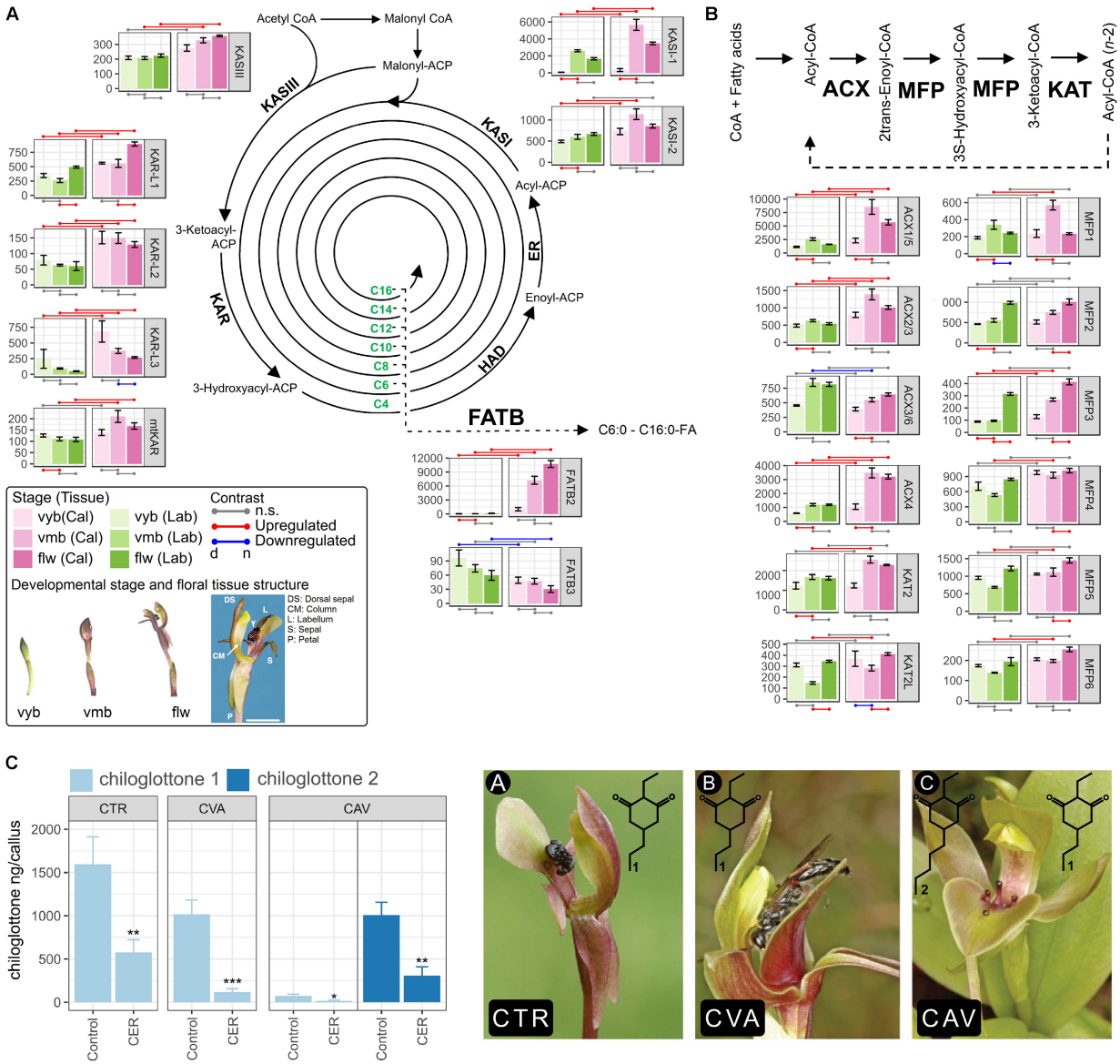

FIGURE 1 | Fatty acid (FA) biosynthesis and degradation pathway gene expression in the callus and labellum tissues of *Chiloglottis trapeziformis* flowers. Differentially expressed genes of the FA **(A)** biosynthesis and **(B)** β-oxidation (degradation) are depicted. Values are normalized transcript counts ± SE. Shades of pink and green colors depict callus and labellum tissues at various flower developmental stage. Developmental stage and floral tissue structure of *C. trapeziformis* are depicted. The arrow indicates the callus tissue at the *flw* stage. Scale bar = 7 mm. Red and blue points connecting any two conditions depict statistically significant (FDR < 0.05, |log2FC| > 0.5) upregulation and downregulation, respectively. Gray points depict no significant differential expression. *n* and *d* represent the numerator and denominator conditions of each comparisons, respectively. **(C)** Chiloglottone amounts in the callus of *Chiloglottis* flowers following incubation with Cerulenin (CER) compared with controls following a 2-h UV-B exposure. Picture A, *C. trapeziformis* flower; Picture B, *C. valida* flower; Picture C, *C.* aff. *valida* flower. CTR, *C. trapeziformis*; CVA, *C. valida*; CAV, *C.* aff. *valida*. The number of flowers used in each treatment group are the following: CTR_{CER} = 10, $CTR_{Control}$ = 9, CVA_{CER} = 12, $CVA_{Control}$ = 8, CAV_{CER} = 6, $CAV_{Control}$ = 7. Bars represent ± SE. Asterisks indicate significant differences between treatments at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ based on Student's *t*-test. Light and dark blue colors indicate chiloglottone 1 and 2, respectively. All images have been reproduced with permission from the respective copyright holders. Please refer to the Section "Acknowledgments" for image credits.

inhibits KAS activity, and thus fatty acid elongation (Shimakata and Stumpf, 1982; Dehesh et al., 1998; Yasuno et al., 2004). We confirm that CtKASIs (CtKASI-2 and CtKASI-1) are potentially susceptible to Cerulenin inhibition as both protein sequence possesses the catalytic Cysteine-Histidine-Histidine triad active site (Wong et al., 2017a). Following a 2-h UV-B exposure of chiloglottone 1-depleted *C. trapeziformis* flowers, the amount of chiloglottone 1 in the calli treated with solvent control was 1,596 ± 317 ng/callus while 100 μM Cerulenin treatment significantly inhibited chiloglottone 1 production by 63.8% ($P < 0.05$) (**Figure 1C**). Motivated by these findings, we extended our test of the effect of Cerulenin on

chiloglottone production to two other *Chiloglottis* species in a different clade to *C. trapeziformis*: *C. valida* that only produces chiloglottone 1 and *C.* aff. *valida* that produces chiloglottone 1 and chiloglottone 2. Following a 2-h UV-B treatment, the mean chiloglottone 1 levels in the controls of *C. valida* was 1,017 ± 165 ng/callus and the treatment significantly inhibited chiloglottone1 production by 88.4% ($P < 0.001$). Similarly, mean chiloglottone 1 and chiloglottone 2 levels in *C.* aff. *valida* controls were 73 ± 21 ng/callus and 1,007 ± 149 ng/callus, respectively. Cerulenin treatment significantly inhibited chiloglottone 1 and chiloglottone 2 production by 77.6% ($P < 0.05$) and 69.5% ($P < 0.01$), respectively (**Figure 1C**).

PERSPECTIVES ON THE BIOSYNTHESIS OF INSECT SEX PHEROMONE-MIMICKING CHILOGLOTTONES

Parallel temporal changes in enzyme activities, protein content, and their corresponding structural gene expression are often pivotal for developmentally regulated and/or tissue-specific volatile production in flowers (Dudareva et al., 2013). Confirmed cases where the biosynthesis of the semiochemicals involved in the sexual mimicry is known in SD orchids is presently limited to the biosynthesis of 7-, 9-, and 12-alkenes in *Ophrys* orchids (Schlüter et al., 2011; Xu et al., 2012; Sedeek et al., 2016) and (S)-β-citronellol in *Caladenia plicata* (Xu et al., 2017). In this study, a strategic developmental stage and tissue differential expression analysis of *C. trapeziformis* floral transcriptomes identified two key transcriptional trends linked to the distribution of chiloglottone in the flowers: (i) During the transition from *vyb* to *vmb*, large suites of FA biosynthesis and β-oxidation genes are upregulated. (ii) In all development stages tested (especially *vmb*), FA pathway genes were consistently induced in the callus compared to the labellum (**Figure 1**). Despite this strong coordinated regulation, no genes from other major biosynthetic pathway (e.g., glycolysis and tricarboxylic acid) showed similar preferential expression (Supplementary Tables 2, 3). Our findings lend further support to the existing hypothesis of chiloglottone formation *in planta* via a FA biosynthetic route and demonstrate a key role of floral developmental transitions (i.e., *vyb* to *vmb*) in the priming of FA pathway gene expression to initiate chiloglottone biosynthesis.

Based on tissue-specific differences in *vmb* and *flw*, we have previously summarized the potential roles of several prioritized FA pathway steps in chiloglottone 1 biosynthesis (Wong et al., 2017a). Considering the new developmental stage contrasts obtained in this study, here we identified a smaller subset of genes (i.e., *CtKASI-2*, *CtFATB2*, *CtACX2/3*, and CtACX4) that may have direct implications for chiloglottone 1 formation (**Table 1**). Of particular interests is the KASI paralog, *CtKASI-2*. We predict that CtKASI-2 may be implicated in both precursor supply (i.e., 3-ketohexanyl-ACP) and directly in the formation of chiloglottone 1 through the condensation of 3-ketohexanyl-ACP and 2-hexenyl-CoA.

TABLE 1 | Potential roles of key fatty acid biosynthesis and β-oxidation pathway genes implicated in chiloglottone 1 biosynthesis prioritized in this study.

Transcript	Enzyme	Pathway	Role	Supporting evidence	Reference
CtACX2/3 and CtACX4	ACX	β-oxidation	Precursor supply (e.g., 2-hexenyl-CoA)	*Arabidopsis* ACXs possess medium-to-long (AtACX2, C14:0 – C20:0; AtACX3, C8:0 – C14:0) and short-to-medium (AtACX4, C4:0 to C8:0) chain substrate specificities. CtACX2/3 and CtACX4 may facilitate 2-hexenyl-CoA production via a continuous passage through the β-oxidation spiral.	(Reviewed in Li-Beisson et al., 2013)
CtKASI-2†	KASI	Biosynthesis	Precursor supply (e.g., 3-ketohexanyl-ACP)	Paralogs of KASI in short FA-accumulating plants (e.g., coconuts) possesses additional short chain length Acyl-ACP substrate specificities (e.g., C4:0). CtKASI-2 may possess the latter preference and facilitate 3-ketohexanyl-ACP production.	Yuan et al., 2015
CtFATB2†	FATB	Biosynthesis	Mid-cycle termination	Tissue (fruit)-specific FATB paralogs in short FA-accumulating species (e.g., oil palm and coconut fruits) possesses short-to-medium chain (C8:0-C14:0) acyl-ACP substrate preference. CtFATB2 may compete between acyl chain elongation and premature cleavage of acyl-ACP.	Jing et al., 2011; Dussert et al., 2013
CtKASI-2†	KASI	Biosynthesis	Condensation	Condensation of activated β-ketoacyl starter with α,β-unsaturated-acyl substrate to form various 2,5-dialkylcyclohexane-1,3-diones in bacteria. CtKASI-2 may be responsible for the condensation of 3-ketohexanyl-ACP and 2-hexenyl-CoA to form 2-ethyl-5-propylcyclohexan-1,3-dion-4-carboxylate, the penultimate precursor to chiloglottone 1.	Fuchs et al., 2013; Mori et al., 2016

†*Paralogs unique to C. trapeziformis.*

To ascertain the role of KASI in chiloglottone biosynthesis, we performed KASI inhibition experiments using Cerulenin. Cerulenin, irreversibly inhibits KASI by forming a covalent bond with the cysteine active site (Moche et al., 1999; Johansson et al., 2008). We show that Cerulenin consistently inhibited chiloglottone production in the callus of three *Chiloglottis* species (i.e., *C. trapeziformis*, *C. valida*, *C.* aff. *valida*) up to 88.4% upon induction with UV-B compared to controls. (**Figure 1C**). Our findings provide the first biochemical evidence supporting FA biosynthesis as the major biosynthetic route for chiloglottones. These findings also indicate that chiloglottone induction by UV-B, tissue-specificity (Falara et al., 2013; Amarasinghe et al., 2015), and molecular pathways (Wong et al., 2017a) involved in chiloglottone formation are potentially conserved across *Chiloglottis*. To ascertain whether co-ordinately regulated patterns of FA pathways linked to chiloglottones in *C. trapeziformis* are also relevant in *C. valida* and *C.* aff. *valida*, systems-based comparative approaches can be adopted (Schilmiller et al., 2012; Wong and Matus, 2017). This could include strategic developmental stage- and tissue-specific differential expression as well as integrated network analysis of metabolites and genes (Wong et al., 2017b).

While in plants there is no evidence that Cerulenin directly/indirectly inhibits FA β-oxidation (Shimakata and Stumpf, 1982; Dehesh et al., 1998; Yasuno et al., 2004), we cannot yet rule out the possibility that FA β-oxidation as an alternative route for chiloglottone biosynthesis (Bohman et al., 2016). This is because any inhibition of KAS enzymes by Cerulenin may have an indirect effect – reducing the levels of *de novo* synthesized FAs (e.g., C16/C18) – and thus restricting further catabolism (via β-oxidation) to yield alternative activated (CoA) FA precursors of appropriate chain lengths for chiloglottone biosynthesis. Transcriptome observations showing coordinated (developmental stage- and tissue-dependent) expression of several *ACX* in *C. trapeziformis* also support the possibility that one or both putative chiloglottone precursors could be obtained by FA β-oxidation. Nonetheless, our results reinforce the current hypothesis that FA biosynthesis serves as the crucial "starting point" for chiloglottones formation. Future studies will require targeted knockdown of pathway candidates and metabolomics analysis for activated (ACP/CoA) FA precursors to ascertain whether chiloglottone production is largely determined by precursor availability (via the FA biosynthesis and/or β-oxidation pathway) or during the condensation of activated precursors that may involve novel KAS activities. Together, these new findings highlight the role of coordinated (developmental stage- and tissue-dependent) FA gene expression and enzyme activities for chiloglottone production in our study species and may have widespread implications for *Chiloglottis* and other orchid genera employing chiloglottones for SD pollination (Peakall et al., 2010).

AUTHOR CONTRIBUTIONS

DW and RA performed the experiments and analyzed the data. RP and EP secured funding, designed the study, and coordinated the experiments and data analysis. DW wrote the article with assistance from EP and RP. All authors have read and approved the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00839/full#supplementary-material

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The Biosynthesis of Unusual Floral Volatiles and Blends Involved in Orchid Pollination by Deception: Current Progress and Future Prospects

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Flowers have evolved diverse strategies to attract animal pollinators, with visual and olfactory floral cues often crucial for pollinator attraction. While most plants provide reward (e.g., nectar, pollen) in return for the service of pollination, 1000s of plant species, particularly in the orchid family, offer no apparent reward. Instead, they exploit their often specific pollinators (one or few) by mimicking signals of female insects, food source, and oviposition sites, among others. A full understanding of how these deceptive pollination strategies evolve and persist remains an open question. Nonetheless, there is growing evidence that unique blends that often contain unusual compounds in floral volatile constituents are often employed to secure pollination by deception. Thus, the ability of plants to rapidly evolve new pathways for synthesizing floral volatiles may hold the key to the widespread evolution of deceptive pollination. Yet, until now the biosynthesis of these volatile compounds has been largely neglected. While elucidating the biosynthesis in non-model systems is challenging, nonetheless, these cases may also offer untapped potential for biosynthetic breakthroughs given that some of the compounds can be exclusive or dominant components of the floral scent and production is often tissue-specific. In this perspective article, we first highlight the chemical diversity underpinning some of the more widespread deceptive orchid pollination strategies. Next, we explore the potential metabolic pathways and biosynthetic steps that might be involved. Finally, we offer recommendations to accelerate the discovery of the biochemical pathways in these challenging but intriguing systems.

Keywords: ***Orchidaceae*, pollination, floral volatile, biosynthesis, semiochemical, deception, volatile organic compounds (VOCs), evolution**

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INTRODUCTION TO DECEPTIVE POLLINATION

Flowers have evolved a diverse array of strategies to secure pollination, with both visual and olfactory cues (i.e., pigmentation and scents) often proving crucial long-distance signals to potential pollinators (Pichersky and Gershenzon, 2002; Raguso, 2008; Muhlemann et al., 2014). Lured by visual and olfactory cues, animal visitors to flowers can be broadly categorized into three

groups: non-pollinator visitors that exploit plant reward – nectar, pollen, or other tissue – but do not pollinate the flower, pollinator visitors that secure reward for their service of pollination; and pollinator visitors that are deceptively exploited by the plant without reward (Schiestl and Schlüter, 2009).

Although by no means exclusive to orchids, deceptive pollination strategies are particularly well-developed in the *Orchidaceae* with an estimated one third of the family (∼10,000 species) using such strategies (Jersáková, 2006). For example, the flowers of some deceptive orchids entice and then defraud their specific (one or few) insect pollinators by emitting volatiles that mimic the sex pheromones of female insects or the presence of the pollinators' prey, oviposition sites, shelter, and rendezvous points. These volatiles can consist of commonly occurring floral compounds in unusual blends and/or as unusual compounds that are uncommon in nature (Bohman et al., 2016). This chemical blends likely serve as sensory private channels that promote specialized plant-pollination relationships in plants, especially in the *Orchidaceae* (Raguso, 2008; Schäffler et al., 2015). The ability of plants to rapidly evolve new pathways or to fine-tune existing pathways for synthesizing these unique floral volatiles blends may hold a key to the widespread evolution of deceptive pollination (Schlüter and Schiestl, 2008; Schiestl and Schlüter, 2009). The mechanisms underpinning this evolution likely include gene duplication and divergence, convergent (and repeated) evolution, and alteration/loss of gene expression and enzyme activities (Gang, 2005).

In this perspective, we first illustrate some examples of the diverse deceptive pollination strategies of the orchids and highlight their chemical diversity. Next, we explore the potential metabolic pathways and biosynthetic steps that might be involved in the production of the often-unusual compounds. Finally, we offer recommendations that may accelerate the discovery of the biochemical pathways in this challenging but intriguing study systems.

VOLATILE DIVERSITY AND POLLINATOR SPECIFICITY IN DECEPTIVE ORCHIDS

Two key features characterize many of the deceptive orchid mimicry examples: (1) Floral volatiles play a pivotal role in the interaction. (2) Pollinator specificity, whereby only one or a few pollinator species are involved, is frequent. Furthermore, while morphology and pigmentation may also play important roles (Schlüter and Schiestl, 2008; Schiestl and Schlüter, 2009), this pollinator specificity is often strongly controlled by chemistry. Below we explore these two themes for some exemplars of deceptive pollination (**Figure 1**).

Although representing just a fraction of the many cases of deceptive pollination, sexual deception is one of the best chemically known pollination system in orchids. In this highly specific system (often just one pollinator), an extraordinary diversity of plant chemicals are now confirmed as the female sex pheromone mimics. These include alkenes and alkanes, uncommon keto and hydroxycarboxylic acids, cyclohexan-1,3-diones, pyrazines, and (methylthio)phenols as active semiochemicals in various sexually deceptive orchids (Bohman et al., 2016, 2017a,b).

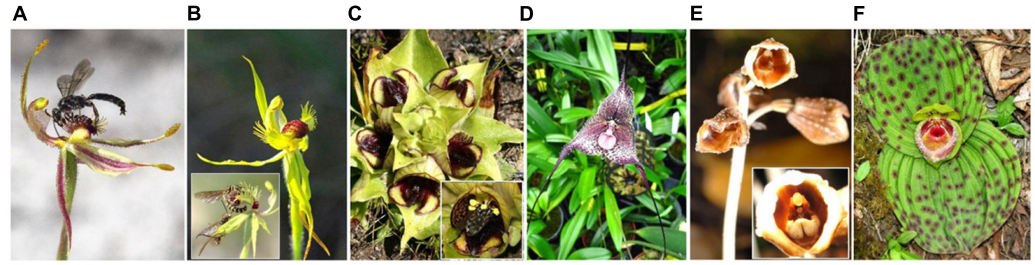
Extreme pollinator specificity has also been reported for orchids that mimic oviposition sites (Martos et al., 2015) and food microbe sources (Ren et al., 2011) via emitting floral volatiles that are associated with rotting fruits and food microorganisms, respectively. Even in cases where pollinator specificity is less extreme, orchids that mimic oviposition, shelter, and rendezvous sites often attract just a specific subset of the many potential pollinators by using floral volatiles associated with carrion or fungi (van der Niet et al., 2011; Policha et al., 2016).

CASE STUDIES OF CANDIDATE FLORAL VOLATILES FOR DECEPTIVE POLLINATION AND FLORAL TISSUE SPECIFICITY

The monoterpene alcohol, β-citronellol, in a unique blend with 2-hydroxy-6-methylacetophenone, a volatile only known from *Caladenia plicata* flowers (**Figure 1A**), play a crucial role in deceptively attracting the mate-seeking male wasp pollinators of just one species (Xu et al., 2017). Production of these compounds is restricted to the sepal tips (clubs) of the flower. In *Caladenia crebra*, flowers emit (methylthio)phenols such as 2-(methylthio)phenol, 2-(methylthio)benzene-1,4-diol, 4-hydroxy-3-(methylthio)benzaldehyde, and 4-(hydroxymethyl)-2-(methylthio)phenol (**Figure 1B**) to sexually deceive its single pollinator, male *Campylothynnus flavopictus* wasp (Bohman et al., 2017a). Productions of these (methylthio)phenols are also restricted to the sepal tips.

Flowers of the fly-pollinated *Satyrium pumilum* orchids emit a cocktail of six compounds (**Figure 1C**) containing sulfurous oligosulfides such as dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS). Emission of these volatiles is also tissue-specific, in this case to the flower spur. Both DMTS and/or DMDS are predicted to be the key olfactory cue for attracting the flesh-eating fly pollinators of *Satyrium pumilum* flowers (van der Niet et al., 2011), consistent with bioassay evidence in other plants (Stensmyr et al., 2002; Shuttleworth and Johnson, 2010; Zito et al., 2014). In the *Dracula* orchid, *Dracula lafleurii,* the labellum acts as both a visual and an olfactory mimic of mushrooms that often grow alongside these orchids (**Figure 1D**) (Policha et al., 2016). Interestingly, the labellum emits an unusual floral volatile blend of mushroom alcohols, especially (*R*)-1-octen-3-ol, which is also emitted by fruiting bodies of co-occurring fungi/mushrooms (Policha et al., 2016). Further experiments revealed that the mushroom-scented labellum is the key lure of the various drosophilid fly species. The involvement of this olfactory signal may be relevant to other *Dracula* orchid species such as *D. chestertonii*, *D. vampira*, *D. chimaera* (Kaiser, 2006).

The above examples highlight floral volatiles of known tissue specificity, and it is likely that tissue specific volatile production will characterize deceptive pollination systems generally. Indeed, tissue-specific emission of floral volatiles is a hallmark of many



	A	B	C	D	E	F
Orchid species	*Caladenia plicata*	*Caladenia crebra*	*Satyrium pumilum*	*Dracula lafleurii*	*Gastrodia similis*	*Cypripedium fargesii*
Mimesis system	SD	SD	OS (Carrion)	S/R (Mushroom)	OS (Rotting fruits)	FM (Fungi)
Pollinator specificity (sp. Per orchid)	1	1	Few	Few	1	1
Pollinator(s)	*Zeleboria* sp	*Campylothynnus flavopictus**	*Sarcophagidae flies** †	*Zygothrica flies* ‡	*Scaptodrosophila bangi* *	*Cheilosia lucida*
Semiochemicals	**1**(18), **2**(1)	**3**(1), **4**(1), **5**(1), **6**(1)	**7**(12), **8**(12), **9**(11),**10**(11), **11**(31)	**12**(13), **13**(7), **14**(3)	**15**(13), **16**(4), **17**(5)	**18**(25), **19**(9), **20**(33), **21**(38)
Chemical bioassay	Yes	Yes	No	No	Yes	No
Tissue specificity	Sepal tips (clubs)	Sepal tips (clubs)	Spur	Labellum	Unknown	Unknown

FIGURE 1 | An illustration of the diversity of orchids employing different deceptive pollination strategies. **(A)** *Caladenia plicata*, **(B)** *Caladenia crebra*, **(C)** *Satyrium pumilum*, **(D)** *Dracula lafleurii*, **(E)** *Gastrodia similis*, and **(F)** *Cypripedium fargesii*. For each orchid species, information on the pollinator(s) (i.e., specificity, species) and volatiles involved (i.e., type, distribution, field bioassay confirmation, tissue specificity) are summarized. Parenthesis for each compounds listed indicate their distribution as floral volatile constituents in angiosperm families surveyed by Knudsen et al. (2006) or as identified in the recent studies discussed in this perspective. A threshold of 50% occurrence (≥45 of the 90 families) determines 'common' floral volatiles as per Knudsen et al. (2006). SD, Sexual deception; FM, Food microorganism; OS, Oviposition site; S/R, Shelter/rendezvous; (**1**) (*S*)-β-citronellol; (**2**) 2-hydroxy-6-methylacetophenone; (**3**) 2-(methylthio)phenol; (**4**) 2-(methylthio)benzene-1,4-diol; (**5**) 4-hydroxy-3-(methylthio)benzaldehyde; (**6**) 4-(hydroxymethyl)-2-(methylthio)phenol; (**7**) dimethyl disulfide; (**8**) dimethyl trisulfide; (**9**) 2-heptanone; (**10**) *p*-cresol; (**11**) indole; (**12**) 1-octen-3-ol; (**13**) 3-octanone; (**14**) 3-octanol; (**15**) ethyl acetate; (**16**) ethyl 2-methylpropanoate; (**17**) methyl 2-methylpropanoate; (**18**) 3-methyl-1-butanol; (**19**) 2-ethyl-1-hexanol; (**20**) 1-hexanol; (**21**) benzyl acetate; * see inset; † (e.g., *Sarcophaga redux*, *Sarcophaga guillarmodi*, and many other indeterminate spp.); ‡ (e.g., *Zygothrica antedispar* and many other indeterminate species of *Zygothrica vittatifrons* group). All images have been reproduced with permission from the respective copyright holders. Please refer to the Section "Acknowledgments" for image credits.

plants, including rewarding species (Muhlemann et al., 2014). Although the precise location of volatile production is unknown, *Gastrodia similis* orchids illustrate an interesting case of rotting fruit mimicry. Flowers emit a scent reminiscent of several host fruits of its sole pollinator, the drosophilid fly *Scaptodrosophila bangi* (**Figure 1E**). The active semiochemicals consist of a blend of three fatty-acid esters, ethyl acetate, ethyl isobutyrate, and methyl isobutyrate (Martos et al., 2015). Meanwhile, *Cypripedium fargesii* has been hypothesized to mimic fungi. Whole flowers emit volatiles normally associated with black mold fungus-infected plant tissue, especially 3-methylbutanol (Ren et al., 2011), and are postulated to be the key cues used to deceptively attract fungi-feeding hoverflies as pollinators. This hypothesis remains to be confirmed by bioassays (**Figure 1F**).

BIOSYNTHESIS OF FLORAL VOLATILES: CHALLENGES AND LESSONS FOR MOVING FORWARD

There has been great progress in deciphering the biochemical and genetic processes underlying the synthesis of floral volatile classes present throughout angiosperms, such as the terpenoids, phenylpropanoids/benzenoids, and volatile fatty acid derivatives (Dudareva et al., 2013). Much, however, remains to be determined, which is not surprising since the number of recognized floral volatiles now stands at ca. 1700 compounds (Knudsen et al., 2006). Furthermore, many other volatiles not yet reported in flowers are known to be synthesized elsewhere in the plant, such as in leaves and roots (Pichersky and Gershenzon, 2002; Dudareva et al., 2013), and it is likely that many of these will eventually be detected in flowers as well (Schiestl, 2010).

Unlocking the biosynthesis of floral volatiles involved in deceptive pollination systems is particularly challenging. Many of the deceptive floral scent compounds in these plants have a limited taxonomic distribution. Genetic resources (e.g., genome and transcriptome sequence databases) that can be invaluable in forward and reverse genetic approaches to elucidate biochemical pathways are rarely available. Often, these plants cannot even be grown in cultivation for a full life-cycle, and biological material has to be collected in nature during the short period of time in the year when the plants are in bloom. Nonetheless, for one recently discovered semiochemical involved in sexual deception, (*S*)-β-citronellol, the complete biosynthetic pathway has now been elucidated in the sexually deceptive orchid *C. plicata* (Xu et al., 2017).

Despite much interest in β-citronellol, given its sporadic but diverse taxonomic distribution in plants, its biosynthesis

remained unknown until the work done in this non-model organism. Earlier work in several model plants such as tomato (Davidovich-Rikanati et al., 2007) and ginger (Iijima et al., 2014) did establish that geraniol was the key precursor of (*S*)-β-citronellol, but subsequent steps were not determined. To identify the genes and enzymes involved in the conversion of geraniol to β-citronellol in *C. plicata*, *de novo* transcriptome assembly and differential expression analysis between club (active) and column (non-active) tissue transcriptomes were carried out. A candidate geraniol synthase gene was quickly identified based both on differential expression and membership in the terpene synthase (TPS) gene family, and the protein encoded by this gene was biochemically demonstrated to catalyze the formation of geraniol from geranyl diphosphate (Xu et al., 2017). The analysis also identified one highly expressed alcohol dehydrogenase (*CpADH3*) and one tissue-specific double-bond reductase (*CpGER1*) transcript as promising candidates. These transcripts belonged to the gene/protein family of interests (i.e., dehydrogenase and reductase) and/or possessed the desired profile of strong differential expression. Contrary to previous predictions of a one-step conversion of geraniol to β-citronellol (Hsiao et al., 2006; Schwab and Wust, 2015), subsequent biochemical assays for *Cp*ADH3 and *Cp*GER1 revealed that β-citronellol biosynthesis from geraniol proceeds in three steps, beginning with the oxidation of geraniol to geranial by *Cp*ADH3, enantioselective reduction of geranial to (*S*)-β-citronellal by *Cp*GER1, and a further reduction of (*S*)-β-citronellal to (*S*)-β-citronellol by *Cp*ADH3 (Xu et al., 2017).

The breakthrough in the elucidation of the biosynthesis of β-citronellol in this non-model plant species was aided by several key factors: (1) Considerable relevant background research in other plants. (2) It was well-established that geraniol was a precursor of β-citronellol. (3) β-Citronellol along with the second active compound, 2-hydroxy-6-methylacetophenone were the dominant floral volatiles. (4) Production of the compound was tissue specific. (5) Thus, strategically targeted contrasting active and non-active tissue transcriptomes were produced, allowing the downstream differential expression analysis, identification of the candidate genes involved, and confirmation of gene function. This combination of just a few dominant components of floral scent (in an often simple floral bouquet) and tissue specific production are common features of the deceptive pollination examples illustrated earlier (**Figure 1**). Thus, differential expression of active and non-active tissue transcriptomes has the potential to rapidly aid identification of candidate genes.

INISIGHTS INTO THE BIOSYNTHESIS OF SEMIOCHEMICALS INVOLVED IN DECEPTIVE POLLINATION SYSTEMS

Motivated by the success of the elucidation of (*S*)-β-citronellol biosynthesis in a non-model system, here we explore the biosynthetic pathways involved in 2-hydroxy-6-methylacetophenone, (methylthio)phenols, dimethyl di- and tri-sulfide, 1-octen-3-ol, ethyl 2-methylpropanoate, and 3-methyl butanol formation (**Figures 2A–F**) by drawing on the literature to establish some plausible hypotheses for the biosynthesis of some compounds involved in deceptive pollination.

Biosynthesis of 2-Hydroxy-6-Methylacetophenone

Compared to β-citronellol, 2-hydroxy-6-methylacetophenone is a rare floral volatile presently only known in the flowers of five *Caladenia* orchids – *Caladenia attingens*, *C. ferruginea, C. pectinata, C. thinicola*, and *C. plicata* (Xu et al., 2017). Two alternative biosynthesis hypotheses have been proposed – a polyketide biosynthetic route based on evidence from radiolabeling studies in an ant (Tecle et al., 1986) or via coenzyme A-dependent β-oxidation of phenylpropanoid precursors (Negrel and Javelle, 2010). Here, we present a hypothesized pathway for the formation of 2-hydroxy-6-methylacetophenone via a polyketide biosynthetic route (**Figure 2A**). The polyketide biosynthetic route deserves attention as a strong tissue-specific differential expression of one polyketide synthase (*CpPKS1*) is present (Xu et al., 2017).

Biosynthesis of (Methylthio)phenol

Plant enzymes catalyzing the methylation of sulfhydryl/thiol (-SH) group have been previously reported, for example, *S*-methyltransferase 1 (CrSMT1) in *Catharanthus roseus* (Coiner et al., 2006). This enzyme is capable of methylating different sulfhydryl-containing aliphatic and aromatic compounds with different efficiencies, and is closely related to plant *O*-methyltransferases (**Figure 2B**). Given that *O*-methyltransferase are widely distributed in plants as large gene families (Gang, 2005) and that only a few substitutions in key residues are sufficient for converting an *O*-methyltransferase into an *S*-methyltransferase (Coiner et al., 2006), close attention should be given to *O*-methyltransferase gene families in *Caladenia crebra*. Under this hypothesis, the biosynthesis of 2-(methylthio)phenol would be achieved by the methylation of 2-hydroxythiophenol (**Figure 2B**). Potential *O*-/*S*-methyltransferase homologs may also evolve unique capacities to accept the different sulfhydryl-containing precursors with different side chains (e.g., –CHO, $–CH_2OH$, –OH) and/or at various positions, which may otherwise interfere with substrate acceptability as demonstrated for CrSMT1 (Coiner et al., 2006).

Biosynthesis of Dimethyl Disulfide and Dimethyl Trisulfide

Biochemical and genetic evidence for the formation of DMDS and DMTS has been established in several plant species (Boerjan et al., 1994; Tulio et al., 2002; Rébeillé et al., 2006; Gonda et al., 2013). First, formation of methanethiol, a highly unstable and reactive compound, requires the sulfur-containing amino acid methionine as precursor. Next, methanethiol autooxidises to DMDS and DMTS (**Figure 2C**). Thus, the enzyme methionine gamma-lyase (MGL) which catalyzes the catabolism of methionine to form α-ketobutyrate, methanethiol, and ammonia (Gonda et al., 2013) is a strong candidate for

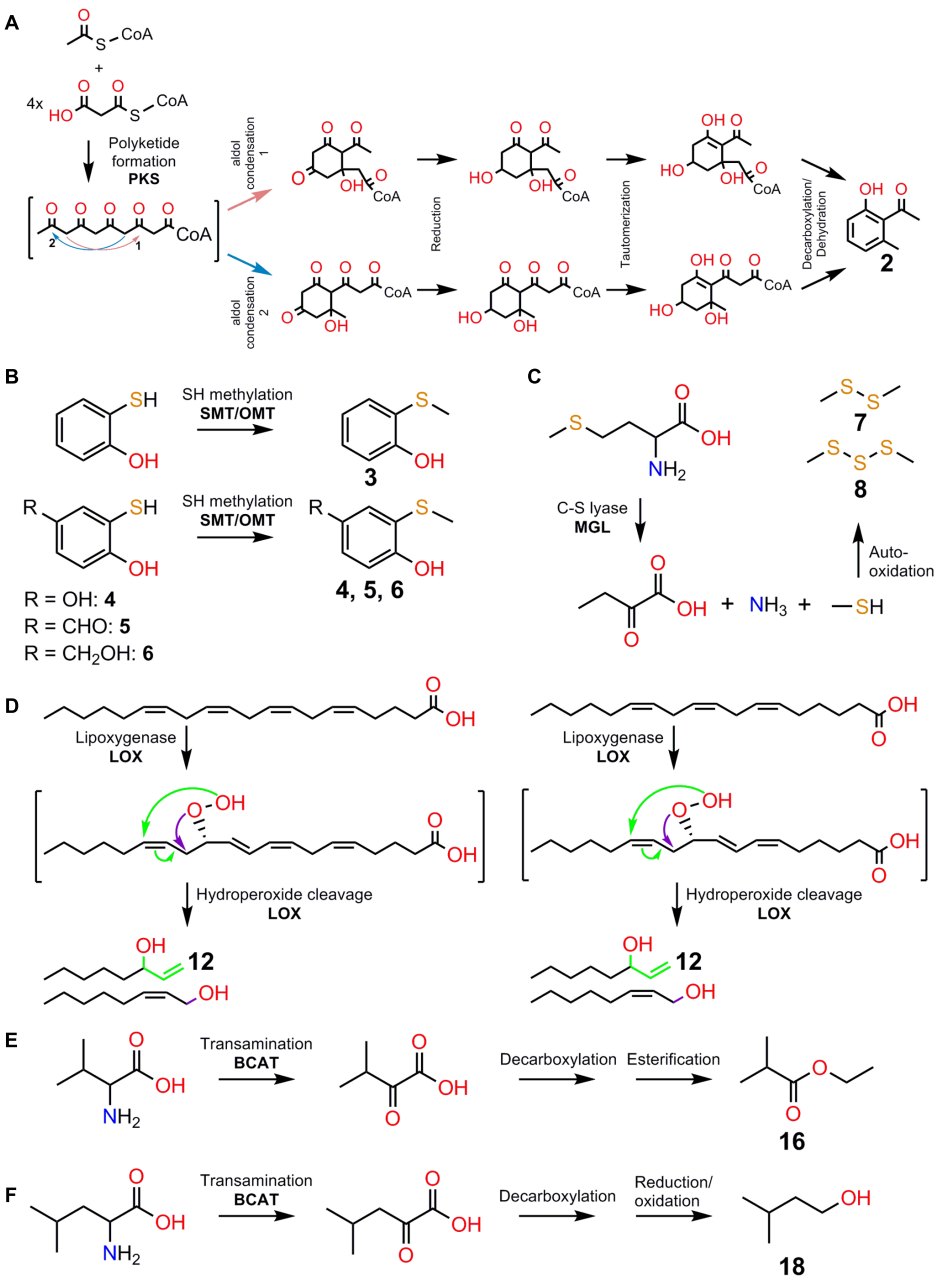


FIGURE 2 | A subset of volatile attractants involved in deceptive orchid pollination and their potential biosynthetic pathways. **(A)** Formation of 2-hydroxy-6-methylacetophenone (**2**) using one acetyl-CoA and four malonyl-CoA starter units via a polyketide synthase pathway involving aldol condensation, reduction, tautomerization, and decarboxylation/dehydration reactions. Involvement of polyketide synthase (PKS) is indicated. **(B)** Formation of the (Methylthio)phenols, 2-(methylthio)phenol (**3**), 2-(methylthio)benzene-1,4-diol (**4**), 4-hydroxy-3-(methylthio)benzaldehyde (**5**), and 4-(hydroxymethyl)-2-(methylthio)phenol (**6**) via sulfhydryl/thiol (-SH) methylation of sulfhydryl-containing precursors. Involvement of *S*-methyltransferase (SMT) and *O*-methyltransferase (OMT) is indicated. **(C)** Formation of dimethyl disulfide (**7**) and dimethyl trisulfide (**8**) via auto-oxidation of methanethiol. The action of C-S lyase activity on methionine produces α-ketobutyrate, ammonia, and methanethiol. Involvement of methionine gamma-lyase (MGL) is indicated. **(D)** Formation of 1-octen-3-ol (**12**) via the lipoxygenase (LOX) pathway with arachidonic acid and γ-linolenic acid as precursors. Involvement of LOX enzymes is indicated. Note that LOX activities on arachidonic acid and γ-linolenic acid precursors can give rise to 12-Hydroperoxyeicosatetraenoic acid (12-HPETE) and 10-γ-hydroperoxyoctadecatrienoic acid (10-γ-HPOTE) products, respectively. 12-HPETE and 10-γ-HPOTE serve as intermediates for the formation of compounds such as **12** and (2*Z*)-octen-1-ol via unusual LOX fatty acid chain-cleaving lyase activities. **(E)** Formation of ethyl 2-methylpropanoate (**16**) via decarboxylation and esterification of α-ketoisovalerate intermediate. **(F)** Formation of 3-methyl butanol (**18**) via decarboxylation and reduction/oxidation of α-ketoisocaproate intermediate. Transamination/deamination of L-valine and L-leucine leads to the formation of α-ketoisovalerate and α-ketoisocaproate, respectively. Involvement of branched-chain aminotransferase (BCAT) enzymes is indicated. Note that α-ketoisovalerate can serve as alternative intermediate for (**18**). The numbering of compounds are kept consistent as listed in **Figure 1** for clarity.

DMDS and DMTS biosynthesis in *Satyrium pumilum* flowers. Alternatively, methanethiol could also be formed via methylation of bisulfide ion ([SH]-) as demonstrated by several *Brassicaceae* thiol methyltransferase (Attieh et al., 2002; Itoh et al., 2009; Nagatoshi and Nakamura, 2009). However, this pathway seems unlikely in orchids which are non glucosinolate-producing plants (Iranshahi, 2012).

Biosynthesis of 1-Octen-3-ol

Plant lipoxygenases (LOXs) belong to large gene families and commonly catalyze the stereo-specific oxygenation of octadecanoid precursors at positions C9 (9-LOX) and C13 (13-LOX) resulting in the formation of various 9- and 13-hydroperoxy intermediates, respectively (Feussner and Wasternack, 2002). Biochemical studies have shown that the moss *Physcomitrella patens* possesses a multi-functional LOX enzyme (*PpLOX1*) with unique fatty acid chain-cleaving lyase activities capable of producing 1-octen-3-ol via 12- and 10-hydroperoxy intermediates (**Figure 2D**), the products of arachidonic acid and γ-linolenic acid precursors, respectively (Senger et al., 2005). 1-Octen-3-ol has also been reported to be specifically induced in reproductive (Savoi et al., 2017) and vegetative (Wichard et al., 2004) tissues of plants during abiotic stress. A tissue- and/or stress-specific expression of a unique *LOX*, may be hypothesized for 1-octen-3-ol biosynthesis in the labella of *Dracula lafleurii*.

Biosynthesis of Ethyl 2-Methylpropanoate and 3-Methyl Butanol

Catabolism of branched chain amino acids (BCAA) such as L-isoleucine, L-leucine, and L-valine, is key to the formation of many BCAA-derived volatiles including ethyl 2-methylpropanoate and 3-methylbutanol (Gonda et al., 2010, 2013; Kochevenko et al., 2012), with respective α-keto acids serving as key intermediates (**Figures 2E,F**). The branched-chain aminotransferase (BCAT) enzymes that catalyze the transamination/deamination of the amino acid precursor to α-keto acids have also been characterized in several fruit crops rich in BCAA-derived volatiles (Gonda et al., 2010; Maloney et al., 2010). Strong evidence implicating BCATs in 3-methylbutanol formation (**Figure 2F**) has also been obtained in transgenic over-expression studies of tomatoes (Kochevenko et al., 2012). Although the tissue-specific distribution of ethyl 2-methylpropanoate and 3-methylbutanol in the flowers of *Gastrodia similis* and *Cypripedium fargesii*, respectively remains to be determined, specific BCATs isoforms may be relevant for their biosynthesis (**Figures 2E,F**).

FUTURE DIRECTIONS AND FINAL REMARKS

High throughput sequencing methodology is an emerging tool for profiling gene expression at a genome-wide scale in non-model plants. The adoption of this technique, especially the sequencing of mRNA from floral tissues and *de novo* transcriptome reconstruction, to prioritize candidate genes and pathways involved in the biosynthesis of deceptive semiochemicals in several orchids have already been carried out (Sedeek et al., 2013; Wong et al., 2017; Xu et al., 2017). To initiate the prioritization of candidate genes and pathways, targeted and strategic transcriptome analysis of active (scent-producing) and non-active flower organs and tissues will often be the first key step. Differential expression analysis between the active and non-active tissues can then be performed, where the latter provides an excellent baseline for identifying differentially expressed genes in the active tissues. This approach was used to the authors' advantage in the breakthrough on (*S*)-β-citronellol biosynthesis (Xu et al., 2017), and may hold potential for elucidating other biosynthetic pathways. When closely related orchid species also employs a common volatile, such as 1-octen-3-ol in some *Dracula* orchids (Kaiser, 2006), transcriptome analysis across species with the goal of identifying shared gene expression and metabolic pathways, may also prove informative.

To provide additional support to the candidates prioritized from targeted/strategic transcriptome analysis highlighted above, an integrated network analysis can be performed. When simultaneous profiling of deceptive volatiles across diverse conditions and their corresponding sample transcriptomes is feasible, such metabolic profiles can be used as 'guides' or 'baits' to infer functionally associated genes that satisfy a given similarity threshold (e.g., correlation and mutual information). This approach is based on the well-established observations that genes and metabolites involved in related processes often have parallel expression/accumulation dynamics across a range of conditions such as tissues and developmental stages (Schilmiller et al., 2012; Wong and Matus, 2017).

A complementary strategy should also include molecular evolutionary analysis. For example, testing for gene duplication and selection signatures on hypothesized pathway genes in a phylogenetic context is often useful. Such an analysis provided critical clues toward the identification of the candidate stearoyl-acyl carrier protein desaturase enzymes involved in 7-, 9-, and 12- alkene biosynthesis in sexually deceptive *Ophrys* orchids (Schlüter et al., 2011; Sedeek et al., 2016).

CONCLUSION

As critical first step, here we have drawn on prior biochemical knowledge from other systems to build plausible hypotheses on the biosynthesis of some volatiles involved in deceptive pollination. We have also highlighted the promising approaches that will allow these hypotheses to be tested. While orchids represent particularly challenging systems, as neither their biochemistry nor genomes and transcriptomes have been extensively characterized, these approaches are making

the biochemical investigation of deceptive chemicals in orchids both feasible and rewarding. Beyond deceptive orchids, these approaches serve as valuable guidelines for other plants, including rewarding species, particularly those species employing unique floral volatile blends for pollinator attraction.

AUTHOR CONTRIBUTIONS

DW conceived the article, planned its structure, discussed the literature, and wrote the article with assistance from EP and RP. All authors have read and approved the paper.

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Nocturnal Bee Pollinators Are Attracted to Guarana Flowers by Their Scents

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Floral scent is an important component of the trait repertoire of flowering plants, which is used to attract and manipulate pollinators. Despite advances during the last decades about the chemicals released by flowers, there is still a large gap in our understanding of chemical communication between flowering plants and their pollinators. We analyzed floral scents of guarana (*Paullinia cupana*, Sapindaceae), an economically important plant of the Amazon, using chemical analytical approaches, and determined the attractiveness of the scent to its nocturnal bee pollinators using behavioral assays in the field. Pollen loads of attracted bees were also analyzed. Inflorescences of guarana emit strong scents, both during day and at night, with some semi-quantitative differences between day- and night-time scents. Synthetic scent mixtures containing some of the identified floral scent components, including the most abundant ones, i.e., linalool and (*E*)-β-ocimene, successfully attracted the nocturnal *Megalopta* bee pollinators. Pollen analyses revealed that many of the attracted bees had pollen grains from previous visits to guarana flowers on their bodies. Overall, our data show that guarana flowers attract nocturnal bee visitors by their strong scents and suggest that the chemical communication between this plant and its pollinators is a key component in crop production of this economically important plant species.

Keywords: flower signal, pollinator attraction, chemical communication, crop pollination by bees, volatile organic compounds, *Paullinia cupana*, Amazon

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INTRODUCTION

Floral scents are important signals for the attraction of pollinators and may be particularly important for plants pollinated at night when visual signals are of limited use. In the last decades, there has been considerable progress in understanding the chemical communication between nocturnal plants and their pollinators (e.g., moths, beetles, bats; Dobson, 2006; Borges et al., 2016). Just recently, however, a new pollination system mediated by floral scent and involving nocturnal bees as pollinators was described (Cordeiro et al., 2017).

The nocturnal/crepuscular habit has arisen in four families of bees, i.e., Andrenidae, Apidae, Colletidae, and Halictidae, comprising at least 250 species (Warrant, 2007). It is hypothesized that night-active bees evolved this habit as a response to competition, parasitism, and predation during the day (Wcislo et al., 2004). Foraging at night and during crepuscular periods may be beneficial

as flowers are often rich in pollen and nectar early in the morning before exploitation by diurnal flower visitors, and late in the evening before night active visitors arrive ("competitor-free space" according to Wcislo et al., 2004; Warrant, 2007). Nocturnal bees must have good vision and a well-developed olfactory system to find their nests and to recognize the flowers at low light intensities (Kelber et al., 2006; Borges et al., 2016). It is likely that they primarily use olfactory floral cues, i.e., floral scents, to efficiently locate appropriate host plants.

Paullinia cupana Kunth (Sapindaceae), popularly named guarana, is an economically important plant of the Brazilian Amazon. The seeds of this plant are used to produce soft drinks, energy drinks, ice creams, creams, pharmaceuticals, and cosmetics (Tavares et al., 2005). Guarana is produced by large and small producers and is one of the most valued products from the Amazon as it is consumed and appreciated by national and international markets. The species is monoecious, but either pistillate or staminate flowers are produced by a given plant individual on a specific day (**Figure 1**). Thus, it depends on cross-pollination to set fruits, with diurnal bees traditionally cited as the main pollinators (Schultz and Valois, 1974; Escobar et al., 1984). Recently, Krug et al. (2015) also reported flower visits by nocturnal and crepuscular bees, which efficiently vector pollen (Krug et al., unpublished data) and are potentially attracted by the floral scent of *P. cupana*.

Here, we collected floral scents of *P. cupana* using dynamic headspace extraction methods and analyzed the samples by gas chromatography/mass spectrometry (GC/MS). We also performed behavioral assays in the field with synthetic compounds and analyzed the pollen load of attracted bees. Specifically, we addressed the following questions: What are the absolute amounts of floral scents released, and does scent differ between day and night in quantitative and compositional (semi-quantitative scent patterns) properties? Are there differences in scent between pistillate and staminate flowers? Are nocturnal/crepuscular bees attracted by the main floral scent components, and if yes, do they carry pollen grains from previous visits to guarana flowers on their bodies?

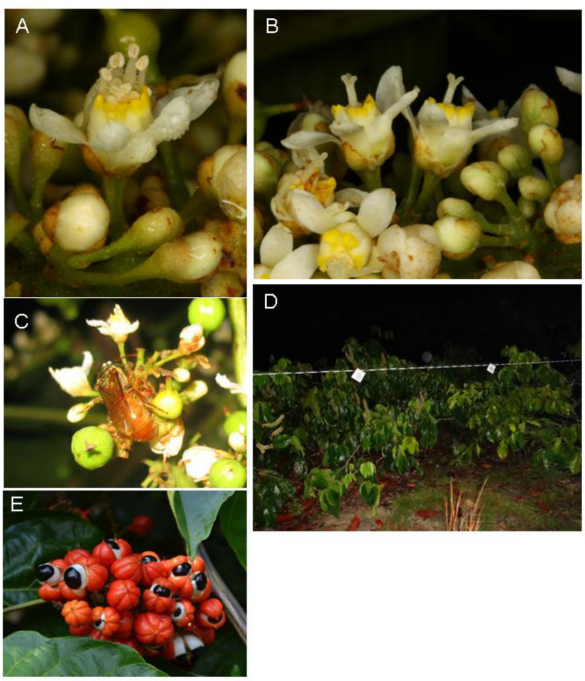

FIGURE 1 | Male **(A)** and female **(B)** guarana flowers. *Megalopta aeneicollis* visiting a guarana flower **(C)**. String and filter papers impregnated with synthetic mixtures or solvent only, as used in the biotests **(D)**. Fruits of guarana **(E)**.

MATERIALS AND METHODS

Study Species

When cultivated in plantations, *P. cupana* Kunth [=*P. cupana* var. *sorbilis* (Mart.) Ducke] is trimmed to a shrubby habit, despite growing originally as a liana (Castro, 1992). Flowering usually occurs between July and September, which corresponds to the least rainy period in the Amazon region and lasts from five to 45 days. Anthesis begins at night at around 2:00, and the flowers remain open until 10:00–12:00 (Escobar et al., 1984). On any given day, most of the flowers of a guarana plant are functionally either female or male; thus, the sex expression of a single plant changes during its flowering period. Overall, a single plant produces five to six times more staminate than pistillate flowers (Schultz and Valois, 1974). When ripe, the fruits have a reddish peel, a white pulp, and a visible black seed (**Figure 1**; Castro, 1992).

Study Area

The study was carried out at three guarana plantations in the Amazonas State, Brazil: the Experimental plantation of Embrapa (Brazilian Agricultural Research Corporation) in the city of Manaus (2°53′13.52″S/59°58′58.71″W), the Experimental field of Embrapa in Maués (3°23′58.20″S/57°40′39.95″W), and a private commercial plantation (3°21′29.8″S/57°42′07.01″W) in Maués. At all three sites, the guarana plantations were surrounded by Amazonian Terra-Firme Forest.

Volatile Sampling and Analyses

Flower volatiles were collected by dynamic headspace extraction methods, as described by Heiduk et al. (2016) in the flowering period of 2014 (October) in Manaus. We bagged inflorescences prior to anthesis on 13 different individuals and collected their scent after the flowers opened. Each inflorescence was sampled twice, at night after flower opening (between 3:30 and 5:30) and during the day (between 7:30 and 9:30), resulting in a total of 26 samples. Seven of the inflorescences had exclusively staminate flowers, and six had exclusively pistillate flowers. To obtain a scent sample, an inflorescence (with 5 to 30 open flowers) was enclosed in a polyester oven bag (10 cm × 20 cm; Toppits®) for 2 min. Then, an adsorbent tube was inserted into the bag to collect the volatiles for 2 min using a membrane pump (G12/01 EB; Gardner Denver Thomas GmbH, Fürstenfeldbruck, Germany). The flow was adjusted at 200 ml/min with a

flowmeter. The adsorbent tubes (quartz vials, length: 25 mm, inner diameter: 2 mm) were filled with 1.5 mg Tenax®-TA (mesh 60–80) and 1.5 mg Carbotrap® B (mesh 20–40, both Supelco). The adsorbents were fixed in the tubes using glass wool. Volatile samples from green leaves were collected with the same method and were used to discriminate between vegetative and flower-specific scent components.

Scent samples were analyzed using GC/MS to (i) identify the flower-specific compounds in the scent samples, and (ii) determine their absolute and relative scent amounts. The system consisted of an automated thermal desorption system (model TD-20, Shimadzu, Japan) coupled to a GC/MS (model QP2010 Ultra EI, Shimadzu, Japan) equipped with a Zebron™ ZB-5 fused silica column (5% phenyl, 95% dimethylpolysiloxane; 60 m long; inner diameter 0.25 mm; film thickness 0.25 μm; Phenomenex), as described in Heiduk et al. (2016). The GC/MS data were processed using the GCMS solution package (Version 4.41, Shimadzu). The identification of compounds was carried out using the Wiley 9, Nist, 2011, FFNSC 2, and Adams (2007) mass spectral libraries, the database available in MassFinder 3, and published data on Kovats retention indices of components. The identity of some of the compounds was confirmed by comparison of mass spectra and retention times with authentic standard compounds.

Field Bioassays

To test if identified floral volatiles attract the nocturnal/crepuscular bee pollinators, 29 two-choice bioassays were carried out: 12 in Maués in August 2015 (five at the commercial plantation and seven at Embrapa) and 17 in Manaus from September to October of 2015 and 2016. The bioassays in Maués were performed between 3:00 and 6:00, before sunrise (4 × Day-scent 1; 3 × Day-scent 2; see also below), and in the evening between 18:00 and 19:00, after sunset (5 × Day-scent 1; see also below). In Manaus, tests were performed before sunrise between 4:30 and 6:00.

We tested three blends of synthetic scent using compounds identified from guarana flowers. The blends had different concentrations and numbers of compounds, and different relative ratios of compounds. Two of the blends (Day-scent 1, Day-scent 2) resembled, in terms of relative amounts of compounds (as determined by dynamic headspace sampling and GC/MS analysis), the first nocturnal sample analyzed in the lab by GC/MS. After analyzing all diurnal and nocturnal samples, however, this sample was found to be an exceptional night sample as its composition was found to be more similar to the mean diurnal than to the mean nocturnal scent composition of the plant. Thus, we prepared another blend (Night-scent), which resembled the mean nocturnal composition in the relative amounts of the compounds used. Given that, there were no differences in scent between staminate and pistillate flowers (see Results), there was no need to consider the sex of flowers in these blends.

When applied to filter paper, Day-scent 1 released (*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%). Day-scent 2 contained the same component mixture as Day-scent 1 except for (*E*)-β-ocimene and epoxy-oxoisophorone, as these were used up when preparing Day-scent 1 and Night-scent. The composition of Day-scent 2 was as follows: (*Z*/*E*)-linalool oxide furanoid (6%), methyl benzoate (19%), linalool (71%), phenylacetonitrile (2%), 4-oxoisophorone (2%), and (*Z*/*E*)-linalool oxide pyranoid (0.4%). Both Day-scent mixtures were used undiluted for bioassays. The mixture resembling nocturnal scent was used 100-fold diluted in acetone (v/v) and consisted of (*E*)-β-ocimene (57%), (*Z*/*E*)-linalool-oxide (6%), linalool (30%), and (*E*)-β-caryophyllene (7%). The compounds used for the experiments were obtained either from Sigma Aldrich in the highest purity available or were available in the reference collection (built up from various sources; purity >90% each) of the Salzburg lab.

For each choice assay, we offered 50–100 μl (depending on availability) of one of the synthetic mixtures on filter paper (diameter 10 cm; Whatman No. 1), and as negative control either just a filter paper (when using an undiluted mixture) or a filter paper with 50–100 μl of acetone (Sigma-Aldrich, 99.8%) solvent (when using the diluted mixture). As determined by dynamic headspace sampling and GC/MS analysis, the total amount of scent released by our scent baits resembled the scent released by ca. two strongly scented plants at night when using the 100-fold diluted sample, and 200 plants, when using the undiluted samples. The filter papers were tied to a string and hung between two flowering guarana plants (**Figure 1**). The two pieces of filter paper were at least 1 m apart and were also separated by at least 1 m from the guarana plants. When choice assays were performed simultaneously at the same plantation, the distance between assay sites was 10–20 m. Bees hovering within 10 cm of the filter papers or landing on them were collected with an insect net. The collected bees were identified following Santos and Melo (2015). To test whether collected bees visited guarana flowers before responding to the synthetic scents, pollen grains from the bodies of 17 of the bees attracted in Manaus (nine *Megalopta aeneicollis*, one *M. cuprea*, one *M. piraha*, one *M. sodalis*, five *Megalopta* sp.) were sampled and identified. Pollen grains on the bees' bodies were removed using pincers and fixed in ethanol for 24 h. The material was then centrifuged, the ethanol discarded, and glacial acetic acid (2 ml) was added for another 24 h, before centrifuging again. The pollen grains were then acetolyzed as proposed by Erdtman (1960), which removes the cytoplasmic content, exposing the morphological features, which are useful for pollen identification using a light microscope. The RCPol's Pollen Collection (online Pollen Catalogs Network[1]) and a specific Amazon Pollen Collection were used for comparison.

Data Analysis

For analysis of total quantitative (total absolute amount of scent) and semi-quantitative (percentage contribution of single compounds to total scent) differences in scent emission between sexual phases and time periods (night and day), we performed PERMANOVA analyses (fixed factors: *time class*, *sex*; random

[1]www.rcpol.org.br

factor: *individual* nested in *sex*; 10.000 permutations) based on Euclidean distances and pairwise Bray–Curtis similarities, respectively (Clarke and Gorley, 2006; Primer 6 Version 6.1.15 and Permanova Version 1.0.5). In addition, SIMPER (factor: *time class*) was used in Primer to determine the compounds responsible for semi-quantitative differences in scent between day and night. A PERMDISP in Primer tested whether dispersion differed between day and night scents.

The field bioassay results were analyzed by exact binomial tests of goodness-of-fit using the spreadsheet provided by http://www.biostathandbook.com/exactgof.html (accessed 20 May 2017). Responses toward specific scent blends (pooled number of attracted bees over replicate assays per blend) were compared to responses toward the negative control. Due to the small number of bees attracted to Day-scent 1 (see Results), no statistical test was performed to compare the attractiveness of this mixture to the control.

RESULTS

Floral Volatiles

The inflorescences emitted an amount of roughly 200 ng of scent per flower per hour (**Table 1**). Overall, we did not find differences in total absolute amounts of scent per flower between the sexes (Pseudo-$F_{1,25} = 0.46$, $p = 0.72$) or between day and night samples (Pseudo-$F_{1,25} = 3.28$, $p = 0.10$), despite some variation in mean and maximum values (**Table 1**). Similarly, there was a non-significant interaction effect of these factors (Pseudo-$F_{1,25} = 1.15$, $p = 0.33$).

In total 41 compounds of six chemical classes and unknowns were found in the night and day samples. The volatiles consisted of monoterpenes (16 compounds), sesquiterpenes (8), unknown compounds (10), irregular-terpenes (3), N-bearing compounds (2), aromatics (1), and miscellaneous (1) compounds (**Table 1**). Most of the compounds were found both in day and night samples, with only one minor compound each being time class specific (**Table 1**).

There were no differences in the percentage contribution of single compounds between the sexes (Pseudo-$F_{1,25} = 1.27$, $p = 0.25$), but differences were evident between samples collected during the day or the night (Pseudo-$F_{1,25} = 9.26$, $p = 0.001$) with a non-significant interaction (sex × time) effect (Pseudo-$F_{1,25} = 0.61$, $p = 0.65$). A PERMDISP analysis revealed that dispersion differed between day and night scents ($F_{1,24} = 30.01$, $p < 0.001$) with night scents being more variable than day scents (see also **Table 1**). Based on a SIMPER analysis, linalool and (*E*)-β-ocimene were responsible for more than 60% of the observed differences among night and day samples with linalool being more abundant during day-time, and (*E*)-β-ocimene being more abundant at night (**Table 1**).

Field Bioassays

Thirty-three bees, all *Megalopta*, from at least four species responded in the field bioassays: *M. aeneicollis* (25 individuals), *M. cuprea* (1), *M. piraha* (1), and *M. sodalis* (1). Five of the attracted individuals could not be assigned to a species. The bees

TABLE 1 | Mean (minimum, maximum) total absolute and relative amount of each compound detected in the night and day floral scent samples of *Paullinia cupana* var. *sorbilis* (*N* = 13 individuals).

Total absolute amount of scent (ng per hour and flower)	**KRI**	**Night Mean (min/max) 60.32 (3.36/454.53)**	**Day Mean (min/max) 360.1 (8.74/2376.35)**
Aromatics			
2-Phenylethanol*	1119	0.00 (0.00/0.03)	0.13 (0.00/0.65)
Monoterpenes			
(*E*)-β-Ocimene*	1050	**46.08** (0.04/94.91)	**19.68** (6.06/31.89)
(*Z*)-Arbusculone	1056	0.11 (0.00/0.64)	0.78 (0.00/3.60)
(*E*)-Arbusculone	1074	0.01 (0.00/0.07)	0.11 (0.00/0.50)
(*Z*)-Linalool oxide furanoid*	1078	3.07 (0.00/38.92)	0.55 (0.00/0.85)
(*E*)-Linalool oxide furanoid*	1093	1.95 (0.00/23.07)	2.42 (0.00/6.05)
Linalool*	1100	**24.16** (2.31/50.10)	**60.97** (38.82/82.43)
1,3,8-*p*-Menthatriene	1125	2.93 (0.00/11.96)	1.14 (0.00/4.58)
Allo-Ocimene*	1131	0.06 (0.00/0.66)	0.18 (0.00/0.41)
Lilac aldehyde A*	1148	0.03 (0.00/0.34)	–
Lilac aldehyde B+C*	1157	0.03 (0.00/0.33)	0.05 (0.00/0.31)
Lilac aldehyde D*	1172	0.00 (0.00/0.02)	0.00 (0.00/0.03)
(*Z*)-Linalool oxide pyranoid*	1177	–	0.01 (0.00/0.04)
(*E*)-Linalool oxide pyranoid*	1180	0.01 (0.00/0.17)	0.08 (0.00/0.39)
Lilac alcohol A*	1208	0.02 (0.00/0.17)	0.18 (0.00/1.06)
Lilac alcohol B+C*	1218	0.63 (0.01/3.79)	1.74 (0.01/3.79)
Lilac alcohol D*	1232	0.04 (0.00/0.20)	0.02 (0.00/0.19)
N-bearing compounds			
Phenylacetonitrile*	1144	0.25 (0.00/1.10)	0.79 (0.00/2.72)
Indole*	1288	0.00 (0.00/0.02)	0.01 (0.00/0.06)
Irregular terpenes			
(*E*)-4,8-Dimethyl-1,3,7-nonatriene*	1117	0.13 (0.00/1.06)	0.38 (0.00/1.62)
4-Oxoisophorone*	1149	0.04 (0.00/0.37)	**2.79** (0.00/20.43)
Epoxy-oxoisophorone*	1137	**5.40** (0.00/30.62)	**4.39** (0.00/8.44)
Sesquiterpenes			
α-Cubebene	1364	4.27 (0.00/42.46)	0.47 (0.00/4.23)
α-Copaene*	1394	2.39 (0.00/23.32)	0.49 (0.00/3.17)
(*E*)-β-Caryophyllene*	1444	**5.28** (0.00/38.56)	1.98 (0.00/15.18)
α-Bergamotene*	1436	0.00 (0.00/0.02)	0.00 (0.00/0.05)
α-Humulene*	1478	0.23 (0.00/1.71)	0.01 (0.00/0.17)
β-Copaene	1485	0.05 (0.00/0.23)	0.01 (0.00/0.12)
Germacrene D*	1504	0.19 (0.00/1.51)	0.04 (0.00/0.61)
(*E*,*E*)-α-Farnesene*	1504	0.07 (0.00/0.94)	0.01 (0.00/0.21)
Miscellaneous			
2,2,6-Trimethyl-3-keto-6-vinyltetrahydropyran	1113	0.02 (0.00/0.25)	0.01 (0.00/0.04)
Unknown compounds			
10 Unknown compounds		0.76 (0.00/4.96)	0.16 (0.00/1.00)

**Identity of compounds was confirmed with synthetic standards compounds. Compounds are listed according to chemical class. KRI, Kovats Retention Index. The four most abundant compounds each in night and day samples are in bold.*

TABLE 2 | Number of *Megalopta* individuals attracted to different scent mixtures in field bioassays performed in Manaus and Maués.

	Day-scent 1	Day-scent 2*		Night-scent*
Bees	**Maués** ***N*** **= 9**	**Manaus** ***N*** **= 9**	**Maués** ***N*** **= 3**	**Manaus** ***N*** **= 8**
Megalopta aeineicollis		9		16
Megalopta cuprea		1		
Megalopta piraha				1
Megalopta sodalis				1
Megalopta sp.	3		2	

*No bees responded to negative control treatments. N refers to the number of trials. *Scent treatment attracted significantly more bees than the control (exact binomial test, $p < 0.01$).*

flew directly to or around the filter papers with synthetic scent and sometimes even landed on them, whereas no bee approached the negative controls. Three bees responded to Day-scent 1 (an un-diluted mixture). Twelve bees were attracted by Day-scent 2 (an un-diluted mixture) and 18 bees by the night-scent (a 100-fold diluted mixture) with these two scent mixtures being significantly more attractive than the controls ($p \leq 0.001$ for both; **Table 2**).

Pollen Analyses

From the 17 *Megalopta* bees whose pollen loads were analyzed, six (three *M. aeneicollis*, one *M. piraha*, two *M.* sp.) carried only pollen grains of *P. cupana* on their body. Four bees carried pollen of *P. cupana* as well as pollen of other species (two carried pollen of Arecaceae and two of *Croton*). No pollen was found on the seven other bees collected.

DISCUSSION

Our study shows that inflorescences of guarana emit strong scents, which do not differ in quantitative and semi-quantitative properties between the sexual phases, but do differ in semi-quantitative properties between day- and night-time. Despite linalool and (*E*)-β-ocimene being the two most abundant compounds in both time periods, the mean value for (*E*)-β-ocimene was higher at night than during the day, and the mean value for linalool was higher during the day than at night. Both synthetic day and night scents attracted nocturnal *Megalopta* bees of different species.

Linalool and (*E*)-β-ocimene are two of the most widespread compounds among floral scents (Knudsen et al., 2006), and this is also true for plants pollinated by nocturnal visitors, such as moths or bats (Dobson, 2006). In several such plants, these two compounds are, similar to guarana, the most abundant compounds (Dobson, 2006). Interestingly, however, the scent of guarana strongly differs from that of the Myrtaceae species *Campomanesia phaea*, which is primarily pollinated by nocturnal bees, such as *Megalopta* and *Ptiloglossa* species. *C. phaea* mainly releases aliphatic and aromatic compounds (e.g., 1-octanol, 2-phenylethanol; Cordeiro et al., 2017) showing that flowers attracting nocturnal bees may have quite different scents.

Our data show that pistillate and staminate flowers release the same scents. Because the bees (females and males) indiscriminately visit pistillate and staminate flowers of guarana exclusively to gather nectar (Krug, unpublished data), this seems to be a good strategy for the plant in order to equally attract the pollinators to flowers of both sexes (Tollsten and Knudsen, 1992). In the bioassays, 33 individuals of nocturnal bees were attracted to the synthetic floral scent mixtures, all *Megalopta*. These bees are the first visitors to arrive at guarana flowers after they open, and efficiently vector pollen (Krug et al., unpublished data). Curiously, *Ptiloglossa lucernarum*, another nocturnal/crepuscular bee which commonly visits guarana flowers (Krug et al., 2015) and was observed visiting flowers during our bioassays, was not attracted to the bait. This suggests that our synthetic mixtures were lacking components needed to attract *Ptiloglossa* bees or that visual cues were missing. This result contrasts with findings of Cordeiro et al. (2017), in which *Ptiloglossa* but no *Megalopta* bees were attracted to synthetic scents of *C. phaea*.

Of the four *Megalopta* species identified among the attracted individuals, two (*M. aeineicollis*, *M. sodalis*) are known visitors of guarana flowers (Krug et al., 2015). Moreover, our pollen analyses show that several of the attracted bee individuals in our bioassays had visited flowers of guarana before, suggesting that the compounds used in the experiments are key signals for attracting *Megalopta* bees to guarana flowers. The bees responded to different compositions and concentrations of compounds, pointing toward an olfactory circuit that is not highly specialized. Indeed, *Megalopta* bees are known to be generalists and visit a large number of plant species from various families as demonstrated by analyses of pollen grains from brood cells in nests of two *Megalopta* species in Panama (Wcislo et al., 2004). Along these lines, some of the bees collected in present study carried pollen from multiple families. We speculate that, like pollen-collecting diurnal bees (Dobson, 2006), nocturnal bees function as pollen dispersers of a diverse group of plants. However, we urgently need more data on nocturnal bee and plant interactions, as well as the mechanisms of communication between nocturnal bees and their host plants to better understand the biology and ecology of this group of insects. Future studies should also determine the relative contribution of diurnal and nocturnal bee visitors to fruit set in guarana, and the importance of the diurnal floral scents of this plant for attracting the diurnal visitors. Given that flowers release high amounts of linalool, a compound known to be an attractant for various diurnal bees (e.g., Dötterl and Vereecken, 2010) during the day, it is very likely that floral scents also play a key role in the attraction of diurnal pollinators to guarana flowers. Thus, available data suggest that the chemical communication between this plant and its pollinators is a key component in crop production of this economically important plant species.

AUTHOR CONTRIBUTIONS

CK, GC, RO, CS, SD, and IAS collected the data and made the biotests in the field. GC, IS, and SD analyzed the chemical data.

CIS analyzed the pollen samples. CK, GC, SD, and IAS wrote the manuscript. All the authors improved the manuscript with comments.

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Divergent Secondary Metabolites and Habitat Filtering Both Contribute to Tree Species Coexistence in the Peruvian Amazon

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Little is known about the mechanisms promoting or limiting the coexistence of functionally divergent species in hyperdiverse tropical tree genera. Density-dependent enemy attacks have been proposed to be a major driver for the local coexistence of chemically divergent congeneric species. At the same time, we expect local soil conditions to favor the coexistence of species sharing similar functional traits related to resource use strategies, while environmental heterogeneity would promote the diversity of these traits at both local and large spatial scales. To test how these traits mediate species coexistence, we used functional trait data for 29 species from the tree genus *Protium* (Burseraceae), collected in 19 plots (2 ha each) in the Peruvian Amazon. We characterized the presence-absence of 189 plant secondary metabolites (SM) for 27 of these species, and 14 functional traits associated with resource use strategies (RUT) for 16 species. Based on these data, we found that SM were significantly more dissimilar than null expectations for species co-occurring within plots, whereas RUT were significantly more similar. These results were consistent with the hypothesis that density-dependent enemy attacks contribute to the local coexistence of congeneric species displaying divergent chemical defenses, whereas local habitat conditions filter species with similar RUT. Using measurements of nine soil properties in each plot, we also found a significant turnover of RUT traits with increasing dissimilarity of soil texture and nutrient availabilities, providing support for the hypothesis that soil heterogeneity maintains functional diversity at larger spatial scales (from 500 m up to ca. 200 km) in *Protium* communities. Our study provides new evidence suggesting that density-dependent enemy attacks and soil heterogeneity both contribute to maintaining high species richness in diverse tropical forests.

Keywords: competitive exclusion, natural enemies, plant community assembly, resource use traits, secondary metabolites, *Protium* (Burseraceae), tropical forest diversity

INTRODUCTION

Local floras in tropical rainforests often contain hundreds of tree species, including genera comprising dozens of species coexisting within the same habitats (Latham and Ricklefs, 1993; Valencia et al., 1994; Vásquez, 1997; Ribeiro et al., 1999; Wright, 2002). The coexistence of so many closely related species poses a considerable challenge to ecological theory, which predicts that, to reduce the effects of negative species interactions, each species is likely to occupy a unique niche (Hutchinson, 1957). Some researchers have speculated that closely related species are functionally similar and thus expected to share similar habitats (Harvey and Pagel, 1991). Hubbell (2005) proposed a hypothesis that consider all species as functionally equivalent (regardless of their phylogenetic relationships) and suggested that their coexistence can be explained by neutral processes and recruitment limitation rather than niche partitioning (Hubbell, 2001). Alternatively, other studies have suggested that local species coexistence is maintained by microhabitat partitioning and density-dependent processes (Janzen, 1970; Connell, 1971; Huston, 1994; Wright, 2002). For example, density-dependent attack from natural enemies (herbivores and pathogens) is a well-known mechanism that promotes coexistence at local scales (reviewed by Comita et al., 2014). If co-occurring plants share many natural enemies, they will suffer from greater density dependent attack than if they have fewer shared enemies, a process that is sometimes called "competition for enemy-free space" (Holt and Lawton, 1994). The fundamental mechanics of this process can be linked to well-known ecological end evolutionary theories like the Janzen-Connell hypothesis (Janzen, 1970; Connell, 1971) or the Resource Concentration hypothesis (Root, 1973). Among close relatives, density-dependent attacks should be more severe because the same species of insect herbivores and fungal pathogens are more likely to be shared within closely related taxa (Novotny et al., 2002; Gilbert and Webb, 2007).

Given that plant chemical defenses can mediate attacks from natural enemies, they represent important plant traits that could determine the abundance and distribution of plant species. Moreover, secondary metabolites (hereafter: SM) can represent 30–50% of a plant's dry weight (Lokvam and Kursar, 2005), emphasizing the potential importance of selective pressures experienced by plants in response to natural enemies. Indeed, most species express dozens of metabolites that function to defend against natural enemies, and these metabolites exhibit important quantitative and qualitative variation across species (Agrawal and Weber, 2015). Because the number of shared enemies of host plants is likely to increase with the plants' chemical similarity, it is expected that coexisting plants with shared enemies will undergo diversifying selection in chemical defenses (Ehrlich and Raven, 1964; Sedio and Ostling, 2013). As a result, at the local scale, the probability of coexistence of closely related plant species is predicted to be related to their divergence in chemical defense traits. This prediction has been supported by several studies carried out on different communities of congeneric species, for instance with the genera *Bursera* (Becerra, 2007), *Inga* (Kursar et al., 2009; Coley et al., 2018), and *Piper* (Salazar et al., 2016ab).

Contrastingly, habitat filtering can counter-balance the coexistence of functionally divergent species and instead select for similar functional traits adapted to local environmental conditions (Kraft et al., 2008; Maire et al., 2012). The traits that are generally used as proxies to investigate the effect of habitat filtering relate to plant biomechanical (e.g., wood specific gravity, bark thickness) or resource acquisition strategies (e.g., specific leaf area, leaf nitrogen content, etc., but see Violle et al., 2007). Habitat filtering can also contribute to species coexistence in highly heterogeneous environmental conditions across spatial scales. Indeed, many studies have emphasized turnover in tree species composition along environmental gradients in tropical forests (e.g., Condit et al., 2013; Baldeck et al., 2016; Vleminckx et al., 2017). Niche theory would predict that turnover in composition would be reflected by turnover in traits associated with different resource use strategies. For example, nutrient-poor white-sand forests select for species with slow growth rates, while nutrient-rich clay forests select for species with fast growth rates, suggesting that functional trait composition should correlate well with soil fertility (Fine et al., 2006). Indeed, Fortunel et al. (2014) demonstrated large-scale community-level functional turnover for 15 traits related to leaf and wood properties reflecting strategies of resource acquisition across a strong edaphic gradient in lowland Amazonian forests in both Peru and French Guiana.

Ideally, studies examining trait variation across environmental gradients would focus on monophyletic groups in which species pairs share a common evolutionary history even though they differ in key traits. Nevertheless, very few studies have investigated both SM and other functional traits within lineages to assess how different traits may contribute to local coexistence and habitat preference (Fine et al., 2013; Endara et al., 2015). An ideal study system to investigate the role of different functional traits in maintaining or limiting species coexistence is the tropical tree genus *Protium* (Burseraceae). This genus, now including the former genera *Tetragastris* and *Crepidospermum*, contains more than 150 published species, with most of them found in Amazonia (Fine et al., 2014; Daly and Fine, 2018). The alpha diversity of Amazonian *Protium* can be extraordinarily high: more than 29 species have been found in a network of 67 0.1 ha plots in the Allpahuayo-Mishana Reserve near Iquitos, Peru (Fine et al., 2005), more than 24 species exist in 25 ha of Amazonian Ecuador (Valencia et al., 2004), and 35 species co-occur in 50 ha north of Manaus, Brazil (Rankin-de-Morona et al., 1992). Moreover, as a member of the frankincense and myrrh family, *Protium* is well known for its chemical defenses, including substantial investment in both terpenes and phenolics (Fine et al., 2013; Salazar et al., 2018).

Here we combine unique datasets on SM (chemical defense traits) and resource use traits (hereafter: RUT) for 29 *Protium* species, together with detailed measures of soil properties, across a network of 19 plots located on contrasting edaphic habitats in the Peruvian Amazon. We integrate these datasets to test the following hypotheses, considering two different spatial scales:

Hypothesis 1: If density-dependent attack by natural enemies promotes coexistence of species that have divergent chemical defenses, we should observe a higher dissimilarity of SM than expected by chance among co-occurring *Protium* species.

Hypothesis 2: Given that local soil conditions are likely to select for *Protium* species with a limited range of values for RUT, we should observe a lower dissimilarity than expected by chance for these traits within plots. Among plots, we predict that turnover in soil properties would be correlated with turnover in RUT.

MATERIALS AND METHODS

Sampling Locations and Species Richness

Between 2008 and 2010, we established 38 permanent plots in the Peruvian Amazon in three river basins: the Nanay, the Morona, and the Ucayali/Tapiche/Blanco (see Supplementary Figure S1, and Baraloto et al., 2011 for more details). These areas have similar climatic conditions and are covered by lowland tropical forest (<500 m a.m.s.l.). Within each area, we selected sites within which three broad habitat classes were found: white-sand forests, seasonally flooded forests, and terra firme forests on clay-rich soils. At each site we established two to six sample plots within different forest stands corresponding to each habitat, with at least 500 m between any two plots. All stands correspond to lowland mature forest with natural gap phase dynamics. Our plot sample method has been called "modified Gentry plots" (Phillips et al., 2003; Baraloto et al., 2013), with each plot corresponding to ten 10 m × 50 m transects that were established within a 2 ha area (see Figure 2 in Rockwell et al., 2014). All stems greater than 2.5 cm at 1.3 m height were inventoried in these transects. We also conducted additional sampling of *Protium* species across the entire 2 ha area of these plots (within or outside of the transects) to obtain one to three stems for each species observed in this genus. Voucher specimens for each collected species have been deposited in the UC herbarium (University of California, Berkeley). This protocol allowed us to be confident of the total number of co-occurring species of *Protium* within each 2 ha area; however, we did not conduct precise exhaustive counts of the number of individuals for each species in each plot, so these data are only appropriate for analyses using species presence–absence.

We tallied a total of 37 species of the genus *Protium* (Burseraceae) in the 38 plots. However, there were only 19 plots, located on white-sand (n = 9) and clay terra firme (n = 10), in which at least two species were present and for which data on both SM and RUT were available (see next sections). Supplementary Table S1 presents the list of all the *Protium* species (29 in total) present in these 19 plots, indicating species for which SM and/or RUT data were available. Six species were present in at least 10 plots (maximum 13 plots): *Protium apiculatum*, *P. calanense*, *P. calendulinum*, *P. crassipetalum*, *P. opacum,* and *P. paniculatum*. Four species were present in two plots only: *Crepidospermum goudotianum*, *P. grandifolium*, *P.* sp. nov. *G5* (Salazar et al., 2018) and *P. urophylliudium* (see Supplementary Table S1 for details).

Soil Analyses

Soils were characterized in white-sand and clay terra firme plots. We analyzed the soil texture (percentages of sand, silt, and clay), the amount of three bioavailable cations (Ca, Mg, and K), C/N ratio, the available phosphorus and the nitrate content. The variables were measured using ten bulked 0–15 cm depth soil cores, one collected in each of the ten transects of each plot. The ten soil cores were combined into a single 500 g sample that was dried at 25°C to constant mass, and sieved to 2 mm. Samples were shipped within 3 months for physical and chemical analyses at the University of California, Davis DANR laboratory (for full details on laboratory protocols, see Baraloto et al., 2011). The edaphic heterogeneity among plots was decomposed using a principal component analysis (hereafter, "PCA_{soil}"), after standardizing and normalizing all variables. Prior to the analysis, we removed variables that were overly collinear with others, based on their variance inflation factor (VIF, Lin et al., 2011). Variables having a VIF > 10 were eliminated (Kutner et al., 2004). The latter procedure generated a soil dataset composed of six remaining variables (P, NO_3^-, C/N, K, Ca and the percentage of silt). The PCA_{soil} revealed a single axis (explaining 43.1% of the overall soil inertia among plots) for which the observed eigenvalue was higher than the one obtained from a broken stick distribution (Cangelosi and Goriely, 2007). Axis 1 scores were significantly correlated to all soil variables except the percentage of silt (Supplementary Table S2). The biplot presenting the projection of the plot scores on axes 1–2 of the PCA_{soil} is shown in Supplementary Figure S2.

Secondary Metabolites

To test how *Protium* community assembly was related to the SM of *Protium* species, we used a new dataset representing an exhaustive characterization of SM for each *Protium* species, using GC/MS for volatile, low-molecular weight compounds and HPLC-MS/ELSD for non-volatile, high-molecular weight compounds (see Appendix S1 and Salazar et al., 2018). Each species was sampled using leaves from six to ten juvenile individuals in the Allpahuayo-Mishana Reserve near Iquitos, Peru (the same Reserve as for two of the plots in this study). For 14 of the species, we also sampled the chemistry of six to ten juvenile individuals from the Ducke Reserve and the Campinas Reserves near Manaus, Brazil (2500 km distant). We found very small intraspecific variation in leaf chemistry in Iquitos and Manaus. Although the relative abundance of some SM changed slightly across sites, all species maintained extremely high qualitative consistency in their chemical composition within species, a pattern also found for other tropical tree genera in a recent study by Endara et al. (2018). Even when 2500 km distant, populations of the same species shared over 95% of SM (Supplementary Figure S3). Nevertheless, it is important to note that our secondary metabolite data for this paper were collected from juvenile *Protium* trees located in a single forest (the Allpahuayo-Mishana National Reserve near Iquitos Peru), and we assume that the shared chemicals that we found in

individuals from Allpahuayo-Mishana apply to all individuals of those species across our plot network.

Previous studies have already emphasized high consistency of leaf chemical composition among conspecific plants in the species-rich genera *Eugenia*, *Inga*, *Ocotea* and *Psychotria* (Bixenmann et al., 2016; Wiggins et al., 2016; Sedio et al., 2017). In addition, at the community-scale, Asner et al. (2014) found high ratios of inter- to intraspecific variation for leaf chemical traits assessed from spectral data. And within the genus *Inga*, Endara et al. (2018) demonstrated the accuracy of chemical profile characterization ("chemocoding") as a tool to identify different species. All of these studies suggest that intraspecific variation in chemical composition remains much lower than interspecific variation. Thus we believe that our approach to compare community surveys using presence-absence of secondary metabolites for each species can be considered appropriate to compare chemical community composition and community similarity, especially given the costly alternative to characterize the defense chemistry of each individual plant sample (Asner et al., 2014).

In addition, for each species, we sampled both young and mature leaves including those that experienced little or no prior herbivore attack as well as those that exhibited evidence of herbivore damage. Thus, our chemical characterization dataset likely includes both constitutive and induced defenses for each species, although it is impossible from our methodology to identify which chemicals are induced and which ones are constitutive.

It is also important to note that our secondary metabolite data were collected from juvenile plants (1–2 m tall) only, while our co-occurrence data includes adult trees in addition to juveniles. We have only limited data on secondary metabolite investment from adult *Protium* trees, yet for two of the species in our dataset we found very similar chemical composition in juveniles compared with adults (Lokvam et al., 2015). Moreover, a recent analysis found highly significant positive correlations for phenolic investment between adults and juveniles for 23 species of *Protium*, suggesting that ontogenetic changes in this important class of secondary metabolites are minor in this genus (Supplementary Figure S4). Nevertheless, further studies are needed to test how chemical composition changes from the juvenile to the adult stage among all *Protium* species.

In order to relate community assembly to secondary chemical composition of *Protium* species, we therefore are making a large assumption that *Protium* chemistry does not substantially vary across the plots in our study nor with ontogeny. It is important to note that the dataset that we generated of six to ten juvenile individuals per species within one forest took over 3 years of work; thus it would be a major undertaking to conduct a complete chemical characterization of all of the individuals from all of the plots included in this study. We acknowledge that geographic, environmental, and ontogenetic variation among plots could indeed influence the secondary metabolite composition of the *Protium* species within the plots; however, we believe that it is valuable to test the species-level secondary metabolite composition with patterns of community assembly because the hypothesis of divergent secondary metabolites is compelling and such detailed secondary metabolite datasets for so many species within a tropical tree genus are so rare. Further details on the chemical extraction protocols are available in Appendix S1.

In order to test the hypothesis that divergent chemical defenses are selected among co-occurring species (hypothesis 1), we used the presence-absence of 189 secondary metabolites (SM) that have been previously characterized for 31 *Protium* species (see Salazar et al., 2018 and Appendix S1). The great majority of SM belonged to two main families of secondary compounds, terpenoids (n = 89) and phenolics (n = 97). 27 out of the 29 species present in the 19 plots had available SM data. These 27 species had, on average, 31.4 different secondary metabolites ($\pm SD$ = 12.8). Each metabolite was present in at least two species (Salazar et al., 2018). Because the great majority of herbivores feed on multiple species, we argue that shared chemicals (present in at least two species) are more relevant to testing how chemical divergence is related to coexistence at the local scale. Unique chemicals were also characterized by our methodology but are by definition only present in one species, and thus including them in our analyses would only introduce noise (even though they potentially could have anti-enemy function). We submit that shared chemicals represent a common currency for species that share natural enemies (Agrawal and Weber, 2015) and provide a more conservative estimate of species divergence than if we included all chemicals. Therefore, we compared only the presence-absence of shared SM among coexisting species to test if plants were more chemically divergent than random expectations (see next sections). Salazar et al. (2018) found that the great majority of insect herbivores that attacked *Protium* in Peru fed on more than one species of *Protium* host plant, with 11 as the average number of host plant species per insect herbivore. We do not present a PCA for the 189 chemicals since their number was so much greater than the total number of species (n = 29) (Legendre and Legendre, 2012), and because the two first PC axes did not represent more than 20% of the SM inertia (not shown).

Functional Traits Related to Resource Acquisition and Mechanical Resistance

In order to test hypothesis 2, we used data on 14 resource use traits (RUT) that have already been characterized for 16 *Protium* species (see Table 1 in Fortunel et al., 2012). Three traits relate primarily to plant mechanical resistance: bark thickness, stem and root wood density. The other traits relate to both resource capture strategies and structural defense against natural enemies. They comprise leaf chlorophyll content, leaf thickness, leaf toughness, leaf tissue density, specific leaf area (SLA), leaf area (LA), leaf contents in carbon, nitrogen, phosphorus and potassium, and leaf $\delta^{13}C$ composition. $\delta^{13}C$ in particular is used as a proxy for water use efficiency (Farquhar et al., 1989; Dawson et al., 2002). The protocol used to measure all the RUT is detailed in Fortunel et al. (2012). In order to make intraspecific measures comparable among traits, each trait was standardized (z-scores, Zar, 1999). They were then normalized, and a principal component analysis on the traits' correlation

matrix was performed in order to decompose the overall RUT inertia into few axes representing composite trait variation ("PCA_{RUT}"). As we did for soil variables, we calculated a variance inflation factor prior to the PCA_{RUT}. However, this procedure only allowed two traits to be removed and did not reduce the number of significant PC axes, while the first PC axis only explained 2% more RUT variation. We therefore retained all RUT in our analyses. Each of the first four axes (representing 26.5, 23.1, 14.1, and 10.7% of the overall RUT variation) of the PCA_{RUT} showed larger variation (eigenvalues) than expected by a broken stick model. Correlations between each trait and each of the three first PC axes are presented in Supplementary Table S3. The fourth axis was not taken into account in our tests as it was only significantly correlated with leaf carbon content (which was significantly correlated to the third axis). The PCA_{RUT} biplots representing correlations between traits and the projection of plots on axes 1–2 and 3–4 are presented in Supplementary Figure S5. There were 14 species that had both SM and RUT data available (50 and 87.5% of the species with SM and RUT data available, respectively; see Supplementary Table S1). These 14 species were present in 18 out of the 19 (95%) plots.

Testing Within-Plot Functional Dissimilarity

To test hypothesis 1 (SM are more dissimilar than expected by chance among co-occurring species), we first calculated the mean observed dissimilarity (1- Jaccard index, Zhang et al., 2016) of SM composition (presence–absence) between co-occurring species within plots. For hypothesis 2 (RUT are more similar than expected by chance among co-occurring species), we calculated the mean distance (Euclidean) of RUT among co-occurring species within plots. This latter distance was calculated for the assemblages of traits that were significantly correlated with each of the three first axes of the PCA_{RUT} (see section "Results" for details), separately. Observed distance values were compared to a distribution of expected distance values obtained by using a null model that does not assume trait divergence or convergence. In this model, we replaced, in each plot, the N observed species by N randomly sampled ones (with replacement) from the list of all species names (each species equally represented, assuming that all of them have an even chance to be present in each plot). The SM or RUT composition of each species was kept intact, thereby assuming that both trait types and have been conserved within species. We then computed the median of the difference between the observed and expected distance values among plots (n = 19). The median was preferred to the mean due to the limited number of plots (n = 19) and to lower the effect of outlying values (but we also controlled for outliers following the method used by Zuur et al., 2010). This procedure was repeated 1000 times, and a p-value was then obtained as the proportion of median values higher (if testing whether within-plot SM are more divergent than expected by chance, hypothesis 1) or lower than zero (if testing whether RUT are more convergent than expected by chance, hypothesis 2).

Testing Functional Turnover With Spatial and Soil Distance

In order to further test hypothesis 2, functional turnover (of SM and RUT) among plots was calculated for each pair of plots using the TAU_{st} statistic. The latter is exactly analogous to the Π_{st} statistic in phylogenetic turnover analyses and has been specifically designed for analyzing functional turnover using presence absence data (Hardy and Senterre, 2007). TAU_{st} is calculated as followed for each pair of plots:

$$TAU_{st} = 1 - D_w/D_a$$

Where D quantifies the mean functional distance between distinct species located within a same plot (D_w) or between distinct species among two different plots (D_a). TAU_{st} thus measures the proportion of functional diversity expressed among plots (compared to within plots). Positive TAU_{st} values indicate functional clustering (species from two different plots are more functionally distant, on average, than species from the same plot), while negative values indicate functional overdispersion (species from two different plots are functionally more similar, on average, than species from the same plot). Pairwise TAU_{st} values were plotted against spatial distance (log transformed) and soil distance (Euclidean) between plots. Soil distance was calculated using the five variables that were significantly correlated with plot scores along the first axis of the PCA_{soil}: C/N, NO_3^-, P, K, and Ca. We then tested the correlation between TAU_{st} and spatial/soil distance using a Mantel test (999 randomizations).

All statistical analyses described in the methods were performed in R statistical environment (R Development Core Team, 2017), using packages *stats* (R Development Core Team, 2017), *vegan* (Oksanen et al., 2015), *car* (Fox and Weisberg, 2011), *usdm* (Naimi, 2013), *ape* (Paradis et al., 2004), *ade4* (Dray and Dufour, 2007), and *SpacodiR* (Eastman et al., 2013). The *Protium* species presence-absence, SM, RUT and soil data as well as the R code used to test within-plot trait dissimilarity and perform turnover analyses are available in Appendices S2–S6.

RESULTS

Within-Plot Functional Dissimilarity

We found significant divergence in secondary metabolites (SM) within plots, as indicated by a significantly positive median of the difference between the observed and expected SM distance values among plots ($P < 0.001$) (**Figure 1**). Conversely, significant convergence (negative median values) within plots was observed for assemblages of resource use traits (RUT) that were significantly correlated with the first and second axes of the PCA_{RUT} ($P = 0.021$ and 0.008, respectively), but not with the third axis ($P = 0.17$). The traits from the first and second axes of the PCA_{RUT} involved every RUT except bark thickness, leaf thickness and leaf carbon content (79% of the traits, see Supplementary Table S3). More particularly, leaf area, leaf phosphorus content, leaf toughness, leaf chlorophyll content, leaf $\delta^{13}C$ and leaf thickness had the highest average correlations with the two

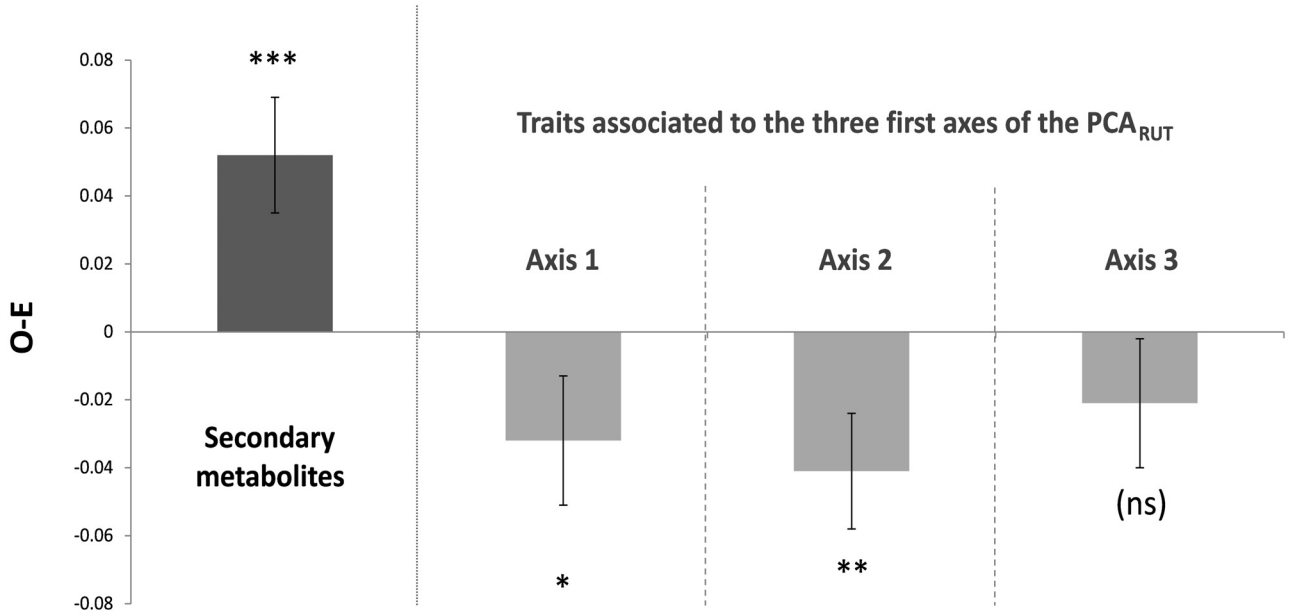

FIGURE 1 | Results of the within-plot functional dissimilarity analyses. O-E: mean (±*SD*) of the 1000 values obtained by calculating the median of the difference (among the 19 plots) between the observed and expected functional dissimilarity. Asterisks indicate whether O-E was significantly positive or negative, or neither of both (ns). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. "axis 1" means that Euclidean distances were computed using resource use traits that were significantly correlated with the first axis of the PCA_{RUT} (idem for axes 2 and 3).

first axes of the PCA_{RUT} when weighting correlations by the eigenvalues of these axes (see Supplementary Table S3).

Functional Turnover Among Plots

Significant turnover among plots was found with soil distance for RUT but not for SM. Pairwise TAU_{st} values quantifying SM turn-over were negatively but not significantly correlated with spatial and soil distance between plots (r-Pearson = −0.12 and −0.10, with P = 0.15 and 0.19, respectively, Mantel test, **Figure 2**). TAU_{st} values calculated using the assemblages of RUT that were significantly correlated with the first and second axes of the PCA_{RUT} (i.e., all traits except bark thickness, leaf thickness and leaf C content) were significantly and positively correlated with soil distance (r-Pearson = 0.41 and 0.39, respectively, with $P < 0.001$, **Figure 2**). They were, however, not significantly correlated with spatial distance between plots (r-Pearson = −0.091 and −0.085, with P = 0.25 and 0.22, respectively, **Figure 2**). No significant correlation of TAU_{st} values with soil or spatial distance was observed with the assemblages of RUT significantly associated with axes 3 and 4 of the PCA_{RUT} (not shown).

DISCUSSION

We found significant divergence in secondary metabolite (SM) composition, but convergence in resource use traits (RUT) for co-occurring *Protium* tree species at local scales. These results were consistent with our hypotheses, which posit (i) that divergent chemical defenses are selected among co-occurring *Protium* species, possibly in response to density-dependent enemy attacks; and (ii) that habitat filtering tends to promote similarity in traits related to resource use strategies. Among plots, we found a significant turnover of resource use traits with dissimilarities in soil texture and nutrient availabilities, providing support for the hypothesis that soil heterogeneity maintains functional diversity patterns in *Protium* at larger spatial scales.

Local Divergence of Secondary Metabolites vs. Convergence of Resource Use Traits

Shared secondary metabolites were significantly more overdispersed than expected by chance among co-occurring *Protium* species (**Figure 1**). This is consistent with the idea that, at the neighborhood scale, species from the local pool which produce defense chemicals that are highly similar to the resident *Protium* community would suffer greater density-dependent attack than chemically divergent species (Becerra, 2007; Sedio and Ostling, 2013), thereby promoting species coexistence (Wright, 2002; Leigh et al., 2004). Other studies on the chemical diversity of tropical woody plants have reported similar results; for example in Coley et al. (2018) for *Inga* (Fabaceae), Sedio et al. (2017) for *Inga*, *Eugenia* (Myrtaceae), *Ocotea* (Lauraceae) and *Psychotria* (Rubiaceae), and in Salazar et al. (2016a,b) for *Piper* (Piperaceae). The consistency of interspecific chemical divergence among congeners in such distantly related Angiosperm families suggests that competitive exclusion mediated by natural enemies may represent a major driver of species assembly at the community level in tropical

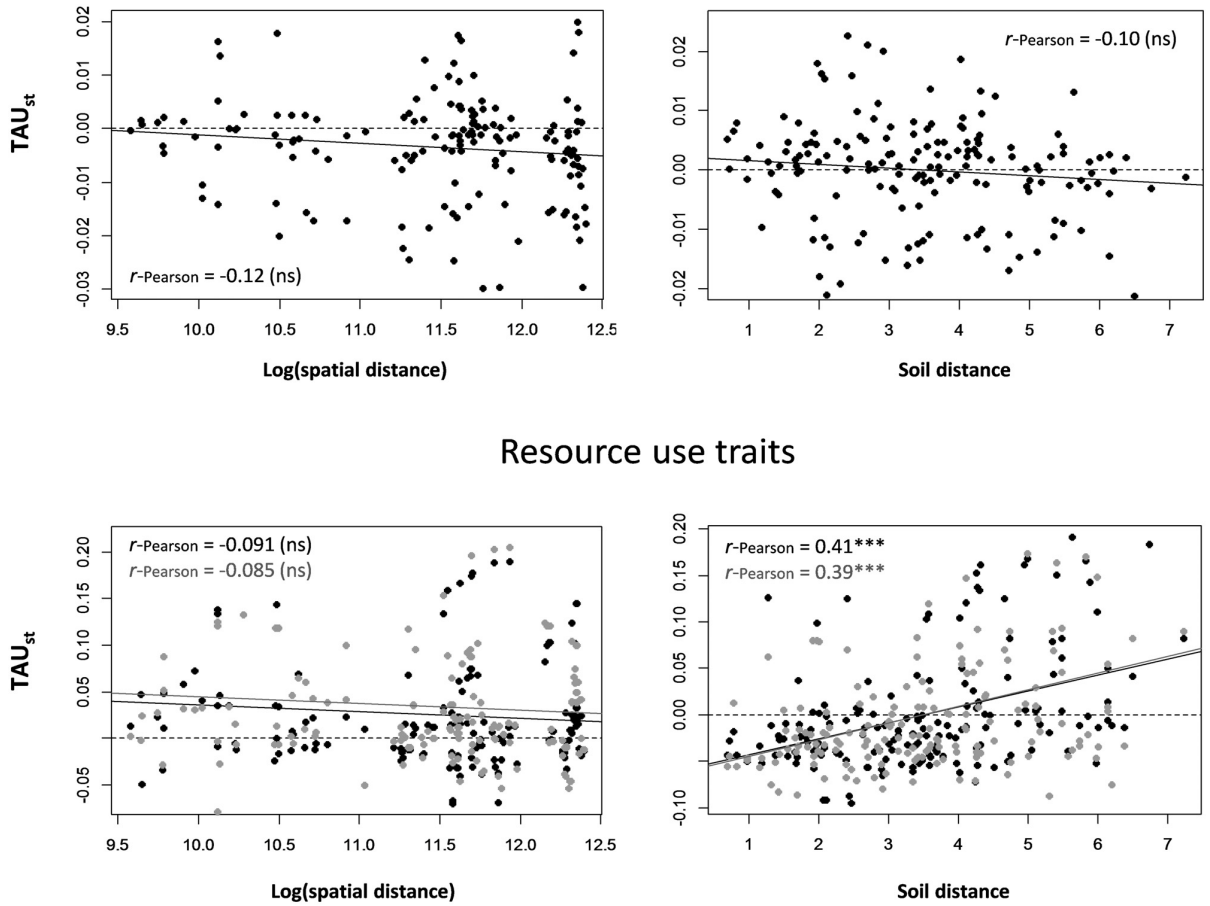

FIGURE 2 | Functional turnover among all pairs of plots (TAU_{st}), calculated for secondary metabolites and resource use traits, plotted against spatial distance (log-transformed) and soil distance (after removing spatial effect) between plots. For resource use traits, black and gray dots correspond to TAU_{st} values calculated using traits that were significantly correlated with axes 1 and 2 of the PCA_{RUT}, respectively. Correlation values between TAU_{st} and spatial/soil distance are represented in each graph. Asterisks indicate whether the Mantel test of each correlation was significant (***$P \leq 0.001$) or not (ns). Soil distance was calculated using the five soil variables (C/N, P, NO_3^-, K, Ca) that were significantly correlated with the first axis of the PCA_{soil}.

forests. Our study further confirms and expands on these results by showing, for the first time, that these processes are also found at larger geographic scales. Here, we show divergent chemical traits within *Protium* communities replicated over 19 forest plots across several hundred square kilometers.

Protium species were characterized by a convergence of RUT associated with the two first axes of the PCA_{RUT} (explaining 49% of the RUT variation among species) (**Figure 1**), suggesting that a limited range of plant strategies related to resource use and tissue protection (mechanical resistance) is selected to face the particular soil conditions occurring within each plot. Looking at the functional composition at the whole community level for 15 traits (including the 14 traits studied here), Fortunel et al. (2014) also found trait convergence within terra firme and white-sand soils, and in particular showed that white-sand communities displayed denser wood, tougher leaves and lower leaf nutrient contents than communities on terra firme soils. In a different community-level study involving 1100 woody plant species, Kraft et al. (2008) found that values of specific leaf area, leaf N content, leaf area and diameter were more evenly distributed than expected by chance for trees located within 20 m × 20 m clayey-soil plots in Ecuadorian rainforest.

Soil Heterogeneity Increases the Diversity of Resource Use Traits

In agreement with our second hypothesis, we found that the turnover of RUT associated with axes 1 and 2 of the PCA_{RUT} was significantly and positively correlated with soil distance (**Figure 2**). The latter distance was computed with all soil variables except the percentage of silt (Supplementary Table S2). The two first axes of the PCA_{RUT} were significantly explained by all traits except bark thickness, leaf thickness and leaf carbon

content. This provides additional evidence that soil filtering contributes to the sorting of functional traits related to hydric stress resistance ($\delta^{13}C$), nutrient capture strategies (leaf K, P, and N contents, chlorophyll content, leaf area and SLA), and to plant tissue resistance traits (bark thickness, stem and root wood densities, leaf toughness) in tropical forests (ter Steege et al., 2006; Kraft et al., 2008; Lebrija-Trejos et al., 2010; Paine et al., 2011; Katabuchi et al., 2012; Liu et al., 2012; Fortunel et al., 2014). However, whereas these studies compared entire tree communities, here we found similar patterns within a single genus (*Protium*). Our result therefore underlines how combinations of traits that are selected as optimal strategies have evolved repeatedly in white-sand and clay specialist species from many angiosperm lineages. It appears that the same processes have influenced speciation over a large window of time, which has resulted in both recently evolved and ancient associations to different soil types in the Amazon (Fine and Baraloto, 2016).

CONCLUSION

Our results support the idea that the local coexistence and assembly of tropical tree species in the hyperdiverse genus *Protium* is influenced by divergent selection of secondary metabolites, which is consistent with the hypothesis of density-dependent attacks by shared natural enemies. At the same time, functional traits associated with resource use strategies and tissue resistance displayed lower variation than expected by chance, showing that soil filtering promotes local trait similarity. Finally, our results suggest that the turnover of functional traits in response to edaphic heterogeneity contributes to species coexistence at the landscape and regional scale.

AUTHOR CONTRIBUTIONS

JV led the data analyses and led the writing of the manuscript with the help of PF, CF, DS, and CB. JV, DS, and PF designed the statistical protocol. CF and CB collected the resource use traits. DS, KB, and JL helped to design protocols for laboratory work and collected valuable data on *Protium* species identity (barcoding) and secondary metabolites. IM, ND, PF, and CB designed field protocols and collected valuable field data on *Protium* species occurrences in the plot network.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00836/full#supplementary-material

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Tracking of Host Defenses and Phylogeny During the Radiation of Neotropical *Inga*-Feeding Sawflies (Hymenoptera; Argidae)

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Coevolutionary theory has long predicted that the arms race between plants and herbivores is a major driver of host selection and diversification. At a local scale, plant defenses contribute significantly to the structure of herbivore assemblages and the high alpha diversity of plants in tropical rain forests. However, the general importance of plant defenses in host associations and divergence at regional scales remains unclear. Here, we examine the role of plant defensive traits and phylogeny in the evolution of host range and species divergence in leaf-feeding sawflies of the family Argidae associated with Neotropical trees in the genus *Inga* throughout the Amazon, the Guiana Shield and Panama. Our analyses show that the phylogenies of both the sawfly herbivores and their *Inga* hosts are congruent, and that sawflies radiated at approximately the same time, or more recently than their *Inga* hosts. Analyses controlling for phylogenetic effects show that the evolution of host use in the sawflies associated with *Inga* is better correlated with *Inga* chemistry than with *Inga* phylogeny, suggesting a pattern of delayed host tracking closely tied to host chemistry. Finally, phylogenetic analyses show that sister species of *Inga*-sawflies are dispersed across the Neotropics, suggesting a role for allopatric divergence and vicariance in *Inga* diversification. These results are consistent with the idea that host defensive traits play a key role not only in structuring the herbivore assemblages at a single site, but also in the processes shaping host association and species divergence at a regional scale.

Keywords: coevolution, defense traits, herbivores, host tracking, *Inga*, plant–insect interactions, sawflies, tropical rain forests

INTRODUCTION

Insect herbivores and their plant hosts dominate terrestrial biodiversity (Hunt et al., 2007), and the processes that drive their interaction and diversification remain an enduring focus of research in ecology and evolution (Futuyma and Agrawal, 2009; Janz, 2011; Hembry et al., 2014; Forbes et al., 2017; Nakadai, 2017). This is especially true in the tropics where most of the species occur.

A central paradigm is that insect-plant associations have been shaped by arms race coevolution between plant defenses and insect countermeasures (Becerra, 1997; Becerra et al., 2009; Volf et al., 2018). Ehrlich and Raven (1964) observed that closely related plants are often attacked by closely related herbivores, a pattern they attributed to an 'escape and radiate' model, in which plant lineages diversify following evolutionary innovation of a key defense trait, and specialist herbivore lineages diversify across the plant radiation through evolution of a key countermeasure (Wheat et al., 2007). Where these traits are phylogenetically conserved in each lineage, we expect some degree of phylogenetic concordance between plant and herbivore lineages, resulting either from simultaneous co-diversification (Cruaud et al., 2012), or delayed herbivore colonization of an existing plant radiation (tracking of host resources; Janz, 2011). Thus, plant defenses play a prominent role in the evolution of host associations (Thompson, 1988), yet they are often not considered in studies of plant-herbivore diversification. Robust analyses require not only phylogenetic histories of both plants and herbivores, but also data on ecologically important traits such as plant defenses.

Although insect herbivores are expected to show evolutionary conservatism in host use (Ehrlich and Raven, 1964; Brooks and McLennan, 2002), many studies show herbivore shifts between distantly related hosts that disrupt any signature of codiversification. Some shifts are between hosts with similar chemical defenses for which herbivore countermeasures are to some extent preadapted, implying a process of host-resource tracking (Janz, 2011; Endara et al., 2017) or ecological fitting (Agosta and Klemens, 2008). Insect herbivores can also radiate across hosts with contrasting defensive traits through diversification of specialist host races, leading to ecological speciation (Nyman, 2010; Hardy and Otto, 2014). These alternative mechanisms of divergence without codiversification do not happen in isolation, and their impacts are expected to reflect the distributions of interacting lineages through time and space (Hoberg and Brooks, 2008; Züst et al., 2012; Calatayud et al., 2016). Assessing the contribution of these alternative mechanisms to observed patterns of interaction and diversity is thus a major challenge (Hembry et al., 2014; Russo et al., 2017). In particular, and with notable exceptions (e.g., Kursar et al., 2009; Wilson et al., 2012; Fine et al., 2013; Marquis et al., 2016; Salazar et al., 2016; Endara et al., 2017; Volf et al., 2018), we know little about the processes driving plant-herbivore diversification in the tropical rainforest areas that harbor most of terrestrial biodiversity (López-Carretero et al., 2018).

Here we explore the factors structuring associations between insect herbivores and neotropical trees in the genus *Inga,* a species-rich radiation that shows high local species richness and abundance in many habitats across the Neotropics, and which is characterized by high diversity of chemical, physical and developmental defenses against insect herbivores (Kursar et al., 2009). Previous analyses support a key role for *Inga* defensive chemistry in structuring lepidopteran herbivore assemblages at a single site (Endara et al., 2017), and non-random combinations of defensive traits across sites imply a role for herbivore avoidance in *Inga* community assembly (Kursar et al., 2009). It remains unclear, however, whether the same *Inga* traits structure herbivore associations in widely separated communities. Previous analyses have also found little phylogenetic pattern in *Inga* defenses (Kursar et al., 2009; Endara et al., 2015, 2017), and related lepidopteran herbivores attack *Inga*s with similar defenses, rather than those that are closely related (Endara et al., 2017). Our hypothesis is that herbivores have driven rapid diversification of defensive traits in *Inga*, with herbivore associations resulting from evolutionary tracking of similar defensive phenotypes (i.e., host-resource tracking) rather than cospeciation (Coley et al., 2018). Here we test this hypothesis using data for four regional communities that span the Amazon Basin, in Panama, Peru, Ecuador, and French Guiana. We focus on sawflies (Hymenoptera; Symphyta) in the superfamily Tenthredinoidea, shown in previous work in other regions of the world to be highly sensitive to (and often dependent on) toxic host plant chemistry (Petre et al., 2007; Boevé et al., 2013; Naya et al., 2016). Thus, they are an excellent candidate taxon in which to explore the impact of diversification in this key aspect of *Inga* defenses. We use novel data on *Inga*-sawfly associations and an analytical approach incorporating phylogenies for both lineages (Hadfield et al., 2014) and defense trait data to address the following questions: (i) Is there phylogenetic patterning in *Inga* defenses? (ii) Does *Inga* phylogeny (cospeciation) or defenses (resource tracking) best predict *Inga*-sawfly associations? (iii) Over what geographic scale have *Inga*-sawfly associations evolved? Are sister sawfly or *Inga* species commonly members of the same regional community, implying local, sympatric diversification? Or are sister taxa dispersed across the Neotropics, suggesting a role for allopatric divergence and vicariance in one or both trophic levels?

MATERIALS AND METHODS

Sampling and Quantification of *Inga* Defensive Traits

We sampled 81 *Inga* species and 3 *Zygia* species (a sister clade of *Inga*) at four sites throughout the Amazon and Panama between July 2010 and September 2014: Panama (January-February 2010; Smithsonian Tropical Research Institute on Barro Colorado Island, 9.150°N, 79.850°W), French Guiana (July–August 2011 and 2012; Nouragues Station, 4.08°N, 52.683°W) Peru (July–October 2010 and 2011; Los Amigos Biological Station, Madre de Dios, 12.567°S, 70.100W) and Ecuador (July–September 2013 and 2014; Tiputini Biodiversity Station, 0.638°S. 76.150°W). In each location, we sampled expanding leaves of 0.5–4 m tall understory saplings. Host associations were recorded on c. 60 young leaf flushes per tree species. Sawfly larvae were found on 34 *Inga* and 2 *Zygia* species comprising from 1 to many gregarious larvae on a specific individual host plant (**Supplementary Table S2**).

We measured multiple defensive traits that capture the entire defensive profile of each species. These include developmental defenses (leaf expansion rate and chlorophyll content), biotic defenses (mean number of ants visiting the leaves and extra-floral nectary size) and chemical defenses. This set of defense traits was measured only on expanding leaves because more than 80% of

the damage accrued during the leaf's lifetime happens during the short period (1–3 weeks) of leaf expansion (Coley et al., 2018).

Developmental Defenses

Young leaves can expand rapidly, which shortens the window of vulnerability to herbivores, and they can delay chloroplast development, which reduces the impact of a given amount of damage (Kursar and Coley, 1992). Leaf expansion rate was determined as the percent increase in area per day for c. 13 individuals per species. Chloroplast development was measured as the chlorophyll content (mg dm^{-2}) of leaves between 30 and 80% of full expansion for c. 30 individuals per species (Endara et al., 2017). Since these two traits are correlated, we treat them as a single defense.

Biotic Defenses

Inga leaves have extra-floral nectaries that produce nectar and attract protective ants only during the short period of leaf expansion. We quantified the diameter of these nectaries and the abundance of ants visiting them (# of ants per nectary) in c. 30 individuals per species.

Chemical Defenses

For chemical analyses, expanding leaves were dried in the field over silica at ambient temperature. Although *Inga* has little quantitative or qualitative induction of young leaf defenses (Bixenmann et al., 2016), samples used for chemical analyses were from plants without sawflies. The chemical defensive profile for each species was determined using metabolomics. Metabolites were extracted at the Coley/Kursar laboratory in the University of Utah in 44.3 mmol L^{-1} ammonium acetate, pH 4.8:acetonitrile (60:40, v/v) and analyzed following the protocol of Wiggins et al. (2016). Metabolites with intermediate polarity were analyzed by ultraperformance C18 liquid chromatography coupled to mass spectrometry (UPLC-MS) in negative mode. Raw data from the UPLC-MS analysis in MassLynx were converted to mzXML format using mzConvert (Chambers et al., 2012) and then processed for peak detection, peak alignment and peak filtering using the R package XCMS (Smith et al., 2006; Tautenhahn et al., 2008; Benton et al., 2010). These results were post-processed in the R package CAMERA to assign the various ions derived from one compound (termed 'features') to that compound (Kuhl et al., 2012), as detailed in **Appendix SII**. This analysis yields 2621 compounds from the 36 plant species. Purification and structure determination by 2-D NMR of several dozen compounds, as well as matching MS-MS spectra from our in-house database to the GNPS databases (Global Natural Products Social Molecular Networking)[1] suggest that, for *Inga,* these compounds are mainly phenolics, saponins and amines. None are primary metabolites (**Supplementary Table S1**). All scripts from this study are deposited in github[2].

Overexpression of the essential amino acid, L-tyrosine, ranges from 5 to 20% leaf DW (Dry Weight) in certain species of *Inga.* At these concentrations, it is highly toxic to non-adapted herbivores, and therefore functions as an important chemical defense (Lokvam et al., 2006). Because tyrosine is insoluble in our extraction buffer, tyrosine concentration as percent of leaf dry weight was determined separately following Lokvam et al. (2006, **Appendix SII**).

Sawfly DNA Barcoding

Taxonomic resources are limited even for adult sawflies (Schmidt et al., 2017), and very few exist for morphological identification of neotropical sawfly larvae to species. We therefore adopted a DNA barcoding approach using sequences for a 645 base pair (bp) fragment of the mitochondrial gene cytochrome oxidase I (*COI*) (For DNA methods, see **Appendix SI**). Every individual was barcoded. For gregarious species, we sequenced a minimum of three in a group and in all cases these belonged to the same MOTU. Sequences were allocated to MOTUs (molecular operational taxonomic units) using two approaches: jMOTU v1.0.8 (Jones et al., 2011) and ABGD (Automatic Barcode Gap Discovery, Puillandre et al., 2012). jMOTU clusters sequences into MOTUs that differ by pre-defined numbers of bases; we examined divergence distances amongst sequences ranging from 1 to 65 bp, with a low BLAST identity filter of 97%. In the presence of a barcoding gap (Puillandre et al., 2012), a plot showing numbers of MOTUs as a function of sequences divergence should form a plateau, with no change in MOTU number across the divergence levels corresponding to the gap (Acs et al., 2010). ABGD defines MOTUs based upon prior values of within-species divergence, and assesses how MOTU number changes as within-species divergence increases. We used prior within-species divergence limits ranging from 0.3 to 6%, split into 30 steps. We used the K2P distance measure, with a transition to transversion ratio of 1.47, as estimated by jModeltest v2.1.7 (Darriba et al., 2012), and the default value of 1.5 for slope increase. Output from the recursive partitioning scheme was used, with the final number of MOTUs chosen at the point where the plot of MOTU versus intraspecific divergence leveled off. Both approaches gave highly concordant results.

Because mitochondrial haplotypes can be shared among species, and hence give misleading indications of species membership in sawflies (Prous et al., 2011; Schmidt et al., 2017) and more widely (Funk and Omland, 2003; Nicholls et al., 2012), we sequenced our candidate *COI* MOTUs for two nuclear loci, *wingless* (coding, 327 bp; n = 75 sequences) and *ITS2* (non-coding, 609 bp; n = 80; for molecular methods, see **Appendix SI**). Sampling incorporated all singleton MOTUs and 2–4 individuals of MOTUs with more extensive sampling. *COI* sequence data were highly effective in resolving relationships between sawfly samples with high posterior probability (**Supplementary Figure S1**). The first barcoding gap using jMOTU was apparently at 7–10 bp (1–1.5% divergence), identifying 42 MOTUs. These were highly concordant with 40 MOTUs identified by ABGD for sequence divergence from 1.03 to 1.41% (**Supplementary Figure S2**), the only difference being that jMOTU split two of the 40 ABGD MOTUs into two. Relationships between MOTUs identified using *COI* data were highly concordant with those based on nuclear *ITS2* and *wingless* (**Supplementary Figures S3, S4**). Our final sawfly MOTU definitions (n = 41) incorporated information from both mitochondrial and nuclear data, with

[1] https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp

[2] https://github.com/ColeyKursarLab/endara_sawflies_2018

COI MOTUs retained if at last one nuclear gene showed the same clustering of individuals. Sawfly MOTUs were allocated to candidate taxonomic families by querying each against voucher sequences in the Barcoding of Life BOLDSYSTEMS database[3].

Gene trees for each of the three loci were generated using MrBayes *v*3.2.2 (Ronquist et al., 2012). Based on relative numbers of variable sites at each codon position, *wingless* was treated as a single partition while *COI* was partitioned between codon positions 1 + 2, and position 3. As a non-coding locus, *ITS2* was treated as a single partition. We used the closest available substitution model in MrBayes as per the recommendation provided by *jModeltest* (Guindon and Gascuel, 2003; Darriba et al., 2012), as follows: *COI*(1,2), GTR+I+G; *COI*(3), GTR+G; *wingless*, GTR+I+G; *ITS2*, GTR+G. MrBayes analyses were run for 20 million generations for *ITS2* and *wingless*, sampling every 2500 generations, with a burn-in of 16 million generations. The analysis for *COI* was run for 40 million generations to achieve convergence, sampling every 5000 generations, with a burn-in of 32 million generations. Likelihood comparisons showed a relaxed IGR clock model to be better supported than either no clock or a strict clock for all loci.

Phylogenetic Relationships Among Sawfly MOTUs

We determined phylogenetic relationships for our MOTUs at two levels. To place our *Inga*-feeding sawfly MOTUs in a wider phylogenetic context, we carried out additional phylogenetic analyses using data for *COI* and an additional coding nuclear locus, *PGD* (496 bp). *PGD* data provided high resolution in a previous wide-ranging phylogenetic analysis of sawflies (Malm and Nyman, 2015), and allow us to place our MOTUs within this taxonomic framework. Analyses for each gene incorporated data on sawfly species from recent phylogenetic (Schulmeister et al., 2002; Malm and Nyman, 2015) and barcoding (Hartsough et al., 2007; Schmidt et al., 2017) surveys of sawflies. These analyses identified related taxa on the basis of nearest matches identified from BOLD. The taxa from the surveys that we added to our analysis comprised 11 species in the family Tenthredinidae, 9 in the family Pergidae and 34 in the family Argidae, none of which are neotropical. We also included a similar number of sequences for neotropical taxa, and the only available *COI* voucher sequence for an *Inga*-feeding sawfly, a specimen of *Ptenos leucopoda* (Argidae) sampled from *Inga oerstediana* (and also recorded from *I. vera*) in Costa Rica (Smith et al., 2013). Metadata and Genbank accession numbers for these reference sequences are provided in **Supplementary Table S3**. We constructed gene trees for each locus using MrBayes, using the closest available substitution model to that identified as appropriate using *jModeltest* (Guindon and Gascuel, 2003; Nylander, 2004; Darriba et al., 2012). Based on relative numbers of variable sites at each codon position, *PGD* data were modeled in two partitions, 1 + 2, and 3, each with a GTR+I+G model, while *COI* was divided into three partitions by codon, each with a GTR+I+G model. For each gene we assumed a relaxed clock, with a birth-death speciation model. To provide an order of magnitude age for *Inga*-associated sawfly lineages, we calibrated the *COI* tree using two alternative estimates: the Brower rate estimate of 0.0115 substitutions per million years (Brower, 1994) and the higher rate of 0.0177 derived by Papadopoulou et al. (2010).

For analysis of evolutionary dynamics in sawfly *Inga* trophic associations, we generated an overall species (MOTU) tree using data for all four loci (*COI, ITS2, PDG, wingless;* 2077 bp) for the 39 MOTUs identified as putative Argidae using the Bayesian *BEAST algorithm (Heled and Drummond, 2010) within BEAST v2.4.1 (Drummond et al., 2012). The *BEAST model used 5 partitions with the following substitution models: *COI* (codon positions 1,2), TN+I+G; *COI* (codon position 3), TN+G; *wingless,* GTR+I+G; *ITS2*, GTR+G; *PGD*, GTR+I+G. We used a Yule speciation model, and compared likelihood support for each combination of relaxed *versus* strict clock models and constant *versus* linearly changing population size. This approach supported a constant population size and an independent relaxed lognormal clock for each partition. We carried out two runs of the *BEAST analysis, with outputs combined in Logcombiner, part of the BEAST suite (Drummond et al., 2012). Each run was for 500 million generations, sampling every 62500 generations, with a burn-in of 300 million generations. Analysis of run diagnostics in Tracer v1.6 (Rambaut and Drummond, 2007) showed all parameters to have an effective sample size of > 100.

Generation of an *Inga* Species Tree

We constructed a species tree for 77 *Inga* accessions representing the taxa from which sawflies were collected, using data for ten coding nuclear loci previously identified as being phylogenetically informative in a wider study of *Inga* phylogenomics (Nicholls et al., 2015) (**Supplementary Table S4**). Aligned sequences for each locus in all *Inga* specimens are available from the Dryad Digital Repository[4]. The ten loci ranged in length from 272 to 2767 bp, with 9–14.7% of sites variable, and spanned a total of 16,125 bp (**Supplementary Table S4**). All ten loci were sequenced in all 77 *Inga* accessions. We co-estimated gene tree topologies and an overall species tree topology using *BEAST, as described above. We used the substitution model previously identified for each locus by Nicholls et al. (2015) (**Supplementary Table S4**). We specified a Yule speciation model and assumed a constant population size. We selected a relaxed lognormal clock over a strict clock model based on very high Bayes factor support (574, estimated as 2Ln harmonic mean likelihood) following criteria in Kass and Raftery (1995). Our analyses ran for 500 million generations, sampled every 62,500 generations, with a burn-in of 50 million generations. Analysis of run diagnostics in Tracer v1.6 (Rambaut and Drummond, 2007) showed all parameters to have an effective sample size of > 100.

Data Analysis

Estimation of Sampling Effort

Sawfly MOTU accumulation curves were generated in the Vegan R package using sampling over *Inga* species [specaccum(data, "random")] and sampling over sawfly individuals [specaccum(data, method = "rarefaction")]. The

[3]http://boldsystems.org

[4]doi: 10.5061/dryad.8403km4

"random" method finds the mean accumulation curve and its standard deviation from random permutations of the data. The "rarefaction" method finds the expected species richness and its standard deviation by sampling individuals instead of sites. It achieves this by applying function "rarefy" with number of individuals corresponding to average number of individuals per *Inga* species – which for our data is 1286 sawflies/34 plant taxa = 38 individuals.

Chemical Similarity Between Species of *Inga*

We analyzed data for phenolics and saponins separately. Saponins were defined as all compounds with chromatographic retention time $>$ 18 min and $m/z >$ 580 for the precursor ion, with the remainder classified as phenolics. For several *Inga* species, early eluting compounds have been purified and their structures elucidated by 2D-NMR (J. Lokvam, unpublished). This shows that the bulk of early eluting compounds are phenolics. For about 10 species, the late-eluting fraction was separated from phenolics, hydrolyzed to remove sugars, the triterpene aglycons isolated and their structures elucidated by 2D-NMR (J. Lokvam, unpublished). This work indicates that the bulk of the late-eluting compounds are saponins. Certainly, we cannot rule out that some peaks may belong to other classes. Compounds that are shared across species were matched based on m/z (mass to charge ratio) and retention time. Because many compounds, 1097 out of 2621, are found in only one species, we also quantified species similarity based on the structural similarity of unshared compounds. This matters because unshared compounds are typically treated as having zero relationship even though they may have significant structural similarity. In metabolomics, molecules can be identified based on whether the MS fragmentation pattern (MS/MS spectrum) of an unknown matches spectra in curated databases. A limitation is that these databases include few secondary metabolites, providing little opportunity to quantify the structural relatedness of similar molecules. A recent advance is to quantify the similarity of the MS/MS spectra of a large number of molecules. These data generate a network using the online workflow at the Global Natural Products Social Molecular Networking site (GNPS)[5]. In the resulting network, each node or circle represents a unique compound, with edges (lines) connecting nodes based on structural similarity. Each pair of compounds is assigned a structural similarity score ranging from 0 (completely dissimilar) to 1 (identical) based on the similarity of their MS/MS fragmentation spectra (Watrous et al., 2012). To accomplish this, we obtained as many MS/MS spectra as possible, for 1925 out of our 2621 study compounds. See **Appendix SII** for MS/MS methods and calculation of the chemical similarity of species from molecular networks.

We constructed a dendrogram of chemical similarity between species by fitting a hierarchical clustering model to the equally weighted chemical similarity matrix with 10,000 permutations using the R package PCVLUST (Suzuki and Shimodaira, 2014). For more details see **Appendix SII**.

Because there are many possible equations and data transformations for calculating species similarity scores, we compared several of these alternatives to lepidopteran dietary preferences following Endara et al. (2017). These analyses validated our method (**Appendix SII**).

Relationship Between Plant Traits and Phylogenetic Signal

Phylogenetic signal was evaluated for continuous host defensive trait data (developmental and biotic defenses), and for the principal coordinates of the chemistry similarity matrix using Blomberg's K (Blomberg et al., 2003). K is close to zero for traits lacking phylogenetic signal, but close to one for traits whose values through the phylogeny match expectations under a Brownian model of evolution. We used the function *phylosig* in the R package *phytools* v.0.6-44 (Revell, 2017).

Analysis of Herbivore–Host Plant Associations

Due to the gregarious habit of sawflies, we use incidence data (presence–absence) for analyses of host associations. Thus, if a specific MOTU was associated with a specific *Inga* host plant in several sampling events on the same plant, it would have been counted only once. To determine the extent to which host phylogeny and/or host defenses structure the associations between sawflies and their hosts, we used maximum likelihood to model the probability of sawfly occurrence *(p)* using a binomial distribution with the number of trials equal to the total number of herbivore species associated with each *Inga* species. These analyses included all *Inga* species, even those on which sawflies were never found, so that we could determine which *Inga* traits predict an association with any sawfly MOTU. We fitted models that incorporated only the intercept, and the effects of one or more *Inga* defensive traits and the principal coordinates of the phylogenetic distance matrix and the chemical similarity matrix using the R packages *bbmle* v.1.0.20 (Bolker, 2017) and *emdbook* v.1.3.9 (Bolker, 2016). For these analyses, we used the whole *Inga* phylogeny (unpublished *Inga* phylogeny, Nicholls et al., unpublished). The models were run using sampling effort as a covariate (number of leaf flushes searched per *Inga* species). We performed model comparison based on Akaike Information Criterion for small sample sizes (AICc).

Evolutionary interactions between sawflies and *Inga* hosts were determined using a Bayesian approach with generalized linear mixed-effects models (GLMM) in the R library *MCMCglmm* (Hadfield and Nakagawa, 2010; Hadfield, 2017). We performed these analyses only with those *Inga* species that are associated with sawflies. Following Hadfield et al. (2014), we partitioned variance in the sawfly incidence data per *Inga* host into the effects of the phylogenetic histories of plants and herbivores, whether in isolation (termed evolutionary effects by Hadfield et al., 2014) or as interactions (a coevolutionary effect), and chemical similarity between *Inga* hosts (a defense effect). This model approach also allows the estimation of other factors, where interactions have evolved independently of the phylogenies and *Inga* chemistry similarity. The magnitude of the effect for each term is determined by the magnitude of the variance. Following Hadfield et al. (2014), the first term in the model captures the effect of the geographic region information

[5]https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp

(here termed Geographical region). The second term determines the contribution of the main effect of the sawfly phylogeny to the covariance and captures the variation in host range explained by the phylogeny (Phylogenetic main effect for sawflies). The third term is the contribution of the main effect of *Inga* chemistry to the covariance and captures the variation in sawfly species richness explained by chemical similarity between *Inga* hosts (Defensive main effect for *Inga* hosts). The fourth term is the contribution of the main effect of *Inga* phylogeny to the covariance and captures the variation in sawfly species richness explained by the phylogeny of the *Inga* hosts (Phylogenetic main effect for *Inga* hosts). The fifth term captures the degree to which related *Inga* have similar sawfly assemblages irrespective of sawfly phylogeny (Phylogenetic *Inga* evolutionary effect). The sixth term captures the degree to which species that are similar in chemistry have similar sawfly assemblages irrespective of sawfly phylogeny (*Inga* defense interaction). The seventh term captures the degree to which related sawflies have similar *Inga* hosts assemblages irrespective of *Inga* phylogeny (Phylogenetic parasite evolutionary effect). The eighth term is the contribution of the coevolutionary interaction to the covariance and captures the degree to which related sawflies feed on related *Inga* (Coevolutionary effect). The ninth term is the contribution of the interaction between *Inga* chemistry and sawfly phylogeny and captures the degree to which related sawflies feed on *Inga* that are similar in chemistry (Defense tracking effect). The last three terms capture interspecific variation in host range (Main effect for sawflies), interspecific variation in sawfly species richness (Main effect for *Inga* hosts) and associations between specific *Inga* hosts and sawflies species (Interaction effect) not due to phylogeny or chemistry.

Phylogeny and chemistry were incorporated into the model as variance-covariance matrices of relatedness and similarity, respectively, in the random effect structure of the generalized linear mixed effect model. We compared models that included site effects (analyses at large spatial scales, as a random factor) and which controlled for sampling effort (as a fixed factor), with models that ignored between-site patterns (hence, analyses at small spatial scales) and sampling effort completely. For the analyses, parameter-expanded priors were used for all variance components following Hadfield et al. (2014). The chain was run for 500,000 iterations with a burn-in of 50,000 and a thinning interval of 450. Because the response variable was incidence data, a Bernoulli error distribution was applied. Models were fitted using the R package *MCMCglmm* v.2.23 (Hadfield, 2017).

Correlations between sawfly phylogenetic relationships with host plant phylogenetic relationships and with host plant chemistry were explored using the function *parafit* (Legendre et al., 2002) in the R package *Ape* v.5.0 (Paradis et al., 2004). We used the global test in *parafit* to test the null hypotheses that (i) the evolution of sawflies and *Inga*, as revealed by the two phylogenetic trees and their trophic associations, has been independent; and (ii) by substituting the *Inga* chemogram for the *Inga* phylogeny that sawfly diversification has been independent of host plant chemistry. Pairwise patristic distances were extracted between sawfly MOTUs from the 4-locus Argidae species tree, and between their corresponding *Inga* host plants from the 10-locus species tree and *Inga* chemogram using the *cophenetic.phylo* command in *Ape*. *Parafit* analyses used 9999 permutations. Matches between the sawfly phylogeny and each of the *Inga* phylogeny and chemogram were optimized using the function *cophylo* in the R package *phytools* (Revell, 2017).

Visualization of the *Inga*-sawfly associations in phylogenetic space was performed using a Principal Component Analysis. Using the function *phylomorphospace* in the R package *phytools* (Revell, 2017), phylogenetic relationships between sawfly MOTUs was mapped onto *Inga* phylospace. For this analysis, we use the whole *Inga* phylogeny (unpublished *Inga* phylogeny, Nicholls et al., unpublished).

RESULTS

Inga Sawflies Are a Diverse Monophyletic Radiation of Specialist Herbivores Within the Family Argidae

Our *COI* barcoding approach identified 41 MOTUs for sawflies feeding on *Inga* and *Zygia* host plants (**Supplementary Figure S1**), differing by 7–10 bp (1–1.5% divergence). Each sawfly MOTU attacked a very narrow range of 1–2 host *Inga* species, and each *Inga* species only hosted a small number of sawfly MOTUs. This pattern is consistent with the MOTU accumulation curve across sampled sawfly individuals, which suggested that adding more *Inga* taxa to the sampling would only add more specialist sawflies (e.g., sawfly MOTU accumulation curve across *Inga* species rise sharply, **Supplementary Figure S5**). In addition, because the sawfly MOTU accumulation curve across sampled sawfly individuals is asymptotic, this indicates that a more extensive sampling would not yield many additional *Inga*-sawfly interactions (**Supplementary Figure S5**). Thirty-nine MOTUs were identified by BOLD query as likely members of the family Argidae, while the remaining two were most similar to sequences for species in the family Tenthredinidae (**Supplementary Table S2**). Phylogenetic analysis showed that the 39 putative Argidae comprise a well-supported monophyletic clade within this family for the nuclear PGD locus (**Figure 1**; clade posterior probability = 1.0) and also for the more extensive taxon set sequenced for mitochondrial *COI* (**Supplementary Figure S6**). The remaining two MOTUs were placed within a strongly supported clade of voucher sequences for the family Tenthredinidae (**Figure 1**; PP = 0.99). Calibrations of the mutation rate for *CO1* estimate the median age of the common ancestor of this Argidae clade at 6.27 (95% confidence interval 4.78–7.93) million years using the Brower (1994) estimate and 5.31 (4.05–6.72) million years using the Papadopoulou et al. (2010) estimate.

Sawflies Feed on a Chemically Distinct Subset of Available *Inga* Hosts

We investigated the specific role of each host trait in predicting sawfly *Inga* associations in analyses including joint-absence information (i.e., analyses including observations where sawflies were never collected on certain species of *Inga*). We found

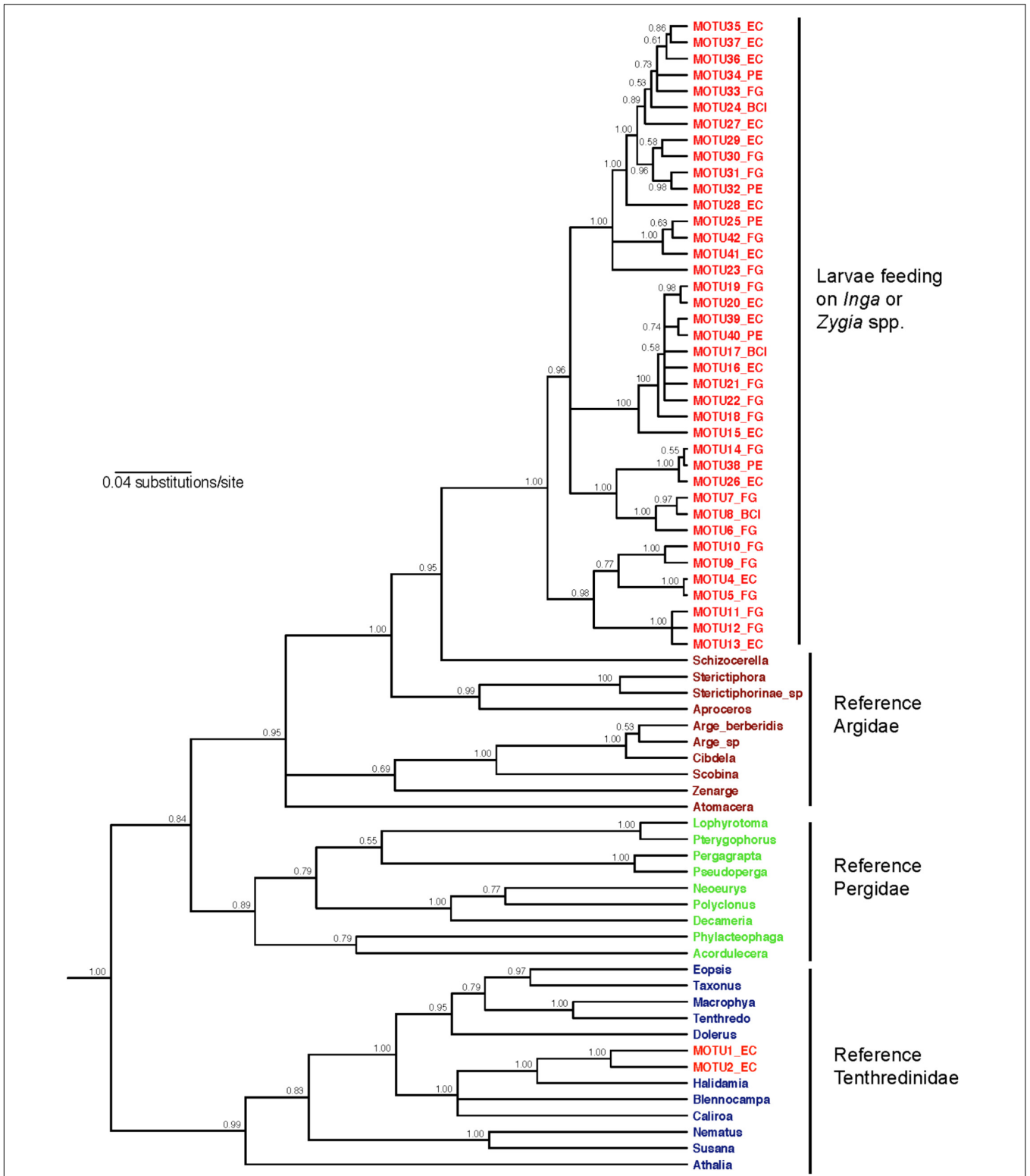

FIGURE 1 | Phylogenetic relationships for the gene *PGD* among the *Inga*-feeding sawfly MOTUs and a panel of voucher sequences for sawflies in the families Argidae, Pergidae (sister group to Argidae; Malm and Nyman, 2015) and Tenthredinidae. The tree shown is a majority-rule consensus tree constructed in MrBayes, using substitutions modeled as GTR+I+G for 1st and 2nd codon positions combined, and GTR+I+G for 3rd positions. We used a relaxed clock, with a birth-death speciation model. Numbers at nodes indicate posterior probability. Taxon labels are colored by sampling source: red MOTU numbers are larvae found feeding on *Inga* or *Zygia*, while other colors indicate reference sequences for adult Argidae, Pergidae, and Tenthredinidae.

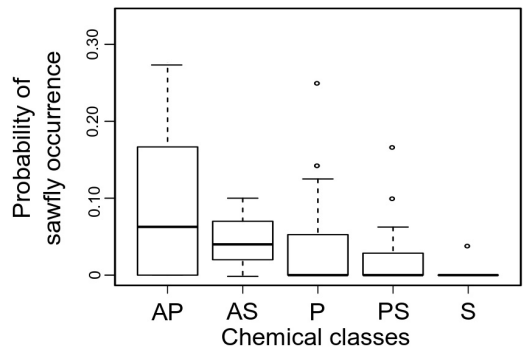

FIGURE 2 | Sawfly occurrence for each *Inga* host chemotype. Shown is the range and distribution of proportion of occurrence of sawfly MOTUs per *Inga* chemotype. Chemistry is represented by the main chemical classes found in *Inga.* AP, Amines + phenolics; AS, Amines + saponins; P, Phenolics; PS, Phenolics + saponins; S, Saponins. The box shows the median and the 25%- and 75% percentiles. The whiskers are the 1.5 × interquartile range; outliers are drawn as individual points.

that similarity in chemical defenses among *Inga* hosts was the most important predictor for the occurrence of sawflies in general [proportional odds estimate for PCO1 = 0.26, (95% CI = 1.3 – 0.04), proportional odds estimate for PCO2 = 0.13, (95% CI = 0.95 – 0.02)]. Specifically, sawflies as a group prefer hosts that are defended by amine metabolites [proportional odds estimate for the presence of amines = 1.52, 95% CI (9.89 to 0.41), **Figure 2**], while the probability of occurrence of sawflies decreases with the presence of saponins [proportional odds estimate for the presence of saponins = 0.18, 95% CI (1.99 to 0.008), **Figure 2**].

Closely Related *Inga* Hosts Fed on by Sawflies Are Similar in Chemical and Developmental Defenses

For the *Inga* that were fed upon by sawflies, we quantified chemical similarity between species based on the similarity of chemical structure and relative abundance of compounds. We found that closely related *Inga* species and geographically separated populations of the same *Inga* species tend to have similar chemical defenses. Principal coordinates of the chemistry similarity matrix showed phylogenetic signal (PCO1 K = 0.71, p = 0.0002; PCO2 K = 1, p = 0.0001, **Table 1**). For example, lineages from the *Inga capitata* species complex (**Figure 3A**, left-hand phylogeny) share a series of tyramine gallates and quinic acid gallates. Similarly, the clade containing *Inga edulis, Inga poeppigiana, Inga ruiziana* and *Inga thibaudiana* share similar chemistry based on gallocatechin/epigallocatechin gallates. However, we find examples of closely related taxa with contrasting chemistry, a typical pattern for the genus as a whole (Kursar et al., 2009). For instance, *Inga umbellifera_no_Y* in French Guiana lacks overexpression of tyrosine in expanding leaves, whereas its sister species, *I. umbellifera* from Panama, contains 10.1% of leaf dry mass as tyrosine.

TABLE 1 | Measure of phylogenetic signal for each *Inga* defensive trait and the principal coordinates of the chemistry similarity matrix (PCO) using Blomberg's *K*.

Defensive traits	*K statistic*	*P* (reps = 9999)
Chemistry PCO1 (39%)	0.71	0.0002
Chemistry PCO2 (17%)	1	0.0001
Leaf expansion rate	0.37	0.05
Chlorophyll content	0.49	0.01
Ant number	0.12	0.58
Extra-floral nectary size	0.09	0.8

For PCO components, values in parentheses represent the percentage of variation explained by each component.

Developmental defenses of *Inga* species fed on by sawflies showed a similar pattern to chemistry. Leaf expansion rate and chlorophyll content showed weak phylogenetic signal (leaf expansion rate K = 0.37, p = 0.05, chlorophyll content K = 0.49, p = 0.001). In contrast, biotic defenses were divergent among close relatives in *Inga* that are sawfly hosts, with no evidence for phylogenetic conservatism in ant visitation and extra-floral nectary size (**Table 1**).

Chemically Similar *Inga* Hosts Are Attacked by Similar Sets of Sawflies

Evolutionary interactions between sawflies and their *Inga* hosts were tested using a four-locus phylogeny for Argidae sawfly MOTUs and a ten-locus phylogeny for their *Inga* food plants. Because only chemistry was selected as an important predictor for sawfly *Inga* associations, the following analyses were performed without the other host defensive traits. Phylogenies for both groups were well resolved, with strong posterior support at many nodes (**Figure 3A**).

Parafit analysis revealed a significant signature of codiversification between these two groups (global correlation, p = 0.015). The 19 sawfly-*Inga* interactions contributing most strongly to this pattern are concentrated in two sawfly and *Inga* clades (**Figure 3A**), and include closely related sawfly MOTUs that feed on geographically separated populations of the same species of *Inga* (see highlighted links in **Figure 3A** for sawflies feeding on *Inga alba, Inga capitata, Inga laurina, Inga leiocalycina, Inga marginata,* and *Inga poeppigiana*). However, there are also multiple examples of a single sawfly MOTU that feeds on phylogenetically divergent host plants (e.g., *Inga auristellae* and *Inga umbratica* attacked by MOTU 37 in Ecuador, *Inga stipularis* and *Inga marginata* attacked by MOTU23 in French Guiana, *Inga retinocarpaFG* and *Inga bourgoniiFG* attacked by MOTU 5), and divergent *Inga* hosts attacked by closely related sawfly MOTUs (e.g., *Inga umbraticaEC* and *Inga auristellaeFG* attacked by MOTUs 11 and 13). A single *Inga* species can also be attacked by phylogenetically divergent sawfly MOTUs (e.g., *Inga marginata*FG attacked by sawfly MOTUs 7, 42 and 23, and *Inga umbratica* attacked by MOTUs 13 and 37).

There is a much stronger correlation between the sawfly phylogeny and *Inga* chemistry (global correlation, p = 0.001) (**Figure 3B**). Many of the links contributing to this pattern (18 of 25 interactions, **Supplementary Table S5**) are the same as

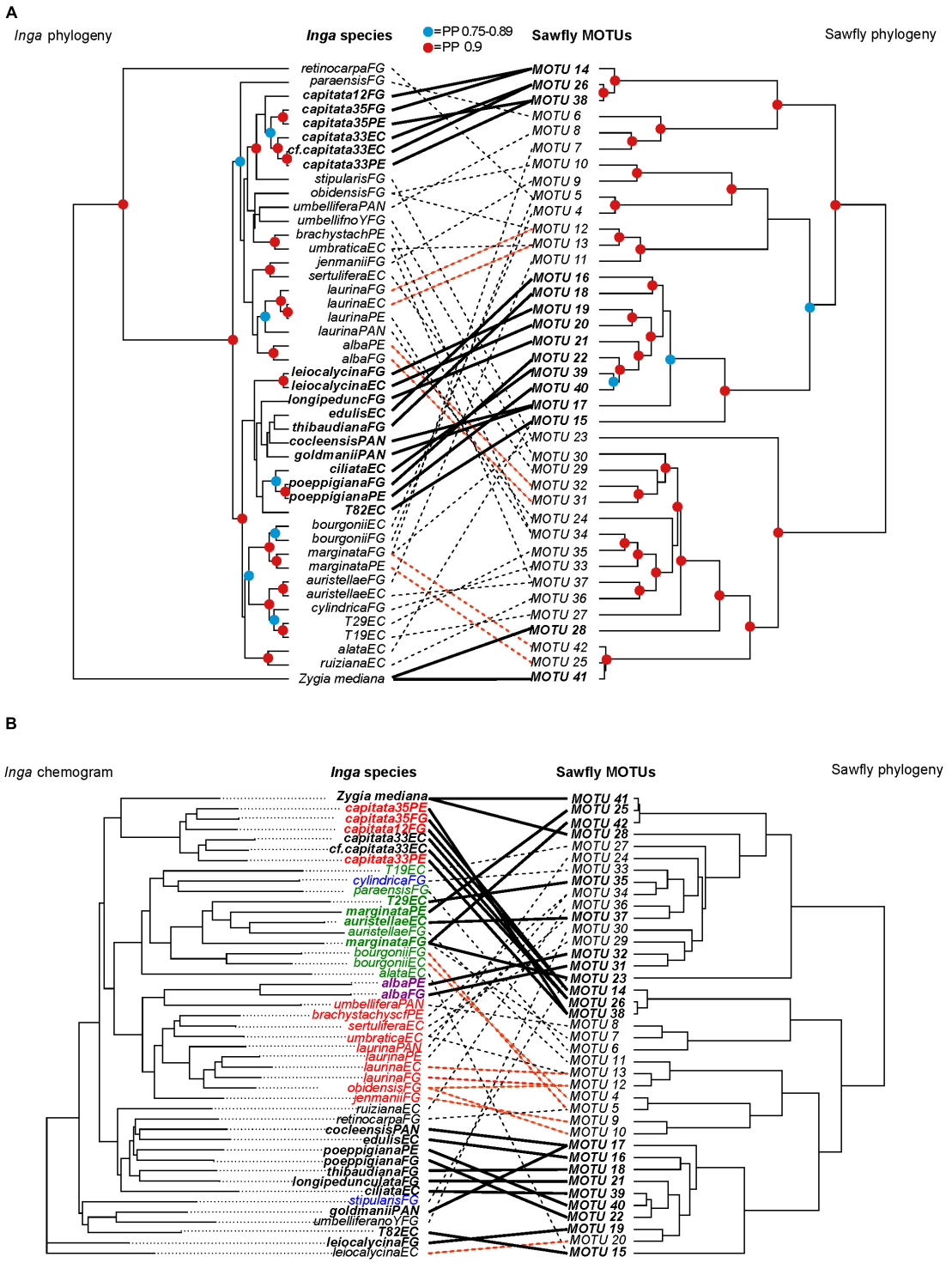

FIGURE 3 | Patterns of diversification in Argidae sawfly MOTUs, mapped against **(A)** the phylogeny of their *Inga* food plants, and **(B)** a phenogram of host chemical defenses ('chemogram'). The match between topologies in each case was optimized using the *cophylo* command in the R *Phytools* package. The sawfly and *Inga* phylogenies are maximum clade credibility species trees produced from multilocus analyses in *Beast, for four and ten loci respectively **(A,B)**. Links and taxon names highlighted in bold are identified as individually significant in *parafit* analyses (see **Supplementary Tables S4, S5**), while links highlighted in red show additional examples of closely related sawflies feeding on geographically separated populations of the same host plant species. The remaining links are indicated as dashed lines. The geographic location of *Inga* populations is indicated in the taxon labels as follows: EC, Ecuador; FG, French Guiana; PAN, Panama; PE, Peru. Colored symbols at nodes on phylogenies indicate posterior probability (PP) support: red = PP from 0.9 to 1.0, blue = PP from 0.75 to 0.89. *Inga* species in **(B)** are color-coded by chemotype: black (phenolics), red (phenolics + amines), green (phenolics + saponins), blue (saponins) and purple (saponins + amines).

TABLE 2 | Proportion of variation in sawfly incidence data attributed to phylogenetic and defensive terms.

	Including geographical region information		Without geographical region information	
	Controlling for sampling effort	**Not controlling for sampling effort**	**Controlling for sampling effort**	**Not controlling for sampling effort**
Geographical region	0.163 (0.000–0.591)	0.165 (0.000–0.596)	0.214 (0.000–0.783)	0.236 (0.000–0.955)
Phylogenetic main effect for sawflies	0.009 (0.000–0.042)	0.003 (0.000–0.015)	0.010 (0.000–0.04)	0.010 (0.000–0.041)
Phylogenetic main effect for *Inga* hosts	0.005 (0.000–0.021)	0.012 (0.000–0.005)	0.006 (0.000–0.029)	0.006 (0.000–0.024)
Defense main effect for *Inga* hosts	0.018 (0.000–0.074)	0.015 (0.000–0.055)	0.011 (0.000–0.449)	0.009 (0.000–0.036)
Inga hosts evolutionary interaction	0.009 (0.000–0.038)	0.007 (0.000–0.03)	0.016 (0.000–0.063)	0.020 (0.000–0.065)
Inga defense interaction	0.537 (0.091–1.009)	0.546 (0.104–0.967)	0.663 (0.188–1.238)	0.650 (0.236–1.039)
Sawfly evolutionary interaction	0.019 (0.000–0.251)	0.019 (0.000–0.071)	0.016 (0.000–0.066)	0.015 (0.000–0.057)
Coevolutionary interaction	0.065 (0.000–0.251)	0.058 (0.000–0.293)	0.015 (0.000–0.10)	0.010 (0.000–0.045)
Defense tracking interaction	0.065 (0.000–0.250)	0.051 (0.000–0.161)	0.026 (0.000–0.10)	0.020 (0.000–0.078)
Main effect for sawflies	0.005 (0.000–0.021)	0.007 (0.000–0.02)	0.005 (0.000–0.023)	0.008 (0.000–0.034)
Main effect for *Inga* hosts	0.006 (0.000–0.026)	0.007 (0.000–0.028)	0.006 (0.000–0.028)	0.004 (0.000–0.015)
Interaction effect	0.063 (0.000–0.241)	0.069 (0.000–0.264)		

Columns contain the posterior modes (with 95% confidence intervals in parentheses) for the estimates. See Materials and Methods for a description of each term.

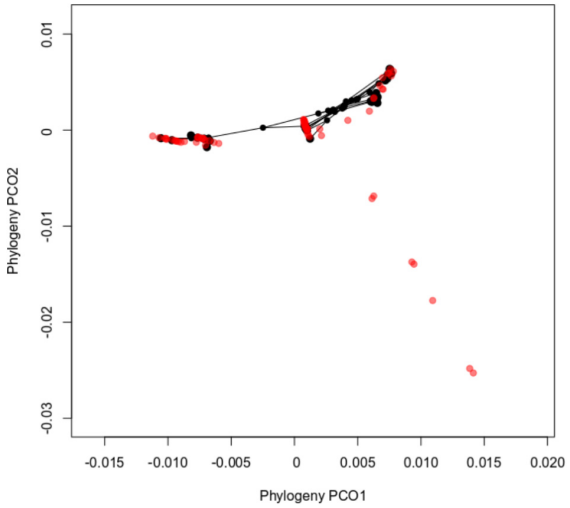

FIGURE 4 | Principal coordinates analyses plots of sawfly MOTUS-*Inga* hosts associations in terms of *Inga* phylogeny. Each point in the figure represents an *Inga* species, including those on which sawflies were never found, colored red. *Inga* that are associated with sawflies are colored in black. Points that are close together in the phylogenetic ordination diagram indicate closely related Inga species. Lines connecting the points represent sawfly phylogenetic relationships. *Inga* species that are located at and below the coordinate −0.01 in the y axis represent basal branches in the *Inga* phylogeny.

those contributing to the correlation between the *Inga* and sawfly phylogenies. There are many examples of closely related sawfly MOTUs attacking chemically similar *Inga* taxa (**Figure 3B**). In some cases, the two chemically similar *Inga* species are not closely related phylogenetically. For example, sawfly MOTU 12 attacks both *Inga laurina* and *Inga obidensis* in French Guiana. These two host plants have similar chemistry (**Figure 3B**), but are quite divergent phylogenetically (**Figure 3A**). There are four examples of the same sawfly MOTU attacking two hosts that are very divergent chemically (sawfly MOTU 15 attacking both *Inga* T82, *Inga alata* and MOTU 37 attacking *Inga auristellae, Inga umbratica* in Ecuador; MOTU 5 attacking *Inga retinocarpa* and *Inga bourgoniii* and MOTU 23 attacking *Inga marginata* and *Inga stipularis* in French Guiana) (**Figure 3B**).

In agreement with *Parafit* analyses, our *MCMCglmm* evolutionary models incorporating phylogenetic and chemical effects showed that the defense interaction term contributed the greatest variation to the sawfly incidence data, suggesting that the association between sawflies and *Inga* hosts is mainly due to chemistry (*Inga* defense interaction term in **Table 2**). The defense interaction term is the only term whose lower confidence limits exclude zero in any model, and this is true for all four models in **Table 2**. Chemically similar *Inga* species are attacked by related sets of sawfly MOTUs, having taken sawfly phylogeny into account. This was true in models with and without between-site information and sampling effort (**Table 2**). At large spatial scales (models with between-site information), coevolutionary and defense tracking effects were moderately large indicating that closely related sawflies are feeding on closely related *Inga*, which also are similar in chemistry (**Table 2**). However, when the models were fitted without controlling for sampling size and at small spatial scales (without between-site information), both the coevolutionary effect and the defense tracking effect decreased.

Geographic region has a large effect in all models (**Table 2**). In some cases, closely related species of sawflies are separated by geography but feed on the same species of *Inga*. For example, MOTU 31 attacks *Inga alba* in Peru, and its sister species MOTU 32 is associated with *Inga alba* in French Guiana (**Figures 3A,B**). MOTU 19 is associated with *Inga leiocalycina* in French Guiana and the sister lineage, MOTU 20, is associated with *Inga leiocalycina* in Ecuador (**Figures 3A,B**). These observations are most consistent with an allopatric mode of sawfly speciation, suggesting that biogeography is an important component in sawfly *Inga* associations.

The ordination diagram of the sawfly *Inga* associations in phylogenetic space (**Figure 4**) supported these findings by clustering sawfly MOTUS associated with *Inga* hosts that are closely related. This graph also shows the level of specialization for sawflies. The portion of *Inga* phylogenetic space towards the bottom right has seven species upon which we did not find any sawflies. These belong to early-diverging lineages of *Inga*. In fact, the sampled sawfly species feed entirely on one clade of *Inga*, albeit a clade that encompasses the large majority of *Inga* species.

DISCUSSION

Sawfly Barcoding

Work on tropical plant-herbivore associations has long been hampered by lack of taxonomic resources. DNA barcoding is well established as a major tool in circumventing this taxonomic impediment in species-rich tropical ecosystems (Janzen et al., 2005; Miller et al., 2016). Our barcoding of sawfly larvae has generated host plant association data for 41 *Inga* or *Zygia*-feeding MOTUs, and represents a substantial extension to what is known for neotropical sawflies. Forty of the full set of 41 MOTUs (38 of the 39 putative Argidae MOTUs) are novel. Two putative Argidae specimens from Barro Colorado, Panama, showed a 99% match to a voucher sequence for the argid species *Ptenos leucopoda*, described from Guanacaste, Costa Rica, and are probably members of this species. Twenty-two other individuals in eight MOTUs showed $\geq$ 90% sequence similarity to voucher sequence for species in the Argidae genus *Ptenos*, and are also probably members of this genus.

Sawfly faunas in many tropical regions of the world remain relatively understudied, and even where adults have been sampled the larval foodplants of most species remain unknown. As an example, the genus *Ptenos,* to which some of our *Inga*-sampled sawflies certainly belong, contains around 31 species from the southwestern United States to Argentina, but to our knowledge, published food plant associations are only known for one species, *P. leucopoda* (Smith et al., 2013). Pairing of adults and larval stages is a major benefit of DNA barcoding (e.g., Stone et al., 2008) – but few voucher barcode sequences for identified adults exist for many groups of sawflies. For example, Schmidt et al. (2017) reported BOLD reference barcode sequences for only 49 of the 918 known Argidae species worldwide. Only one of our specimens showed a high match to an identified voucher, for *Ptenos leucopoda* from Costa Rica. While sequence match places the other 40 MOTUs confidently within the families Argidae (n = 38) and Tenthredinidae (n = 2), their species status remains to be determined. The sequence divergence threshold we have used, at 1.5%, is slightly lower than the 2% applied by Schmidt et al. (2017) for the same sequence region in their Europe-focused barcode study of sawflies. However, Schmidt et al. (2017) found sequences for 13 of 49 Argidae voucher taxa to differ by less than 2%, suggesting that our empirically determined lower threshold is appropriate for this group.

Inga-Sawfly Evolutionary Associations

Our results extend Ehrlich and Raven's main prediction that closely related plants are associated with closely related herbivores (Ehrlich and Raven, 1964). Colonization of *Inga* by sawflies seems to have been restricted to two events: (1) once by the ancestor of the *Inga*-associated Argidae clade, and (2) once by the common ancestor of the two *Inga*-associated Tenthredinidae MOTUs (**Figure 1**). Here we focus on Argidae. Given the high phylogenetic conservatism for chemical defenses in the species of *Inga* associated with sawflies (**Table 1**), we would predict high topological congruence between *Inga* and sawfly phylogenies. Evolutionary analysis suggested a significant congruence between both topologies (**Figure 3A**). This result is further supported by the monophyly of the argid sawflies associated with *Inga.* Most *Inga* and *Zygia*-associated sawflies belong to a single clade that can be confidently placed in the family Argidae with reference to identified reference material – including a sequence match with Costa Rican sequences for the species *Ptenos leucopoda*. It is possible, however, that the monophyly of the Argidae group of 39 MOTUs could be an artifact resulting from undersampling of alternative host plant groups in the Neotropics. Nevertheless, the fact that related sawflies have not been found on other hosts in Guanacaste, Costa Rica[6] despite many years of sampling, suggests that this sawfly clade is genuinely restricted to *Inga* and close relatives.

The genus *Inga* is thought to represent a geologically young radiation, with a common ancestor between 4 and 10 million years ago (Richardson et al., 2001). If associated sawflies have co-diversified with their *Inga* hosts, we expect the ages of the two radiations to be similar. Because there are no fossil records for the *Inga*-associated Argidae clade, we used independent estimates for beetles and butterflies in order to calibrate the *Inga*-associated sawfly phylogeny. Comparisons with fossil-calibrated phylogenies for other sawfly taxa suggests that these calibrations are broadly applicable to sawflies (Nyman et al., 2006; Malm and Nyman, 2015). Based on these data, the estimate we obtained for the age of the common ancestor of the *Inga*-associated Argidae clade suggests that this group diversified at broadly the same time, or more recently, than their plant hosts [mean of 6.27 (between 4.78 and 7.93) million years ago using the Brower (1994) estimate and a mean of 5.31 (between 4.05 and 6.72) million years ago using the Papadopoulou et al. (2010) estimate]. Given the uncertainty in the date of the *Inga* radiation, these results are consistent with *Inga*-associated sawflies having diversified alongside their hosts, a conservative pattern of host plant use also found in other sawfly clades (Nyman et al., 2010;

[6]janzen.sas.upenn.edu/index.html

Schmidt and Walter, 2014) and in leaf-feeding beetles, seed predators and many other insect herbivore groups (Farrel and Mitter, 1998; Janz and Nylin, 1998; Winkler and Mitter, 2008; Edger et al., 2015). Alternatively, the radiation of Argidae might be younger than *Inga*, a pattern consistent with host-resource tracking or ecological fitting.

Ehrlich and Raven (1964) hypothesized that any taxonomic correspondence between plants and herbivores was the result of herbivore tracking of phylogenetically conserved host plant traits. Several lines of evidence suggest that defensive chemistry plays the key role in structuring sawfly associations with *Inga*. First, among all host traits, chemistry was identified as the most important predictor in sawfly *Inga* associations, with sawflies preferring *Inga* hosts that express amines (**Figure 2**). Second, after controlling for phylogenetic effects, we find that host associations in sawflies are more strongly correlated with *Inga* chemistry than *Inga* phylogeny (**Table 2** and **Figures 3A,B**). The significant concordance between the topologies of *Inga* and sawfly phylogenies could thus be explained as the result of phylogenetic conservatism in *Inga* chemistry for the set of species attacked by sawflies. Chemistry is better able to explain *Inga*-sawfly associations than the *Inga* phylogeny alone because some sawfly sister taxa are associated with hosts that are chemically similar but not closely related (**Figures 3A,B**), while there are very few cases of sawfly sister MOTUs associated with chemically divergent hosts.

Phylogenetic concordance between plants and herbivores could represent either a signature of codiversification or a radiation onto existing *Inga* (delayed resource tracking). The facts that host-shifting in sawflies is more strongly determined by *Inga* defenses than by *Inga* phylogeny (**Table 2** and **Figures 3A,B**), and that most examples of shifts between *Inga* hosts include species that are similar in defensive chemistry, regardless of relatedness (**Figures 3A,B**), support delayed host tracking. Nevertheless, it is striking that none of the more basal species in the *Inga* phylogeny are attacked by sawflies (**Figure 4**). This strongly implies cospeciation, that the ancestors of both the argid and tenthredinid sawflies now associated with *Inga* colonized, and then codiversified alongside an already ongoing radiation of *Inga*. In the end, which hypothesis is correct depends on the relative ages of the *Inga* and sawfly radiations. Our best estimate of the age of the common ancestor of the *Inga*-associated Argidae is fairly constrained (4.02–7.93 million years). In contrast, our estimate for the age of the common ancestor of *Inga* ranges from 4 to 10 million years, with the further caveat that the more derived *Inga* that are sawfly hosts are younger by an unknown extent. While the dates used here are consistent with codiversification, delayed resource tracking cannot be ruled out until the dates of origin for both crown groups, particularly *Inga*, are known with more certainty.

Although the significance of defensive traits in plant-herbivore diversification has been recognized (Futuyma and Agrawal, 2009), it is often not included in coevolutionary studies. Most studies compare the congruence between the ages and topologies of insect and host-plant phylogenies with the expectation that closely related hosts will share closely related herbivores (reviewed in Suchan and Alvarez, 2015). Alternative hypotheses, such as tracking of host defenses, cannot be tested. We argue that in order to understand the process and factors that influence the evolution of herbivore host ranges, characterization of relevant host traits is essential.

Inga-Sawfly Patterns of Diversification

Previous work suggests that modes of speciation vary among sawfly lineages with different life history strategies. Analyses of temperate nematine sawflies suggest that lineages with externally feeding larvae tend to feed on multiple host plant species (Nyman et al., 2006, 2010), and, as a result are more likely to diversify through allopatric speciation than via host shifts. In contrast, gall-inducing sawfly lineages, which are more intimately associated metabolically with their hosts, are both more likely to feed on a narrow host range and to diversify by shifts among willow host species (Nyman et al., 2006).

Although they are external feeders, the narrow host ranges observed for *Inga*- and *Zygia*- feeding sawflies (1-2 hosts per MOTU) more closely match patterns seen in specialist gall-inducing sawflies than the wider host associations seen in externally feeding sawflies on willow. This high host specificity could result from constraints or adaptations related to host use, such as host-finding capabilities, avoidance of larval predators, and avoidance or sequestration of host toxins (Brooks and McLennan, 2002). For the sawflies associated with *Inga*, a control choice experiment in a previous study suggested that host preference is primarily driven by leaf secondary metabolites and possibly nutrition (Endara et al., 2015). Although much of the available literature concerns the superfamily Tenthredinidae in the northern hemisphere, and the families Pergidae and Argidae in Australia, many sawflies show adaptations for dealing with, and using the host plant chemistry. Many can sequester and modify toxic host compounds for use in their own anti-predator defense [e.g., Diprionidae (Eisner et al., 1974); Tenthredinidae (Boevé et al., 2013); Argidae (Petre et al., 2007)], particularly against ants (Boevé and Schaffner, 2003; Petre et al., 2007; Boevé et al., 2013). This is particularly relevant in *Inga,* many species of which recruit ant guards through secretion of extrafloral nectar. The lack of any significant association between the presence of ants and sawflies on *Inga* suggests that sawflies may not be highly sensitive to ants that provide some defense against other herbivores (Endara et al., 2017). In *Inga,* we observed that when contacted by ants, sawfly larvae raised their abdomen, and ants generally retreated immediately (MJ Endara, personal observation). In addition, most of the sawfly MOTUs found on *Inga* are gregarious, a characteristic often considered a sign of chemical defense (Boevé et al., 2013). Thus, sawflies associated with *Inga* may have an intimate relationship with their host chemistry.

Although the specialized relationship between sawflies and *Inga* would suggest a mode of speciation similar to the specialist, gall-inducing sawflies, our phylogenetic analysis reveals that the predominant mode of speciation is allopatric, similar to external sawfly feeders on willow (Nyman et al., 2010). Results from the evolutionary analysis that included phylogenetic and chemical effects show that the coevolutionary effect best explained variation in sawfly incidence when between-region information

was included (**Table 2**). This suggests that pairs of sister *Inga* host populations and sawfly MOTUs occur in non-overlapping geographic regions (Hadfield et al., 2014). This pattern can be seen throughout the whole sawfly phylogeny, with more than 60% of lineage splits potentially caused by non-ecological factors in allopatry. For example, MOTU 31 attacks *Inga alba* in Peru, and its sister species MOTU 32 is associated with *Inga alba* in French Guiana (**Figures 3A,B**). This is evidence for allopatric speciation between sawfly sister taxa associated with the same *Inga* host (Barraclough and Vogler, 2000). Thus, *Inga*-feeding sawflies could have diverged and speciated in allopatry either directly because of *Inga* speciation or because the same ecological and geographical factors that facilitated *Inga* speciation could have facilitated the speciation of its sawfly herbivores. Alternatively, although species accumulation curves show that further sampling would not yield many additional *Inga*-sawfly interactions, we may have missed collecting sister sawfly species at the same site, meaning that speciation in sympatry cannot be totally ruled out.

The finding that the speciation process in the *Inga*-sawflies is largely non-ecological in allopatry does not exclude the possibility that some diversification events may have an ecological basis (i.e., host shifts). Along the phylogeny, four instances of lineage splits can potentially be ecologically based, with two host shifts to novel hosts in sympatry (MOTU 36 is associated with *Inga ruiziana* in Ecuador which produces phenolics, whereas the sister species MOTU 37 is associated with *Inga auristellae* which produces saponins, **Figures 3A,B**) and in allopatry (MOTU 7 is associated with *Inga marginata* in French Guiana which produces saponins, and the sister lineage MOTU 8 attacks *Inga umbellifera* in Panama which produces amines, **Figures 3A,B**). The other two host shifts simply involved range expansion (i.e., switch to a different host but with a similar chemistry), with one example in sympatry (in French Guiana, MOTU 7 is associated with *Inga obidensis* and MOTU 8 is attacking *Inga jenmanii,* both hosts produce amines, **Figures 3A,B**) and the other in allopatry (MOTU 16 is associated with *Inga edulis* in Ecuador and MOTU 18 is associated with *Inga thibaudiana* in French Guiana, with both hosts producing phenolics, **Figures 3A,B**). Excluding few exceptions, none of these switches involved phylogenetically closely related hosts, but rather chemically similar ones (**Figures 3A,B**), highlighting the importance of plant chemistry in ecological speciation.

CONCLUSION

Our phylogeny- and trait-based analysis of the interactions between *Inga* and Argidae sawflies indicates the importance of including ecologically relevant traits for host selection in studies of herbivore-host plant coevolution. For example, closely related sawfly species often shift to *Inga* that are similar chemically but not closely related phylogenetically. Our results suggest a major role for host chemistry in explaining both the observed concordance between *Inga* and sawfly phylogenies, and in explaining the deviations from this pattern resulting from evolutionary tracking of defensive traits by sawflies.

Our analyses suggest two modes of diversification of sawflies: (i) allopatric divergence between sawfly sister taxa associated with the same *Inga* food plant and (ii) niche shifts. The vast majority of lineage splits in these sawflies seem to have occurred non-ecologically in allopatry, a pattern that may well be true for other groups of insect herbivores (Nyman et al., 2010). Thus, sawflies primarily speciate allopatrically, but descendent species are constrained to use the same host species or others with similar chemistry. Closely related sawflies very rarely attack chemically dissimilar *Inga* species, implying that, for the most part, these herbivores have not experienced the niche shifts thought to promote diversification in other insect herbivores, and particularly in highly specialized taxa (Rundle and Nosil, 2005; Dyer et al., 2007; Futuyma and Agrawal, 2009).

AUTHOR CONTRIBUTIONS

M-JE, JN, PC, GS, and TK designed and conducted the research. M-JE, KD, and GS designed and performed the data analysis. DF and GY contributed to the metabolomic analysis. JN, RP, KD, CK, and GS contributed the next-generation DNA sequence data and phylogenies. M-JE, JN, PC, KD, DF, GY, RP, CK, GS, and TK wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01237/full#supplementary-material

Sawfly sequence data can be found in GenBank, accessions MH206848 – MH207017 for COI, MH206768 – MH206847 for ITS2, MH206617 – MH206691 for *wg* and MH206692 – MH206767 for *PGD*.

FIGURE S1 | MrBayes majority-rule consensus tree for the mitochondrial *COI* DNA barcode fragment. Numbers above nodes indicate posterior probabilities. Taxon label colors indicate membership of 1.5% sequence divergence jMOTU taxa, indicated by the labels at right.

FIGURE S2 | Results of MOTU identification analyses of *Inga*- and *Zygia*-feeding sawflies, using a 645 bp fragment of the mitochondrial *COI* DNA barcoding region for **(a)** jMOTU and **(b)** ABGD.

FIGURE S3 | MrBayes majority-rule consensus tree for the nuclear locus *ITS2,* sequenced for exemplars of each of the selected 41 jMOTU 1.5% *COI* MOTUs. Numbers above nodes indicate posterior probabilities. Taxon labels are colored to indicate membership of different MOTUs.

FIGURE S4 | MrBayes majority-rule consensus tree for the nuclear locus *wingless,* sequenced for exemplars of each of the selected 41 jMOTU 1.5% *COI* MOTUs. Numbers above nodes indicate posterior probabilities. Taxon labels are colored to indicate membership of different MOTUs.

FIGURE S5 | Sawfly MOTU accumulation curves when sampling over *Inga* host plant taxa, and when sampling over individuals. For each curve, the mean estimate is shown as a dark blue line and the standard deviation as a pale blue shaded region either side. The total numbers of *Inga* taxa and sawfly specimens in these analyses were 34 and 1286, respectively.

FIGURE S6 | Phylogenetic relationships for the gene *CO1* among the *Inga*-feeding sawfly MOTUs and a panel of voucher sequences for sawflies in the families Argidae, Pergidae (sister group to Argidae; Malm and Nyman, 2015) and Tenthredinidae. The tree shown is a majority-rule consensus tree constructed in MrBayes, using substitutions modeled as GTR+I+G for each of 1st and 2nd codon positions, and GTR+G for 3rd positions. We used a relaxed clock, with a birth-death speciation model. Numbers at nodes indicate posterior probability. Taxon labels are colored by sampling source: red MOTU numbers are larvae found feeding on *Inga* or *Zygia*, while other colors indicate reference sequences for adult Argidae, Pergidae and Tenthredinidae. The taxon label MOTU17_BCI marked with two asterisks is a voucher sequence for a specimen of *Ptenos leucoopoda* (Argidae) sampled from *Inga oerstediana* (and also recorded from *I. vera*) in Costa Rica (Smith et al., 2013).

TABLE S1 | List of compounds putatively identified through matches to reference MSMS spectra on the Global Natural Products Social Molecular Networking database (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp). The cosine score is a measure of the similarity of MS/MS-derived fragments between two compounds.

TABLE S2 | Metadata for all sawfly specimens collected in this study, including host plant and collection location, MOTU allocation (1.5% jMOTU taxa), and Genbank accession numbers for all sequenced gene fragments. Note that in our sampling system, each study site has independent collection numbers. Thus, it is possible for two *Inga* plants to have the same host plant number, but only because they were sampled at different sites.

TABLE S3 | Metadata for additional reference sawfly sequences, with species name, country of origin, Genbank accession numbers for *COI* and *PGD* gene fragments, and source reference.

TABLE S4 | Information on the ten sequence loci used for construction of the *Inga* species tree. Locus number, reference transcript, functional annotation and the substitution model used in phylogenetic analyses all refer to Nicholls et al. (2015).

TABLE S5 | (A) *Parafit* analysis output for sawfly and *Inga* phylogenies, for sawfly MOTUs in the family Argidae. **(B)** *Parafit* analysis of concordance between sawfly phylogeny and Inga chemogram. In **(A)** and **(B)** herbivore-*Inga* associations that are identified as individually significant are highlighted in yellow.

APPENDIX SI | Molecular methods for PCR amplification of sawfly sequences.

APPENDIX SII | Detailed chemical methods for construction of a chemical similarity matrix.

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Across Multiple Species, Phytochemical Diversity and Herbivore Diet Breadth Have Cascading Effects on Herbivore Immunity and Parasitism in a Tropical Model System

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Terrestrial tri-trophic interactions account for a large part of biodiversity, with approximately 75% represented in plant–insect–parasitoid interactions. Herbivore diet breadth is an important factor mediating these tri-trophic interactions, as specialisation can influence how herbivore fitness is affected by plant traits. We investigated how phytochemistry, herbivore immunity, and herbivore diet breadth mediate plant–caterpillar–parasitoid interactions on the tropical plant genus *Piper* (Piperaceae) at La Selva Biological station in Costa Rica and at Yanayacu Biological Station in Ecuador. We collected larval stages of one *Piper* generalist species, *Quadrus cerealis,* (Lepidoptera: Hesperiidae) and 4 specialist species in the genus *Eois* (Lepidoptera: Geometridae) from 15 different species of *Piper,* reared them on host leaf material, and assayed phenoloxidase activity as a measure of potential larval immunity. We combined these data with parasitism and caterpillar species diet breadth calculated from a 19-year database, as well as established values of phytochemical diversity calculated for each plant species, in order to test specific hypotheses about how these variables are related. We found that phytochemical diversity was an important predictor for herbivore immunity, herbivore parasitism, and diet breadth for specialist caterpillars, but that the direction and magnitude of these relationships differed between sites. In Costa Rica, specialist herbivore immune function was negatively associated with the phytochemical diversity of the *Piper* host plants, and rates of parasitism decreased with higher immune function. The same was true for Ecuador with the exception that there was a positive association between immune function and phytochemical diversity. Furthermore, phytochemical diversity did not affect herbivore immunity and parasitism for the more generalised herbivore. Results also indicated that small differences in herbivore diet breadth are an important factor mediating herbivore immunity and parasitism success for *Eois* at both sites. These patterns contribute to a growing body of literature that demonstrate strong cascading effects of phytochemistry on higher trophic

levels that are dependent on herbivore specialisation and that can vary in space and time. Investigating the interface between herbivore immunity, plant chemical defence, and parasitoids is an important facet of tri-trophic interactions that can help to explain the enormous amount of biodiversity found in the tropics.

Keywords: tropics, *Piper*, tri-trophic interactions, phytochemical diversity, parasitism, diet breadth, chemodiversity

INTRODUCTION

Tri-trophic interactions are an important feature of biotic communities and contribute to the maintenance of biodiversity as well as mediate ecosystem processes (Price et al., 1980; Hunter and Price, 1992; Agrawal, 2000; Price, 2002; Whitham et al., 2006). For instance, terrestrial plant–insect–predator/parasitoid interactions may make up approximately three quarters of the diversity of multicellular organisms (Price, 2002). Ecologists have found that tri-trophic interactions can shape community parameters, such as species diversity, functional diversity, primary productivity, and consumer abundance (Hairston et al., 1960; Ives et al., 2005; Singer and Stireman, 2005; Crutsinger et al., 2006; Johnson, 2008; O'Connor et al., 2016). Many tri-trophic studies have focused on how primary producers affect biotic communities through effects on densities or population dynamics of herbivores, mutualists, and natural enemies (Crutsinger et al., 2006; Crawford et al., 2007; Barbour et al., 2015). Plant chemical defence is one of the most important components of these bottom-up effects, and there is a rich literature documenting how chemistry affects plant–insect interactions (Fraenkel, 1959; Ehrlich and Raven, 1964; Schoonhoven et al., 2005; Hunter, 2016), via both negative and positive physiological and behavioural effects on herbivores and natural enemies (Smilanich et al., 2016). One clear gap in our knowledge of how phytochemistry influences tri-trophic interactions is empirical data that consider the entire suite of plant secondary metabolites in a species instead of focusing on one or two major compounds (Richards et al., 2010, 2016; Smilanich et al., 2016). Given that herbivores are exposed to the full array of compounds during their larval development and as adults, significant consideration should be given to the diversity of secondary metabolites found in plants (Hay et al., 1994; Richards et al., 2015). Here, we use phytochemical diversity as a metric of plant defence to investigate the effects on herbivore performance as measured by immune strength, and whether effects on the immune response cascade to impact parasitism success (Smilanich et al., 2009b; Richards et al., 2015; Hansen et al., 2017).

Research on the role of herbivore immunity as a mediator of tri-trophic interactions has been expanding over the last decade (Bukovinszky et al., 2009; Smilanich et al., 2009a; Richards et al., 2012; Singer et al., 2014; Lampert and Bowers, 2015). However, the majority of this work has been performed in temperate systems (but see: Smilanich et al., 2009b; Smilanich and Dyer, 2012; Hansen et al., 2017), where plant chemistry is typically less diverse and compounds may be less toxic (Coley and Barone, 1996; Dyer and Coley, 2002). In general, increased concentrations or mixture complexities of plant chemical compounds have a detrimental impact on herbivore immunity (Haviola et al., 2007; Smilanich et al., 2009a; Richards et al., 2010, 2016; Lampert, 2012; Hansen et al., 2017), but these effects can differentially influence the success of predators and parasitoids (Dyer et al., 2004; Bukovinszky et al., 2009; Richards et al., 2015). For instance, specialist caterpillars (*Junonia coenia*: Nymphalidae) have a weakened immune response due to sequestering higher concentrations of secondary metabolites, and this has been termed the 'vulnerable host hypothesis' (Smilanich et al., 2009a; Lampert and Bowers, 2015). More generally, specialised herbivores should be better adapted to diverse mixtures of secondary metabolites in their specific host plants, which may also protect specialists from natural enemies (e.g., Dyer, 1995); however, the energetic costs that accompany sequestration may be toxic to immune cells or may lead to reallocation of resources away from immune functions, rendering specialists more susceptible to parasitism (Smilanich et al., 2009a). Chemically defended or immune-compromised specialists may provide a 'safe haven' for parasitoids because they are less likely to be attacked or consumed by other natural enemies, which tend to avoid toxic specialist hosts (Dyer, 1995). Indeed, generalists are often better protected than specialists against parasitoids (Dyer and Gentry, 1999). The vulnerable host and safe haven hypotheses suggest that phytochemically defended plants may host specialist herbivores that are immunocompromised and more likely to be attacked by parasitoids (Smilanich et al., 2009a; Lampert et al., 2010).

The effect of host plant chemistry on the immune response also depends on the physiological ecology of the organism: herbivores that utilise metabolically expensive strategies, such as detoxification or sequestration, to tolerate host plant chemistry may incur physiological costs to eating toxic diets and experience compromised immune systems (Smilanich et al., 2009a). For example, the immune response of *Eois nympha* and *Eois apyraria* (Geometridae) caterpillars was suppressed when feeding on *Piper cencocladum* (Piperaceae) compared to other *Piper* host plants, and *P. cenocladum* is more phytochemically diverse than other *Piper* host species (i.e., *Piper imperiale*) (Hansen et al., 2017). Furthermore, Richards et al. (2010) found that a mixture of plant secondary metabolites from a neotropical shrub in the genus *Piper* (Piperaceae) affected a naïve generalist noctuid caterpillar (*Spodoptera*) differently from adapted specialist geometrid caterpillars (*Eois*), with *Spodoptera* experiencing high mortality through direct toxicity, and indirect negative effects of chemistry on *Eois* via increased levels of parasitism. Increased parasitism associated with host plant toxicity is also consistent with the hypothesis that higher phytochemical diversity may weaken a caterpillar's immune response, leading to increased parasitoid

success. This hypothesised association is best tested when direct effects of chemistry on adult parasitoids are ruled out, which is the case in experiments where caterpillars are naturally exposed to parasitoids first in the field and then subsequently assigned to feeding treatments in the laboratory (e.g., Smilanich et al., 2009b; Richards et al., 2010; Hansen et al., 2017). Similarly, iridoid glycosides sequestered by buckeye caterpillars (*J. coenia*) negatively affected the efficacy of encapsulation by these specialists (Smilanich et al., 2009a) but did not affect this same (encapsulation) measure of the immune response in the generalist caterpillar, *Grammia incorrupta* (Erebidae: Arctiinae) (Smilanich et al., 2011). Overall, there is growing evidence that plant chemistry may mediate herbivore susceptibility to parasitoids via the herbivore's immunity and the strength or direction of this relationship is dependent on the level of specialisation of the plant–herbivore interaction. While previous studies have included how diet breadth may affect the ecoimmunology of tri-trophic interactions, there are other axes of variation that are likely to be important for modifying this relationship, including biogeographical differences among sites. For example, plant chemistry and tri-trophic interactions vary across elevations (Rodríguez-Castañeda et al., 2016) and with rainfall intensity (Cunningham et al., 1999), thus the same herbivore species may be affected differently by host plant chemistry and parasitoids across elevational and precipitation gradients, due to differences in chemistry and in enemy communities (Rodríguez-Castañeda et al., 2016).

In this study, we used the tropical plant genus, *Piper,* the associated specialist herbivore genus, *Eois* (Lepidoptera: Geometridae), and a *Piper* generalist, *Quadrus cerealis* (Lepidoptera: Hesperiidae), to investigate whether variation in phytochemical diversity influences the strength of the herbivore immune response and associated levels of parasitism (**Figure 1** and **Table 1**). In addition to examining variation across these different herbivore species, we examined these relationships in two distinct ecosystems – a lowland wet forest in Costa Rica (La Selva, Sarapiqui) and a cloud forest in Ecuador (Yanayacu, Napo), which differ dramatically in temperature means and variance, annual rainfall, and elevation. Specifically, we designed our study to address the following questions: (1) How does phytochemical diversity influence herbivore immunity and levels of parasitism and how are these relationships affected by diet breadth? (2) How do these effects vary across different herbivore species and different locations?

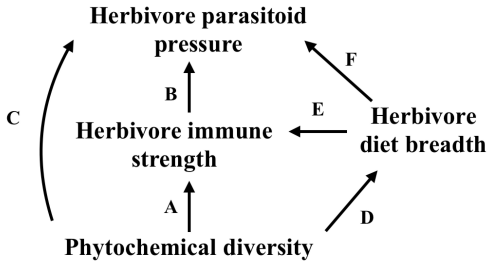


FIGURE 1 | Meta-model that structured our *a priori* hypotheses. Letters over paths are associated with hypotheses in **Table 1**.

MATERIALS AND METHODS

Study Sites

Our study took place at two different field stations in the neotropics: (1) La Selva Biological Station, Heredia Province, Costa Rica (10° 26′ N 83° 59′ W) and (2) Yanayacu Biological station, Napo Province, Ecuador (00° 36′ S 77° 53′ W). The La Selva Biological reserve is 1600 ha of lowland rainforest and ranges from 35 to 140 m in elevation and is surrounded by a combination of disturbed, agricultural habitat, and natural forest. The mean annual precipitation is approximately 4200 mm. Sampling at Yanayacu Biological Station included the 100 ha owned by the station as well as thousands of hectares of surrounding cloud forest on the slopes of the eastern Andes. The elevation at the station is 2100 m and the annual precipitation is approximately 2624 mm.

Piper–Eois, *Piper–Quadrus* System

The plant genus *Piper* (Piperaceae) is an emerging tropical model system for studying tri-trophic interactions because of the growing knowledge on its evolutionary history, genomics, plant chemistry, distribution, and insect communities (Marquis, 1991; Greig, 1993; Dyer and Palmer, 2004; Richards et al., 2015; Glassmire et al., 2016; Salazar et al., 2016). Currently there are over 2000 species of *Piper* that have been identified pantropically, with approximately 1300 species occurring in the neotropics, 50 species present at the La Selva Biological station and 20 present at the Yanayacu station. *Piper* is a phytochemically diverse genus, including compounds from at least 15 classes, and a total of 667 individual compounds have been discovered (Richards et al., 2016). In this study, we used previously published data quantifying phytochemical diversity for multiple *Piper* species (Richards et al., 2015). Each of the *Piper* species in this experiment had a fixed diversity value and therefore no intra-specific variation was quantified. Briefly, phytochemical diversity is an effective number of functional groups, transformed from a Simpson's diversity entropy calculated from proton nuclear magnetic resonance (^{1}H–NMR), which incorporates both mixture complexity and structurally complexity, the two key components of chemical diversity (Richards et al., 2015).

Piper species host diverse lepidopteran herbivore communities that vary in diet breadth (Dyer and Palmer, 2004). Caterpillars in the genus *Eois* (Lepidoptera: Geometridae) are *Piper* specialists that feed exclusively on 1–4 different *Piper* species (Connahs et al., 2009). They are one of the most well studied and abundant genera of caterpillars found on *Piper*, and over 80% of *Eois* species are found in the neotropics with others in Africa, Asia, and Australia (Rodríguez-Castañeda et al., 2010; Brehm et al., 2011). In contrast, the *Piper* skipper, *Q. cerealis* (Lepidoptera: Hesperiidae), has been recorded feeding on 23 *Piper* species; in this paper we categorise this skipper as a *Piper* generalist[1] (Dyer et al., 2010).

[1]http://www.caterpillars.org

TABLE 1 | Description of the hypotheses and predictions behind each path in our supported SEM models.

Explanatory variables	Response variables	Paths	Hypotheses and predictions	References
Plant quality (phytochemical diversity)	Herbivore fitness (Immunity)	A	Plants with high phytochemical diversity are more likely to contain compounds that decrease herbivore fitness.	Berenbaum and Neal, 1985; Jones and Firn, 1991; Smilanich et al., 2009b; Diamond and Kingsolver, 2011; Lampert and Bowers, 2015
Herbivore fitness (immunity)	Herbivore parasitism	B	The immune system provides important protection against parasitoids, thus as the strength of the immune system decreases, parasitism increases.	Bukovinszky et al., 2009; Smilanich et al., 2009a; Quintero et al., 2014
Plant quality (phytochemical diversity)	Herbivore parasitism	C	Low plant quality caused by toxic secondary metabolites, and higher phytochemical diversity are more likely to weaken herbivores via the presence of bioactive compounds and/or toxic synergies, increasing parasitoid success.	Lill et al., 2002; Bukovinszky et al., 2009; Richards et al., 2010; Sternberg et al., 2012; Hunter, 2016
Plant quality (phytochemical diversity)	Herbivore diet breadth	D	Plants with greater diversity of phytochemical compounds are more likely to host specialised herbivores that have adapted to bioactive compounds and/or toxic synergies.	Becerra, 2007, 2015; Dyer et al., 2003, 2007; Richards et al., 2015
Herbivore diet breadth	Herbivore fitness (immunity)	E	Specialist herbivores are adapted to detoxifying or sequestering toxic plant compounds and will perform better on their host plants than generalists.	Coley et al., 2006; Richards et al., 2010; Lampert, 2012
Herbivore diet breadth	Herbivore parasitism	F	Herbivores that feed on a greater number of plants are exposed to a greater variety of toxic plant compounds which weaken herbivores, increasing parasitoid success.	Barbosa et al., 1991; Carvalheiro et al., 2010; Lampert et al., 2011; Reudler et al., 2011

Long Term Rearing Databases

Since 1991, principal investigators, students, volunteers, and technicians have been collecting plant–herbivore–parasitism interaction data in Costa Rica (e.g., from Dyer and Floyd, 1993; Hansen et al., 2017). We used data from 1996 to 2015 in this database (these years included the most complete parasitism data) to determine simple taxonomic diet breadth for herbivores (number of host plants documented for a caterpillar species) and parasitism frequency, quantified as the total number of parasitized caterpillars divided by the total number of caterpillars reared to adult plus parasitoid (parasitoids)/(healthy adults + parasitoids) (Gentry and Dyer, 2002; Hansen et al., 2017; **Tables 2**, **3**). Data consisted mainly of entries from La Selva Biological Station, but also from other areas nearby such as Braulio Carrillo National Park and the Tirimbina Biological Reserve. Primarily third instar caterpillars were collected year-round in all forest types and reared on the host plant from which they were collected in ambient conditions until they pupated and eclosed into adulthood, or if parasitized prior to collection, until they succumbed to parasitism. Data were collected on the caterpillar species, the host plant it was found on, and whether it reached adulthood or was parasitized (for detailed methods see Gentry and Dyer, 2002). Using these data, we evaluated herbivore immunity for four different *Eois* species collected from five different *Piper* species (**Table 2**). For these species, we found a total of 2011 records in our database with 900 caterpillars successfully reaching adulthood (**Table 2**). Additionally, we collected *Q. cerealis* from 10 different *Piper* species, though we have records of larvae feeding on 23 different *Piper* species (**Table 3**). We recorded 117 instances of *Q. cerealis* on these 10 *Piper* species with 75 caterpillars successfully reaching adulthood (**Table 3**). **Tables 2**, **3** summarise the sample sizes of larvae collected for immune assays and long-term parasitism sample sizes for those same species.

The same data collection procedure was utilised at the Ecuador site, where the database spans 15 years (2001–2015). Larvae were collected in the cloud forest surrounding Yanayacu Biological Station. At this site, we measured the immune response from eight *Eois* morphospecies feeding on three different *Piper* species (**Table 2**). We had 2079 records of our *Eois* morphospecies in our database with 809 caterpillars successfully reaching adulthood (**Table 2**). We calculated diet breadth and levels of parasitism using the same method at both sites. Diet breadth was calculated as the number of *Piper* species on which a caterpillar species was found feeding and successfully reared to adult moth or parasitoid. As with the Costa Rica data, parasitism frequency was calculated as the number of parasitism events for each caterpillar species divided by the total number of successfully reared adults + parasitoids.

Immune Assay

Phenoloxidase (hereafter PO) is an important enzyme for triggering the melanization process, a mechanism of innate immunity involving deposition of pigments on foreign bodies (Beckage, 2008; González-Santoyo and Córdoba-Aguilar, 2012). It is typically stored in hemolymph cells in a non-activated form called prophenoloxidase (proPO) since active PO can have locally toxic effects (Cerenius et al., 2008). Upon infection or natural enemy attack, proPO is converted to the active form, PO, which catalyses the cascade to produce melanin. Phenoloxidase has been shown to be an important part of the immune response in arthropods, protecting them from

TABLE 2 | *Eois* caterpillars and their host plants collected for immune assays.

Site	*Eois* spp.	*Piper* spp.		Database		
			n	Records	Adults	% parasitized
Costa Rica	*Eois nympha*	*Piper biseriatum*	9	44	7	29
		Piper cenocladum	28	921	317	18
	Eois apyraria	*Piper cenocladum*	1	328	164	8.4
		Piper imperiale	7	616	359	1.4
	Eois russearia	*Piper sancti-felicis*	12	48	24	4
	Eois mexicaria	*Piper umbricola*	13	54	29	0
Total			70	2011	900	
Ecuador	Six black two pink spots	*Piper baezanum*	2	6	1	0
		Piper kelleyi	16	1792	700	14
		Piper lancifolium	1	1	0	0
	Lime slime	*Piper baezanum*	1	3	1	0
		Piper kelleyi	7	9	0	0
	Two black spots	*Piper kelleyi*	27	83	29	3.3
		Piper lancifolium	1	1	0	0
	Eois viridiflava Dognin	*Piper baezanum*	1	2	0	0
		Piper lancifolium	20	36	0	0
	Pink spots funk	*Piper kelleyi*	3	86	37	8.1
		Piper lancifolium	1	1	0	0
	Eight black blur	*Piper baezanum*	1	1	9	0
	Eois beebei Fletcher	*Piper kelleyi*	1	36	19	14
	Eois ignefumataPdfLatex Dognin	*Piper kelleyi*	1	22	13	19
Total			83	2079	809	

Sample size of the immune assays is indicated by "n". Host plant-caterpillar species information from two multi-year databases includes all collection records, specified in the 'records' column, caterpillars that made it to adulthood and parasitism percentage.

TABLE 3 | *Quadrus cerealis* caterpillars and their host plants collected for immune assays.

Site	*Piper* spp.		Database		
		n	Records	Adults	% parasitized
Costa Rica	*Piper arboreum*	3	2	2	0
	Piper cenocladum	1	4	3	25
	Piper colonense	13	16	13	38
	Piper garagaranum	1	3	2	33
	Piper imperiale	6	2	1	50
	Piper multiplinervium	19	26	26	7.7
	Piper pseudobumbratum	1	1	1	0
	Piper reticulatum	18	62	26	68
	Piper trigonum	2	0	0	0
	Piper umbricola	1	1	1	0
Total		65	117	75	

Sample size of the immune assays is indicated by "n". Host plant-caterpillar species information from a 19-year database includes all collection records, specified in the 'records' column, caterpillars that made it to adulthood and parasitism percentage.

bacteria, viruses, and parasitoids (Cerenius et al., 2008). We measured the activity of the PO enzyme as an indicator of the strength of the herbivore immune response (González-Santoyo and Córdoba-Aguilar, 2012). We collected four species of early instar caterpillars from five different plant species and reared them on the host plant in which they were found in ambient conditions until they reached 5th instar. To measure PO activity (modified from Adamo, 2004), we took 2 μL of hemolymph from each *Eois* caterpillar (Costa Rica: $N = 70$, Ecuador: $N = 83$) and 5 μL from each *Q. cerealis* caterpillar (Costa Rica: $N = 65$), collected by puncturing the caterpillar with a pin and extracting hemolymph with a pipette. The volume of hemolymph was

divided the into two Eppendorf tubes—one for cell-free PO found in the hemolymph at the time the hemolymph is taken (standing PO), and one for cell-bound PO, which is artificially activated by adding a chemical activator (total PO). The aliquots of hemolymph were added to 50 μl of phosphate buffered saline for *Eois* individuals and 100 μl PBS for *Q. cerealis* individuals. For the total PO in both species, 35 μl of chymotrypsin (1mg/mL) was added to the PBS-bound hemolymph, vortexed for 2 s, then incubated at room temperature for 20 min. During incubation, the substrate, dopamine, (0.0284 g/10 mL distilled water) was prepared. Since this compound is light sensitive, fresh dopamine was prepared daily. For *Eois*, we added 300 μl of dopamine to each Eppendorf tube, vortexed for 2 s, then added 25 μl of the dopamine-hemolymph mixture to a well plate. For *Q. cerealis*, we added 500 μl of dopamine to each Eppendorf tube, vortexed for 2 s, then added 200 μl of the dopamine-hemolymph mixture to a well plate. We used a spectrophotometer (BIO-RAD: iMark Microplate Absorbance Reader) at a wavelength of 490 nm to measure the activity of PO every 30 s for 45 min. We measured the slope, which was the rate of reaction, from the first 10 min because it was a linear increase. PO assays were performed in Costa Rica from January 2013 to December 2015 and in Ecuador from December 2015 to January 2016.

Statistical Analyses

We used structural equation models (SEM) to evaluate 7 *a priori* hypotheses, which tested for bottom-up effects of phytochemical diversity and herbivore diet breadth on herbivore immunity and parasitism success (**Figure 1** and **Table 1**). We used the global estimation method in the R packages piecewiseSEM v.1.2.1 (Lefcheck, 2016) and lavaan v.0.5–23 to run our SEMs (Rosseel et al., 2017) in R v3.4.2 (R Core Team, 2017). We were not able to normalise the residuals of our data, so we chose a more robust estimator to account for non-normality and unequal variance instead of the default maximum likelihood method; this method is based on the Satterthwaite approach and is called the maximum likelihood estimation with robust standard errors and a mean and variance adjusted test statistic (Rosseel et al., 2017). Lastly, we used the same 7 hypotheses in our Ecuador dataset as we had no reason to believe that our systems should operate differently (**Figure 1** and **Table 1**).

For each site, we used a Bayesian mixed linear model to examine effects of phytochemical diversity on immune response. This approach allowed us to incorporate prior distributions from earlier studies using the same methodology (Smilanich and Dyer, 2012), also to account for Type II error (i.e., reporting actual probabilities of null hypotheses) and to test the generalizability of our results. Caterpillar species were a random effect in the model. Priors were generated from *E. nympha* and *E. apyraria* caterpillars collected on *P. biseriatum, P. cenocladum, P. imperiale,* and *P. urostachyum* at La Selva Biological Station in Costa Rica. The Bayesian model was estimated using SAS 9.4 (v13.1) procedure MCMC. We chose the quasi-Newton algorithm, convergence was assessed via visual examination of the trace plot, and the first 2,000 (burn-in) out of 10,000 samples were discarded, yielding robust posterior distributions for parameters. We report the posterior distributions of B_1 parameter estimates from this model for the effects of phytochemical diversity on rate of total PO absorbance per minute.

RESULTS

Summary Statistics

Average immune response for *Eois*, as measured by total PO absorbance per minute (ΔAbs), was approximately equal across sites (*Eois:* Costa Rica: 0.03 ± 0.004 ΔAbs; Ecuador: 0.02 ± 0.001 ΔAbs; here and elsewhere, error is 1 SEM), and between specialist *Eois* and *Piper* generalist, *Q. cerealis* (*Q. cerealis*: 0.02 ± 0.002 ΔAbs). However, average parasitism level was higher for *Q. cerealis* (0.34 ± 0.03 parasitism frequency) compared to *Eois* at both sites (Costa Rica: 0.12 ± 0.01 parasitism frequency; Ecuador: 0.04 ± 0.01 parasitism frequency). Parasitoid families attacking the caterpillars also differed between sites and species. *Q. cerealis* parasitism was entirely tachinid parasitoids, while *Eois* parasitism in Costa Rica was 80% braconids, 8% tachinids, and 12% parasitism by other families. *Eois* parasitism in Ecuador was 24% tachinids, 41% braconids, and 35% parasitism by other families. Increases in phytochemical diversity had negative effects on the immune response in both Costa Rica and Ecuador, with posterior distributions of parameter estimates (from the mixed Bayesian model) similar to those reported previously for effects of diet on immune response. The negative effects of phytochemical diversity on immune response yielded parameter estimates at both sites that did not include a slope of zero; the combined mean slope for effect of NMR bin diversity on ΔAbs was −0.46.

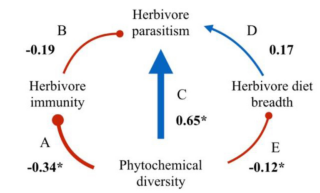
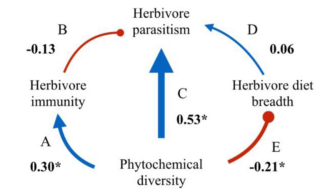
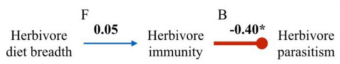
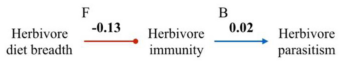
Structural Equation Models

Overall, the best fit structural equation models supported the hypotheses that both phytochemical diversity and herbivore diet breadth are important factors shaping herbivore immunity and parasitism for *Eois* species in both Ecuador and Costa Rica, however, for some relationships, the directions of the effects were reversed from one site to another (**Table 4** and **Figures 2**, **3**). Tests of seven a priori models to explain the relationships of our measured variables were completed for both sites. In addition, we tested our models by bootstrapping missing data to even out sampling effort (see Supplementary Tables S1, S2). This analysis yielded only one of the same models as our initial analysis without the bootstrapped data for Costa Rica but not Ecuador (Model II, the diet breadth regulation hypothesis; Supplementary Table S3).

Model I: Phytochemical Diversity Regulation Hypothesis

The phytochemical diversity regulation hypothesis (Model I) for our Costa Rica *Eois* data included phytochemical diversity as an exogenous variable with direct paths to herbivore immunity, herbivore diet breadth, and herbivore parasitism; the model also included effects of herbivore immunity and diet breadth on herbivore parasitism (Costa Rica model fit: Robust test statistic = 0.004, df = 1, P = 0.95, scaling factor = 2.08). This model supported the hypothesis that there is a strong direct

TABLE 4 | Structural equation model (SEM) results from Costa Rica *Eois* and *Q. cerealis* study systems.

Model	Structure	Robust test statistic	*P*	DF	Scaling factor
Site: Costa Rica **(I)** Phytochemical diversity regulation hypothesis		0.004	0.95	1	2.08
Site: Ecuador **(I)** Phytochemical diversity regulation hypothesis		0.28	0.60	1	1.34
Site: Costa Rica **(II)** Diet breadth regulation hypothesis		0.81	0.37	1	1.13
Site: Ecuador **(II)** Diet breadth regulation hypothesis		0.16	0.69	1	0.60

Our hypotheses tested for: (I) 'Phytochemical diversity regulation hypothesis' – Phytochemical diversity having direct and indirect effects on higher trophic levels and which are mediated by both herbivore immunity and herbivore diet breadth (model fit: Robust test statistic = 0.004, df = 1, P = 0.95, scaling factor = 2.08), (II) 'Diet breadth regulation hypothesis' – Herbivore diet breadth is the main driver of herbivore immunity which in turn influences herbivore parasitism (model fit: Robust test statistic = 0.81, df = 1, P = 0.37, scaling factor = 1.13). SEM results from Ecuador Eois system. Our hypotheses tested for: (I) 'Phytochemical diversity regulation hypothesis' – Phytochemical diversity having direct and indirect effects on higher trophic levels and which are mediated by both herbivore immunity and herbivore diet breadth (model fit: Robust test statistic = 0.28, df = 1, P = 0.60, scaling factor = 1.34), (II) 'Diet breadth regulation hypothesis' – Herbivore diet breadth is the main driver of herbivore immunity which in turn influences herbivore parasitism (model fit: Robust test statistic = 0.16, df = 1, P = 0.69, scaling factor = 0.60). Asterisks represent significant path coefficients (P < 0.05).

positive effect of phytochemical diversity on herbivore parasitism (**Figure 2C**, standardised path coefficient (hereafter, spc) = 0.65, $P < 0.01$, slope (B_1) = 1.13), showing that herbivores feeding on plants with high phytochemical diversity had higher parasitism rates. This model also showed that phytochemical diversity decreases herbivore immunity (**Figure 2D**, spc = −0.34, $P < 0.01$, $B_1 = -0.14$). It supports the hypothesis that higher herbivore immunity decreases herbivore parasitism frequency (**Figure 2B**, spc = −0.19, $P = 0.08$, $B_1 = -1.52$). Lastly, this model shows a negative effect of phytochemical diversity on herbivore diet breadth (i.e., *Piper* species with greater phytochemical diversity are consumed by more specialised *Eois* species; spc = −0.12, $P = 0.03$, $B_1 = -2.66$). In turn, herbivore diet breadth has a weak, positive effect on herbivore parasitism (i.e., generalists have higher levels of parasitism; spc = 0.17, $P = 0.11$, $B_1 = 0.001$). The same model was strongly supported by our Ecuador *Eois* data, however, the directions of some of the relationships were reversed (Ecuador model fit: Robust test statistic = 0.28, df = 1, $P = 0.60$, scaling factor = 1.34). Consistent with the Costa Rica data, phytochemical diversity has a strong positive effect on herbivore parasitism (**Figure 3C**, spc = 0.53, $P < 0.01$, $B_1 = 1.93$), however, in contrast to the Costa Rica data, phytochemical diversity has a positive effect on herbivore immunity (**Figure 3D**, spc = 0.30, $P < 0.01$, $B_1 = 0.30$). Phytochemical diversity has a negative effect on herbivore diet breadth (spc = −0.21, $P < 0.01$, $B_1 = -9.55$), and herbivore immunity negatively affects herbivore parasitism (**Figure 3B**, spc = −0.13, $P = 0.22$, $B_1 = 0.08$). Lastly, diet breath has no effect on herbivore parasitism (spc = 0.06, $P = 0.62$, $B_1 = -0.003$). Models for *Q. cerealis* caterpillars in Costa Rica did not fit the data, for example, a model where phytochemical diversity affects herbivore immunity, which in turn influences herbivore parasitism, was a poor fit to the data (model fit: Robust test statistic = 28.37, df = 1, $P < 0.01$, scaling factor = 0.50). However, a separate regression analysis showed that phytochemical diversity had a negative relationship with *Q. cerealis* parasitism [$B_1 = -4.39$, $F_{(1,63)} = 15.25$, $P < 0.01$].

Model II: Diet Breadth Regulation Hypothesis

The diet breadth regulation hypothesis (model II) is a simpler model focusing on the effects of diet breadth on herbivore immunity and parasitism (Costa Rica model fit: Robust test statistic = 0.81, df = 1, $P = 0.37$, scaling factor = 1.13). In Costa Rica, this model shows that greater diet breadth (measured as *Eois* species that are documented feeding on a greater number of host plants) had a weak positive effect on herbivore immune response (**Figure 2A**, spc = 0.05, $P = 0.76$, $B_1 = 0.001$) and that immune function reduces parasitism success (**Figure 2B**, spc = −0.40, $P < 0.01$, $B_1 = -1.52$). The diet breadth regulation hypothesis was again supported by our Ecuador data (model II) (Ecuador model fit: Robust test statistic = 0.16, df = 1, $P = 0.69$, scaling factor = 0.60),

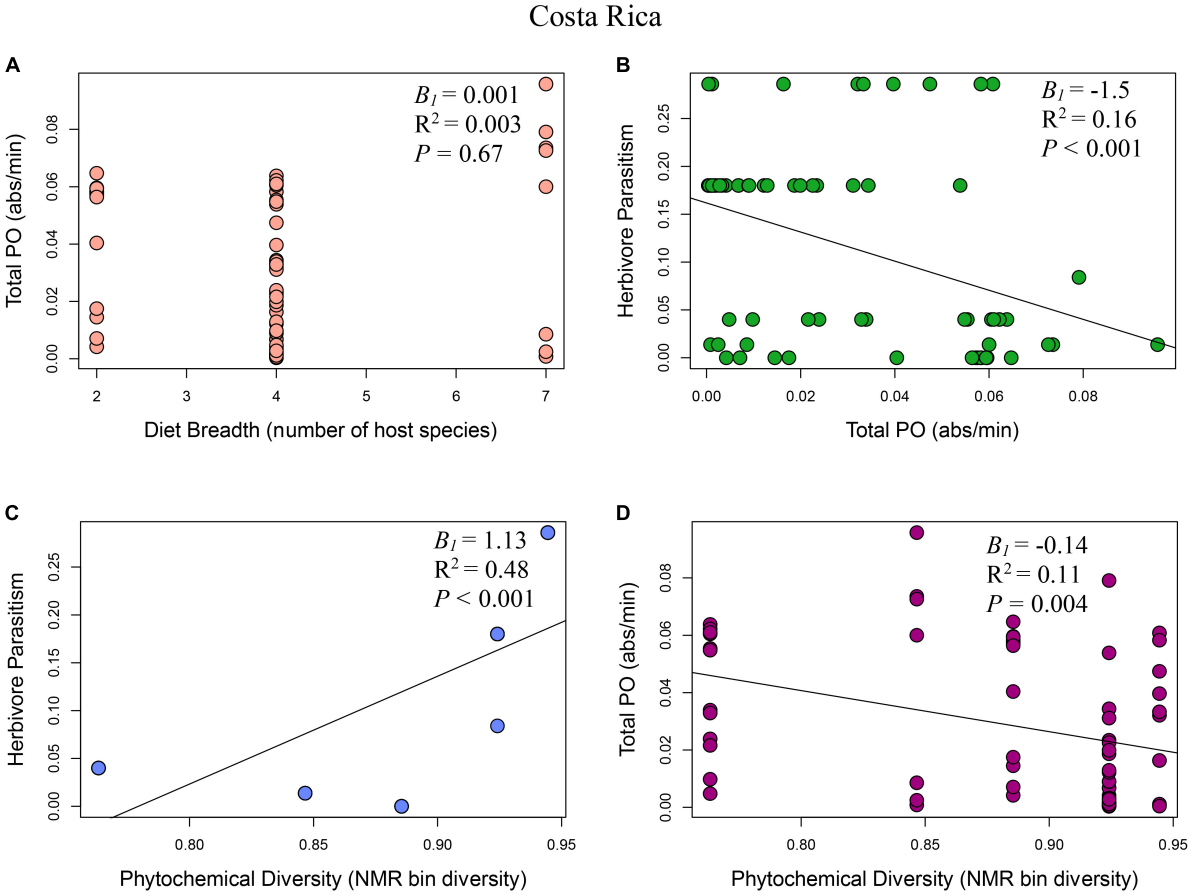


FIGURE 2 | Multi-panel regression plots of *Eois* ecoimmunological parameters in Costa Rica: **(A)** Relationship between diet breadth, measured as number of host species, and *Eois* immune response, measured as total phenoloxidase absorbance per minute (B_1 = 0.001, R^2 = 0.003, $F_{1,68}$ = 0.18, P = 0.67). **(B)** *Eois* immune response and percent *Eois* parasitism (B_1 = –1.5, R^2 = 0.16, $F_{1,68}$ = 12.95, P < 0.001). **(C)** Phytochemical diversity, measured as NMR binned peak diversity, and *Eois* percent parasitism (B_1 = 1.13, R^2 = 0.48, $F_{1,68}$ = 63.78, P < 0.001). **(D)** Phytochemical diversity and *Eois* immune response (B_1 = –0.14, R^2 = 0.11, $F_{1,68}$ = 8.67, P = 0.004).

but for this site, a greater diet breadth had a weak negative association with herbivore immunity (**Figure 3A**, spc = −0.13, P = 0.30, B_1 = −0.003), and herbivore immunity has no effect on herbivore parasitism (**Figure 3B**, spc = 0.02, P = 0.84, B_1 = 0.08).

DISCUSSION

Our results corroborate many other studies demonstrating that the chemistry of herbivore host plants, as well as herbivore diet breadth have strong effects on multiple aspects of herbivore ecology (Berenbaum and Neal, 1985; Haviola et al., 2007; Diamond and Kingsolver, 2011; Lampert and Bowers, 2015), including immunity and parasitism (Smilanich et al., 2009a; Hansen et al., 2017). A focus on the immune response allows for investigation of an important physiological parameter that is directly linked to protection against natural enemies (Smilanich et al., 2009b), putting our results in a strong tri-tropic context. It is also interesting that the relationships between phytochemical diversity, immunity, and parasitism were dependent upon the diet breadth of the specialist herbivores and that relationships varied across herbivore taxa and site. In Costa Rica, *Eois* feeding on *Piper* species with high phytochemical diversity had a weakened immune response, while the immune response of *Q. cerealis* was unaffected. It is important to note that the sample size for some herbivore species in Ecuador was small, which weakens the strength of our results (**Table 2**). For instance, three different herbivore species were only collected once (**Table 2**) – collecting many replicate herbivore species in the tropics can be difficult, depending on their abundance and distribution. Nevertheless, our results correspond with the importance of diet breadth in other results with this system (Richards et al., 2015). *Eois* data in Ecuador fit the same two models as in Costa Rica, however, some relationships were reversed. For example, in Costa Rica, individuals with a strong immune response had lower parasitism frequency (model II), however, in Ecuador herbivore immunity had almost no effect on parasitism frequency. This difference may be due to the differences in parasitoid pressure between the two sites.

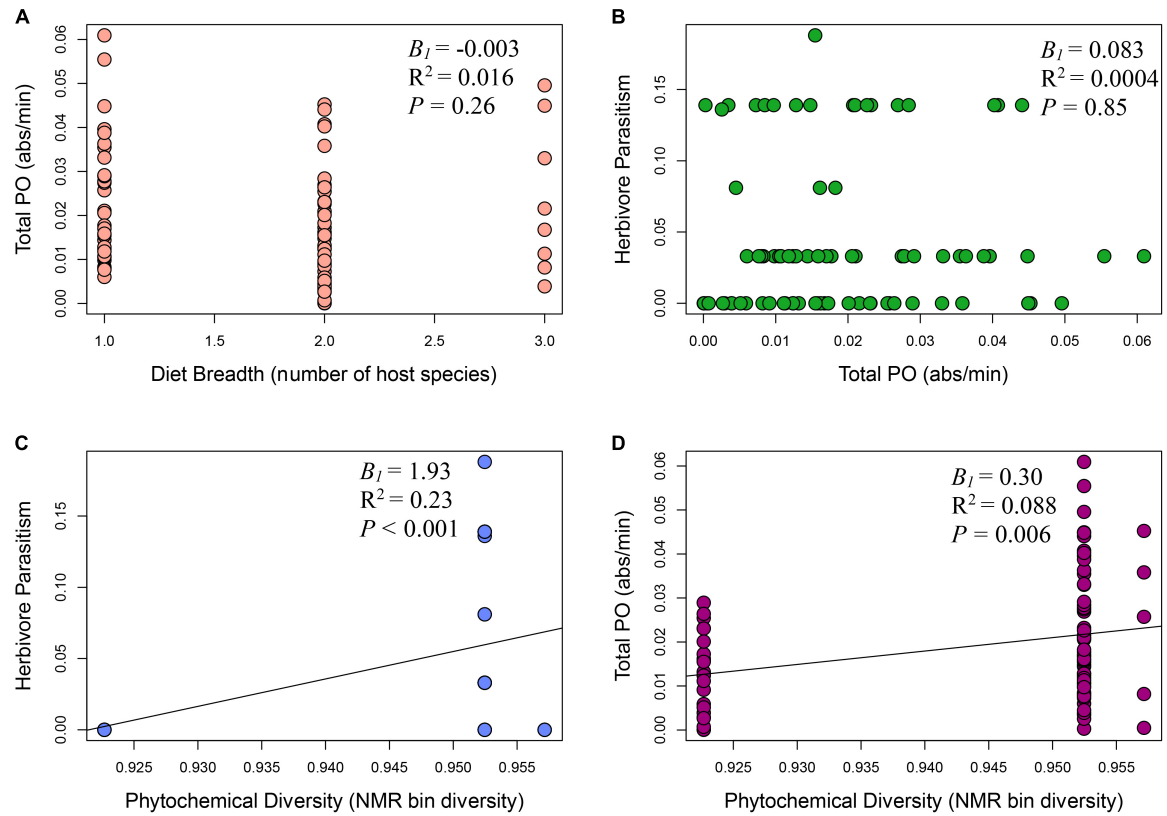


FIGURE 3 | Multi-panel regression plots of *Eois* ecoimmunological parameters in Ecuador: **(A)** Relationship between diet breadth, measured as number of host species, and *Eois* immune response, measured as total phenoloxidase absorbance per minute (B_1 = –0.003, R^2 = 0.016, $F_{1,81}$ = 1.28, P = 0.26). **(B)** *Eois* immune response and percent *Eois* parasitism (B_1 = 0.083, R^2 = 0.0004, $F_{1,81}$ = 0.036, P = 0.85). **(C)** Phytochemical diversity, measured as NMR binned peak diversity, and *Eois* percent parasitism (B_1 = 1.93, R^2 = 0.23, $F_{1,81}$ = 23.82, P < 0.001). **(D)** Phytochemical diversity and *Eois* immune response (B_1 = 0.30, R^2 = 0.088, $F_{1,81}$ = 7.82, P = 0.006).

Compared to Ecuador, the database shows that *Eois* in Costa Rica have three times more parasitism by a relatively more specialised parasitoid community (Braconidae). Our Ecuador data include plant-caterpillar species pairs that are not well represented in our historical database and which have 0% parasitism as a result. We ruled out that this was driving our observed patterns by re-running our SEMs without plant-caterpillar species pairs that had low representation in our database, but found the same qualitative result. We therefore included these data points in our final analysis. Other possible particulars of the taxa and sites used for our study, such as degree of specialisation and elevation of the site, may also be responsible for these differences, but greater insight into those variables will require further experimentation using carefully selected taxa and locations.

Untangling relationships between plant chemistry, herbivores, and natural enemies has been a focus of insect ecology for decades (Price et al., 1980; Bernays and Graham, 1988; Dyer, 1995, 2011) and our results with *Eois* in Costa Rica are consistent with emerging paradigms of the importance of phytochemistry in mediating multi-trophic interactions. Most notably, we provide further support for the 'safe haven hypothesis' (Lampert et al., 2010) and the 'vulnerable host hypothesis' (Smilanich et al., 2009a). *Eois* data from Costa Rica support all aspects of this 'safe haven hypothesis' and data from both sites support the more general concept that changes in chemistry are likely to alter herbivore immunity and parasitism – the positive effects of phytochemical diversity on herbivore immunity in Ecuador are not inconsistent with this hypothesis, and they simply require further investigation to determine mechanisms causing this relationship. Furthermore, both SEM models (**Table 4**, Hypotheses I and II) are consistent with the growing body of evidence that the ability of an insect herbivore to mount an immune response is negatively associated with herbivore parasitism (Bukovinszky et al., 2009; Quintero et al., 2014), which is an important component of the 'safe haven hypothesis,' and some have argued that this is the best predictor of parasitism (Smilanich et al., 2009b; Greeney et al., 2012).

Other studies that support the 'safe haven hypothesis' (Gentry and Dyer, 2002; Lampert et al., 2010) or related hypotheses (i.e., 'nasty host hypothesis' Barbosa et al., 1991; Gauld et al., 1992)

have focused on detoxification or sequestration of individual compounds or entire classes of compounds and have measured relative concentrations of those compounds (e.g., Haviola et al., 2007; Smilanich et al., 2009a; Lampert and Bowers, 2015). We utilise a different approach and consider the fact that phytochemical mixtures are complex and herbivores may be as susceptible to mixture complexity, synergies, or additive effects rather than just increases in concentrations of individual compounds or classes, such as tannins (Richards et al., 2015). One shortcoming of this approach is that results will require further investigation to get at mechanism. In Costa Rica, the immune responses of *Eois* species were negatively affected by increases in phytochemical diversity (**Table 4**, Hypothesis I). Another study with *Eois* on *Piper* found that changes in mixture complexity are associated with synergistic effects on parasitoid success (Richards et al., 2010). It is possible that host plants with higher phytochemical diversity are more likely to have synergistic effects on herbivores, impairing immune function, regardless of whether the mixtures are sequestered.

It is interesting to note that the results depended on taxon (*Quadrus* versus *Eois*) and site (Ecuador versus Costa Rica). Such variation is expected, and it is worth further investigation to determine conditions that are favourable for these chemically mediated tri-tropic interactions. Site and taxon were treated as random effects in the broader sense and were not statistically compared; nevertheless, it is interesting to consider possibilities for some of the differences across the two taxa and the two sites. Specialist *Eois* caterpillars in Costa Rica support our predictions, whereas, the same genus of caterpillars in Ecuador do not support any of our a priori models. Elevation is one clear difference between these sites, with the cloud forest in Ecuador situated 2,000 m higher than the lowland forest in Costa Rica. It is well known that herbivore development rates, herbivory, levels of predation, and herbivore diversity are lower at higher elevations, while parasitism and parasitoid diversity increase with elevation (Rodríguez-Castañeda et al., 2011, 2016), so it is not surprising that the specifics of chemically mediated tri-tropic interactions would vary with elevation. Reasons for the positive effect of phytochemical diversity on immunity at higher elevation are not obvious, but given the higher levels of parasitism and slow development rates, it is possible that maximised immunity is enhanced with slow development rates since larvae are exposed to parasitoids for longer periods of time. Similarly, there are many differences between the geometrid and hesperiid caterpillars utilised in our study, including diet breadth; however, one large difference is that *Quadrus* is a concealed feeder, and concealed feeders are affected less by phytochemical defence (Sandberg and Berenbaum, 1989; Berenbaum, 1990) and experience very high levels of parasitism (Gentry and Dyer, 2002). As such, *Q. cerealis* appeared to be unaffected by changes in chemistry and experienced extremely high levels of parasitism. There are likely unmeasured variables that influence immunity of hesperiids and more generally of concealed feeders, and it is certainly possible that the greater diet breadth played a role in the differences noted here.

In summary, our research builds on previous work investigating the effects of phytochemical diversity and herbivore diet breadth on ecoimmunology and tri-trophic interactions. These results support the hypothesis that variation in phytochemical diversity, rather than individual compounds, was a predictor of tri-trophic interactions and herbivore immunity (Richards et al., 2010, 2016). These patterns are also particularly important for understanding tropical systems, which are typically characterised by intense biotic interactions and high levels of diversity (Dyer and Coley, 2002; Novotny et al., 2006). Future work should investigate how much intraspecific phytochemical variation exists within these *Piper* species, how intraspecific variation compares across different *Piper* species, and what is driving that variation. Further, how does this intraspecific variation affect higher trophic levels and what are the differences in responses between predators and parasitoids. A field experiment evaluating the susceptibility of herbivores to parasitoids feeding on *Piper* of varying phytochemical profiles would greatly add to our understanding of the consequences of phytochemical diversity on herbivore immunity. Lastly, as the effects of global change worsen, including loss of tropical forests, the diversity of plant secondary metabolites is predicted to decrease, and understanding this diversity is a key part of documenting these losses.

AUTHOR CONTRIBUTIONS

LR, AS, and LD designed the experiments. HS performed the field work. HS and LD analysed the data. LD, LR, AS, and PH provided advice for the data analysis. HS wrote the first drafts. All authors contributed to additional draft of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00656/full#supplementary-material

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frontiers in Plant Science

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Bacteria and Competing Herbivores Weaken Top–Down and Bottom–Up Aphid Suppression

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Herbivore suppression is mediated by both plant defenses and predators. In turn, plant defenses are impacted by soil fertility and interactions with soil bacteria. Measuring the relative importance of nutritional and microbial drivers of herbivore resistance has proven problematic, in part because it is difficult to manipulate soil-bacterial community composition. Here, we exploit variation in soil fertility and microbial biodiversity across 20 farms to untangle suppression of aphids (*Brevicoryne brassicae*) through bottom–up and top–down channels. We planted *Brassica oleracea* plants in soil from each farm, manipulated single and dual infestations of aphids alone or with caterpillars (*Pieris rapae*), and exposed aphids to parasitoid wasps (*Diaeretiella rapae*) in the open field. We then used multi-model inference to identify the strongest soil-based predictors of herbivore growth and parasitism. We found that densities of *Bacillus* spp., a genus known to include plant-growth-promoting rhizobacteria, negatively correlated with aphid suppression by specialist parasitoids. Aphid parasitism also was disrupted on plants that had caterpillar damage, compared to plants attacked only by aphids. Relative abundance of *Pseudomonas* spp. bacteria correlated with higher aphid growth, although this appeared to be a direct effect, as aphid parasitism was not associated with this group of bacteria. Non-pathogenic soil bacteria are often shown to deliver benefits to plants, improving plant nutrition and the deployment of anti-herbivore defenses. However, our results suggest that these plant growth-promoting bacteria may also indirectly weaken top–down aphid suppression by parasitoids and directly improve aphid performance. Against a background of varying soil fertility, microbial biodiversity, competing herbivores, and natural enemies, we found that effects of non-pathogenic soil microbes on aphid growth outweighed those of nutritional factors. Therefore, predictions about the strength of plant defenses along resource gradients must be expanded to include microbial associates.

Keywords: tritrophic interactions, *Brassica oleracea*, *Bacillus* spp., *Pseudomonas* spp., *Brevicoryne brassicae*, growth-defense tradeoff

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INTRODUCTION

Chemical defenses induced by plants in response to herbivore attack often trade-off with plant growth due to resource allocation costs, genetic costs, and opportunity costs of prioritizing defense-associated physiological processes over growth (Züst and Agrawal, 2017). Growth/defense investments are traditionally discussed along a continuum of resource availability, where

investments in defense diminish under high nutrient conditions (Coley et al., 1985; Herms and Mattson, 1992). Defense induction is also governed by herbivore community composition (Stam et al., 2014) and microbial associates of host plants (Pineda et al., 2017), among numerous other ecological factors. For example, production of secondary metabolites induced by chewing herbivores can be constrained when chewers and suckers co-occur, due to antagonism between defense signaling pathways (Thaler et al., 2012), while plant-growth-promoting rhizobacteria (PGPR) can prime inducible defenses in advance of herbivory (Pineda et al., 2010). *Pseudomonas simiae* (formerly known as *P. fluorescens*), a well-known PGPR, increases resistance to chewing herbivores (Pangesti et al., 2015a), but has been found to interfere with volatile-mediated attraction to parasitoids of sucking herbivores (Pineda et al., 2013). Indeed, complex interactions between soil microbes, plants, herbivores, and natural enemies suggest that simple predictions made about defense investments along resource gradients are inadequate, but the importance of each of these numerous drivers of herbivore defense remain unclear.

While rapid advances have been made in understanding how microbe-mediated plant defenses influence multi-trophic interactions, knowledge has largely emerged from experiments with single-strain inoculations of defense-priming bacterial taxa (Pangesti et al., 2013; Pineda et al., 2017). Manipulating multiple bacterial taxa is a considerable challenge, but efforts to move from examining single PGPR species to whole bacterial and fungal communities suggest that rhizobiome diversity may reduce the vulnerability of plants to herbivore attack (Hol et al., 2010). However, effects of naturally diverse soil microbes on plant defenses in multi-herbivore communities have yet to be elucidated. Interactions between diverse microbes, plants and diverse herbivores are likely to be complex because microbe-mediated defense can act on herbivores directly, via production of secondary metabolites, and also indirectly, via production of herbivore-induced plant volatiles that attract natural enemies, such as predators and parasitoids (Pangesti et al., 2013). Examining these microbe-mediated tritrophic interactions in field environments is critical to understanding the function, context-dependence, and utility of PGPRs for plant protection in agricultural systems.

While communities of rhizobacteria remain challenging to manipulate, the recent affordability of next-generation sequencing (NGS) technology enables an observational approach to quantifying effects of the ambient rhizobiome on herbivores. Here, we use 16S sequencing to describe the diversity and relative abundance of soil bacteria across a gradient of soil fertility using soil collected from 20 organic farms, evaluating the predictive strength of microbial and fertility-based predictors of herbivore growth and suppression by natural enemies in the field. Then we examine effects of single and dual infestations of chewing and sucking insects on herbivore growth on *Brassica oleracea* plants along this same gradient in soil quality. We use this correlative approach to examine the relative importance of microbial symbionts and resource availability in top–down and bottom–up suppression of herbivores in diverse field environments.

MATERIALS AND METHODS

Natural History

At our field site, broccoli plants (*Brassica oleracea* var. capitata, cvs. Arcadia) are attacked by herbivorous aphids and caterpillars, while the aphids (but generally not the caterpillars) are in turn attacked by parasitoid wasps (Snyder et al., 2006; Blubaugh et al., 2018). The cabbage aphid (*Brevicoryne brassicae*) is the dominant aphid on these plants, while *Pieris rapae* is the most common caterpillar species. The most abundant aphid parasitoid in the system is *Diaeretiella rapae*, which limits cabbage aphid population growth in the U.S. Pacific Northwest (Blubaugh et al., 2018); however, parasitism of *P. rapae* is rare in the region (Blubaugh et al., 2018). Caterpillars (*P. rapae*) used in our experiments came from a lab colony, and aphids (*B. brassicae*) came from a wild colony collected from a single kale plant (*Brassica oleracea* var. acephala) on April 12, 2016.

Soil Collection

On April 20, 2016, 19 L of soil from the 1–15 cm profile were collected from 20 organic mixed-vegetable farms in eastern Washington and northern Idaho (**Figure 1**). Map was composed in QGIS (QGIS Development Team, 2018), using data from the USA Web Soil Survey (Soil Survey Staff et al., 2018). Soil cores (10-cm diameter) were collected every 1 m in beds designated for *Brassica* plantings by our cooperating growers, timed to

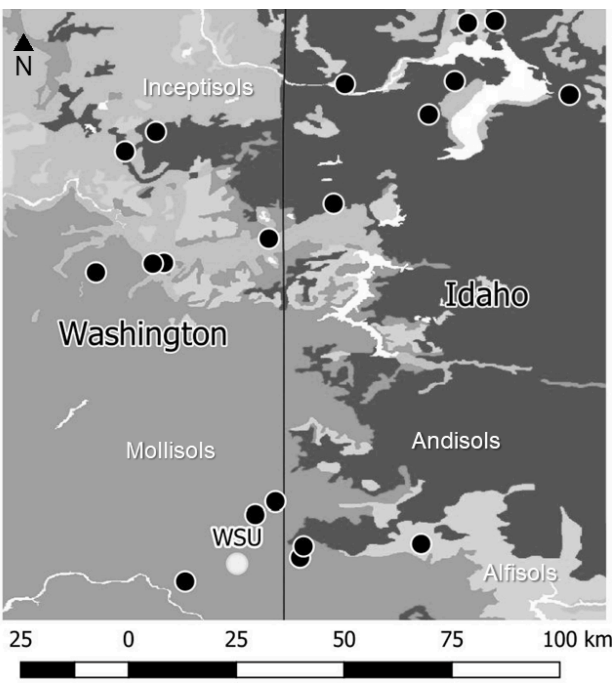


FIGURE 1 | Map of 20 sites and underlying soil orders in Eastern Washington and Northern Idaho, United States, where soil was collected from collaborating organic vegetable farms for a common garden experiment. The experiment took place at the Eggert Family Organic Farm at Washington State University, indicated in white.

TABLE 1 | Hypotheses and associated evidence for each of the soil-based predictors of aphid growth and parasitism examined in our global models.

Factor	Hypothesis	Reference
Soil NO_3, Soil NH_4, P, K, SO_4	N, P, K, and S availability increases aphid growth and parasitism	Coley et al., 1985; Price, 1991
pH	Acidic soils decrease aphid growth and decrease parasitism	Birkhofer et al., 2008
Organic matter	Organic matter decreases aphid growth and increases parasitism	Altieri and Nicholls, 2003
Microbe biomass	Microbial activity decreases aphid growth and increases parasitism	Birkhofer et al., 2008
Bacillus spp.	*Bacillus* spp. decrease aphid growth and increase parasitism.	Gadhave and Gange, 2016; Gadhave et al., 2016a
Pseudomonas spp.	*Pseudomonas* spp. increase aphid growth and decrease parasitism	Pineda et al., 2012, 2013

synchronize with growers' bed preparation for transplanting. Because microbial communities change rapidly following soil disturbance events (Doran, 1980), all samples included in our study were collected in a single day and immediately deposited in cold storage, and seedlings were transplanted into it the following morning. Unfortunately, the necessity of sampling in a single day limited the number of sites (20) and the number of replicates within sites (2) that were possible for our team to acquire for this study. Soil samples from each farm were sent to Soiltest Farm Consultants (Moses Lake, WA, United States) immediately following collection. There, soil samples were passed through a 2-mm sieve and analyzed for the following properties according to recommended soil-testing methods by Gavlak et al. (2003): nitrate-nitrogen (N) using the chromotropic acid method; ammonium-N with the salicylate method; Olsen phosphorus; NH_4OAc extractable potassium; DTPA-Sorpitol extractable sulfur; soil pH in a 1:1 w/v water saturated paste; and percent soil organic matter by loss on ignition method. Microbial biomass was estimated using a Solvita test (Haney et al., 2008). The remaining soil was stored overnight at 4 degrees C before planting in the greenhouse (described next).

Aphid Population Growth and Parasitism in the Field

To evaluate the effects of soil fertility and soil bacteria on aphid growth and parasitism on plants with or without caterpillar herbivory, we measured aphid colony growth and parasitism across all 20 farm soils in a common garden at Eggert Family Organic Farm at Washington State University in Pullman, WA, United States. The morning after soil samples were collected (April 21), broccoli seedlings (*B. oleracea* var. *Capitata* svs. *Arcadia*) were transplanted into 2.8-L pots of soil collected from each farm (2 pots × 20 replicate farms = 40 plants), and completely randomized in the greenhouse. After growing in farm soils for 5 weeks in the greenhouse on 16/8 h light/dark cycles at 26 degrees C, pots were completely randomized and placed

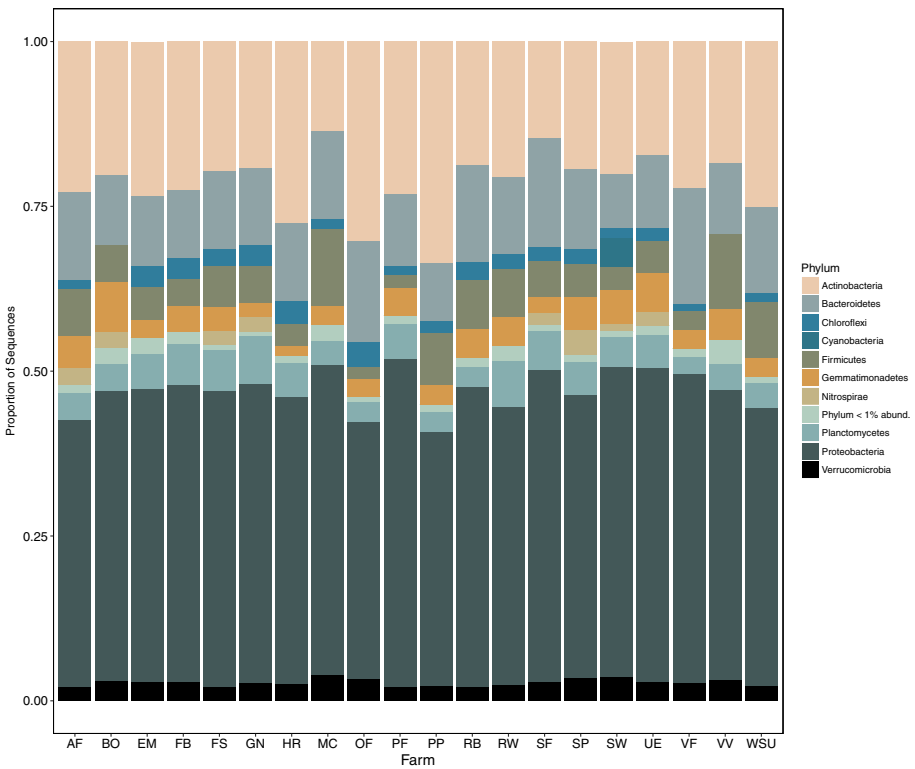
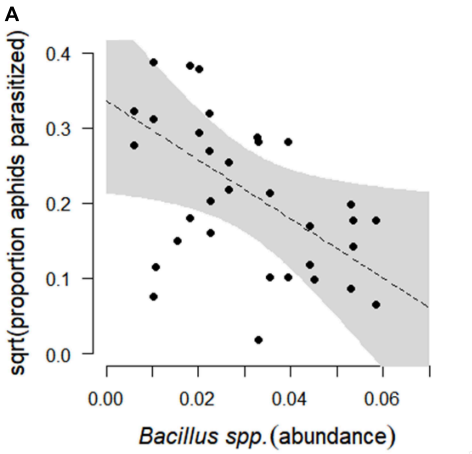


FIGURE 2 | Relative abundance (proportion of sequences) of the 10 most common soil bacterial phyla across the 20 farms surveyed.



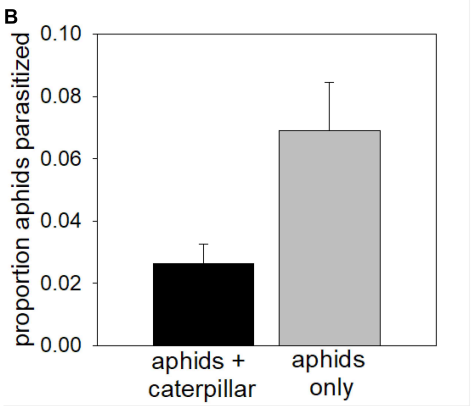


FIGURE 3 | Relationship between **(A)** *Bacillus* spp. relative abundance and **(B)** caterpillar co-herbivory and parasitism of cabbage aphids (*B. brassicae*) in a common garden field assay across 20 organic farm soils. Proportions of aphids parasitized were square root transformed prior to analysis to meet model assumptions; the scatterplot represents output from the mixed-effect model and the shaded gray area indicates a 95% confidence interval. Error bars indicate standard error of the mean.

in the field on June 1, 2016. Twenty-five aphids were placed on all plants, and 2nd instar *P. rapae* caterpillars were added to one plant from each farm soil treatment (2 caterpillar treatments × 20 replicate farms = 40 total replicates). Aphids were counted every 96 h and after 2 weeks (the amount of time required for parasitoid development), proportions of aphids parasitized were estimated by counting aphid mummies (mummies are hardened pupal cases left behind after parasitoids emerge from killed aphids). For the first week, the mixed-age aphid colonies were controlled to keep aphid densities relatively similar (within 50 aphids/plant) by gently brushing aphids off plants with colonies that grew more quickly than others.

Soil Bacterial Community Profiling

The soil microbial community was destructively sampled from an extra control plant (with no herbivore damage) from each farm soil replicate on June 8, 2016 by gently shaking roots loose from the soil, and then shaking soil from the fine root region through a 2-mm mesh sieve to remove small root parts. Microbial DNA from two 0.25-g subsamples was extracted immediately following using MoBio soil extraction kits (Qiagen, Inc., Germantown, MD, United States).

The bacterial amplicon library was generated using PCR primers 341F/785R (Klindworth et al., 2013), with Illumina adapter overhang sequences, to target the V3–V4 hypervariable regions (∼464 bp) of the 16S rRNA gene. The library was prepared using a two-step PCR protocol, following the Illumina Metagenomic Library Prep Guide[1] and Nextera XT index kit (Illumina, Inc., San Diego, CA, United States) for sample multiplexing. PCR products were cleaned with an Agencourt AMPure XP kit (Beckman Coulter, Brea, CA, United States), quantified with a Quant-IT HS-DNA dsDNA assay (Thermo Fisher Scientific, Inc., Waltham, MA, United States), then normalized and pooled at equimolar concentration. The pooled library was then sequenced on an Illumina MiSeq at the Center for Genome Research and Biocomputing (Oregon State University, Corvallis, OR, United States) using 2 bp × 300 bp paired-end V3 chemistry. Raw sequences are available on the NCBI Sequence Read Archive (SRA) under study accession SRP152350.

Demultiplexed sequences were initially trimmed of trailing low-quality bases and then merged using Trimmomatic v.0.36 (Bolger et al., 2014) with the following settings: LEADING = 3, TRAILING = 3, HEADCROP = 15, SLIDING WINDOW = 5:15 and MINLEN = 100. Merged reads were then fed into the *DADA2* pipeline (v.1.6.0; Callahan et al., 2016) in R (v. 3.4.0; R Core Development Team), error-corrected, and assembled into amplicon sequence variants (ASVs). ASVs have many benefits over traditional operational taxonomic unit (OTU) methods, such as revealing cryptic diversity through identification of exact biological sequences that differ by a single nucleotide, among others (Callahan et al., 2017). Once assembled, chimeras were detected, removed, and taxonomic information was then assigned to each ASV using the RDP Naïve Bayesian Classifier (Wang et al., 2007), trained to the RDP training set (v.14). ASV read counts were then averaged across the two subsamples for each farm, and those that failed to classify to kingdom or were identified as chloroplast or mitochondrial sequences, respectively, were discarded. Moreover, singletons, as well as rare, low abundance taxa were also filtered from the dataset prior to analyses, with ASVs with a minimum read count of 5 and occurring in at least 10% of our samples being retained. Post-filtering, *DADA2* inferred 4,621 unique, error-corrected ASVs representing 1,294,852 sequences, on which analyses below were performed.

Post-processing, ASV richness and diversity (Shannon index) were calculated at the farm level using the *phyloseq* package (McMurdie and Holmes, 2013). Calculations were performed on rarefied data (39185 reads per farm). These metrics were also used to estimate microbial community evenness, following Pielou's index (Pielou, 1969). Finally, as we were interested in the role of known PGPRs in mediating aboveground tritrophic

[1]https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

interactions, we also calculated the relative abundance of *Bacillus* and *Pseudomonas* spp. (ASVs agglomerated at the genus level) observed at each farm.

Analyses

All analyses were performed in R (v. 3.4.0; R Core Development Team), and data used in analyses are available in **Supplementary Table S1**. To examine herbivore resistance across farms, we applied an information theoretic approach to identify the strongest uncorrelated soil-based predictors of herbivore performance and rates of parasitism. Prior to running global models, regressions of all potential pairs of predictor variables were plotted in R using the *pairs* function, and only one variable from collinear pairs was selected for inclusion in the global model. Our first model used the *lme* function in the *nlme* package (Pinheiro et al., 2014) to predict parasitism of *B. brassicae,* and the global model contained the following variables: concentrations of nitrate-N, ammonium-N, plant available P, K, SO_4, pH, organic matter, *Bacillus* spp. relative abundance, *Pseudomonas* spp. relative abundance, microbial community evenness, and microbial biomass (mg/kg soil). We selected our variables and hypotheses for the global model based on known relationships between soil nutrient pools, soil microbes, host plant quality, and herbivore defenses (**Table 1**). To simplify models, we analyzed means of proportions of aphids parasitized across the five sample dates, and square root transformed them to meet normality and variance assumptions. We included 'farm' as a random intercept because the model included two replicates from each of the 20 farms (both herbivory treatments were included). One outlier replicate plant (from the 40 replicates) was removed from our models because its aphid colony count was more than 3 standard deviations higher than the mean of the whole group, and twice as high as the next largest colony. We expect that such rapid growth was due to confounding factors unrelated to plant quality (i.e., immigration from adjacent areas). Competing models were evaluated using the *dredge* function in the *Mumin* package (Bartoń, 2014), and best-fit models were chosen by selecting the model that included the fewest variables within two corrected Akaike information criterion (AICc) values of the minimum (Burnham and Anderson, 2004). Because we experimentally manipulated caterpillar co-herbivory, we did not include this variable in the correlative model selection process. Instead, we ran a separate model to more robustly test effects of our herbivory treatments on aphid parasitism across the 20 farms. Two replicates were removed from the caterpillar model because the caterpillars died on these plants without consuming any leaf tissue.

To examine effects of the same soil-based predictor variables on aphid colony growth, we performed another model predicting mean aphid counts, pooled across seven sample dates. Random effect structure and predictors in the global model were the same as described above for the model predicting aphid parasitism, and the best-fit subset of predictors was identified in a similar manner. A separate model tested the effects of caterpillar co-herbivory on aphid growth. All scatterplots were made using function *visreg* in the *visreg* package (Breheny and Burchett, 2016) and were fit using output from best-fit models.

RESULTS

Soil Microbial Gradients

A total of 18 bacterial phyla were found, of which *Proteobacteria* was most dominant, ranging from 38.6 to 49.7% of total sequences across farms sampled (**Figure 2**). Other abundant phyla observed included *Actinobacteria* (range: 13.6–33.6%), *Bacteroidetes* (range: 8.2–17.6%), *Firmicutes* (range: 1.9–11.6%), *Planctomycetes* (range: 2.6–7.2%), and *Gemmatimonadetes* (range: 1.5–7.5%). We identified known PGPR *P. fluorescens* at the species level, while the majority of other *Pseudomonas* ASVs failed to match beyond the genus level. *Pseudomonas* spp. include both PGPR and pathogenic species (Haney et al., 2017); however, we found little/no evidence of pathogenic *P. syringae* presence in our sequences during ASV-level analyses. *Bacillus* taxa identified at the species level, included *B. coagulans, B. lentus, B. drentensis*, and *B. acidiceler*. Because none of the well-known pathogenic species were identified in our samples, our subsequent analyses

TABLE 2 | Model selection table for the top soil-based predictors of aphid parasitism across 20 organically managed farm soils (*Bacillus* spp. relative abundance, *Pseudomonas* spp. *relative abundance,* bacterial community evenness).

Model	Intercept	*Bacillus* spp.	*Pseudomonas* spp.	Bacterial community evenness	AICc	Delta	Weight
1	0.2640	−2.48	0.3489		−42.5	0	0.297
2	0.5705	−2.441	0.698	−0.342	−42.0	0.49	0.232
3	0.2660	−2.458			−40.6	1.85	0.118
4	0.5002	−2.409		−0.2595	−40.1	2.36	0.09
5	0.2024		−0.9867		−39.6	2.93	0.068
6	0.7055		−0.3513	−0.5599	−39.4	3.06	0.064
7	0.1945				−37.4	5.07	0.026
8	0.7278			−0.5876	−37.3	5.16	0.022

Delta indicates the difference in AICc from the best-fit model, and the AICc weight of each model, which represents the likelihood of the model relative to competing ones. Retained top model is shaded gray, and factors that were not retained in any of the top eight candidate models were eliminated from tables. Original global model included the following factors: concentrations of nitrate-N, ammonium-N, plant available P, K, SO_4, pH, organic matter, Bacillus spp. relative abundance, Pseudomonas spp. relative abundance, microbial community evenness, and microbial biomass (mg/kg soil).

TABLE 3 | Model selection table for the top soil-based predictors of aphid growth across 20 organically managed farm soils (*Bacillus* spp. relative abundance, *Pseudomonas* spp. relative abundance, bacterial community evenness, NH_4, percent organic matter, and pH).

Model	(Intercept)	*Bacillus* spp.	*Pseudomonas* spp.	Bacterial community evenness	NH_4	% Organic matter	pH	AICc	Delta	Weight
1	191.60	72.28	1667	−127.7			−11.06	276.3	0	0.263
2	178.30	77.66	1772	−116		1.365	−11.6	276.4	0.13	0.246
3	194.60	71.78	1834	−129.4	−0.21		−11.53	278.4	2.16	0.089
4	178.50	77.62	1780	−116.1	−0.01	1.36	−11.63	278.8	2.57	0.073
5	20.25		1709					333.6	26.13	0
7	29.92	133.60						342.2	34.75	0
8	9.619			26.67				342.9	35.43	0
9	73.45						−6.11	349	41.48	0
10	33.82							352.9	45.45	0

Delta indicates the difference in AICc from the best-fit model, and AICc weights represent the likelihood of the model relative to competing ones. Retained best-fit model is shaded gray, and factors that were not retained in any of the top five candidate models were eliminated from tables. Models 5–10 are single-parameter and intercept-only models for reference. Original global model included the following factors: concentrations of nitrate-N, ammonium-N, plant available P, K, SO_4, pH, organic matter, Bacillus spp. relative abundance, Pseudomonas spp. relative abundance, microbial community evenness, and microbial biomass (mg/kg soil).

included relative abundances of *Bacillus* spp. and *Pseudomonas* spp. ASVs agglomerated at the genus level.

Aphid Colony Growth and Parasitism

The top model predicting aphid parasitism contained only *Bacillus* spp. (**Figure 3A** and **Table 2**; marginal R^2 = 0.0928, conditional R^2 = 0.113). Relative abundance of *Bacillus* marginally negatively correlated with aphid parasitism (Coefficient = −1.445, *SE* = 1.277, T = −1.974, *P* = 0.0639). Across diverse soil communities, caterpillar co-herbivory reduced aphid parasitism by 57% (**Figure 3B**, Coefficient = 0.089, *SE* = 0.036, T = 2.461, *P* = 0.0242).

The top model predicting aphid colony growth contained *Bacillus* spp., *Pseudomonas* spp., bacterial community evenness, and pH (**Table 3**; conditional R^2 = 0.2177957, marginal R^2 = 0.272799). Of these, only *Pseudomonas* spp. and pH significantly correlated with aphid growth. Aphid colony growth increased with *Pseudomonas* spp. relative abundance in the soil, and marginally decreased with increasing soil pH (**Figures 4A,B** and **Table 4**). Despite the strong negative effect of caterpillar co-herbivory on aphid parasitism, caterpillars did not significantly increase aphid colony growth (**Figure 4C**, Coefficient = −6.27325, *SE* = 6.837, T = −0.917, *P* = 0.372).

DISCUSSION

We provide new evidence of the importance of plant–microbe associations in tritrophic interactions by examining them in naturally diverse soil communities across a gradient of resource availability. Here, we trade experimental control for ecological relevance by evaluating numerous correlative predictors of herbivore performance across 20 different farm soils and their unique bacterial communities in a common garden. In contrast to experiments using inoculations of isolated PGPR strains (Zehnder et al., 1997; Gadhave and Gange, 2016), we found no evidence of herbivore suppression associated with either *Bacillus* spp. or *Pseudomonas* spp. relative abundance in the ambient soil microbial community. Rather, our data suggest that these bacterial genera may increase susceptibility of *B. oleracea* to aphids in field environments. None of the soil nutrients we examined associated with aphid growth. This suggests that plant–microbe interactions might have stronger effects on cabbage aphids than nutrient limitation.

Bacillus spp. negatively associated with aphid parasitism rates (**Figure 3A**). This pattern from soil communities collected from the field is consistent with another controlled laboratory experiment showing that PGPRs can interfere with volatile signaling that enables parasitoids to locate prey (Pineda et al., 2013). While *Bacillus* spp. are effective at priming chemical defenses induced by the jasmonic acid (JA) and ethylene defense signaling pathways (Pozo et al., 2008; Pangesti et al., 2013), as well as increasing parasitoid attraction to caterpillars (Pangesti et al., 2015b), they have also been found to induce susceptibility to phloem-feeding insects (Shavit et al., 2013). In contrast, other studies found that single-strain inoculations of different *Bacillus* strains reduced aphid growth (Valenzuela-Soto et al., 2010) and parasitism (Gadhave et al., 2016a); however, negative effects may be neutralized in mixed-strain treatments (Herman et al., 2008; Gadhave et al., 2016a,b) that more accurately reflect the diverse microbial communities of organically managed soils we examine here (Li et al., 2017; Schmid et al., 2017). We suspect that one or more *Bacillus* taxa, including *B. coagulans, B. lentus, B. drentensis*, and *B. acidiceler* identified in our study, may have interfered with parasitoid attraction.

Co-infestation with caterpillars also reduced aphid parasitism (**Figure 3B**). A competing chewing herbivore might dilute or constrain volatile signals exploited by aphid parasitoids to find their prey (Vos et al., 2001), reducing top–down suppression. Indeed, our recent fieldwork in this system showed that co-herbivory by chewing herbivores reduced aphid parasitism as well as concentrations of secondary metabolites important in aphid-specific volatile signaling (Blubaugh et al., 2018). This consistent pattern detected across a gradient of microbial diversity further

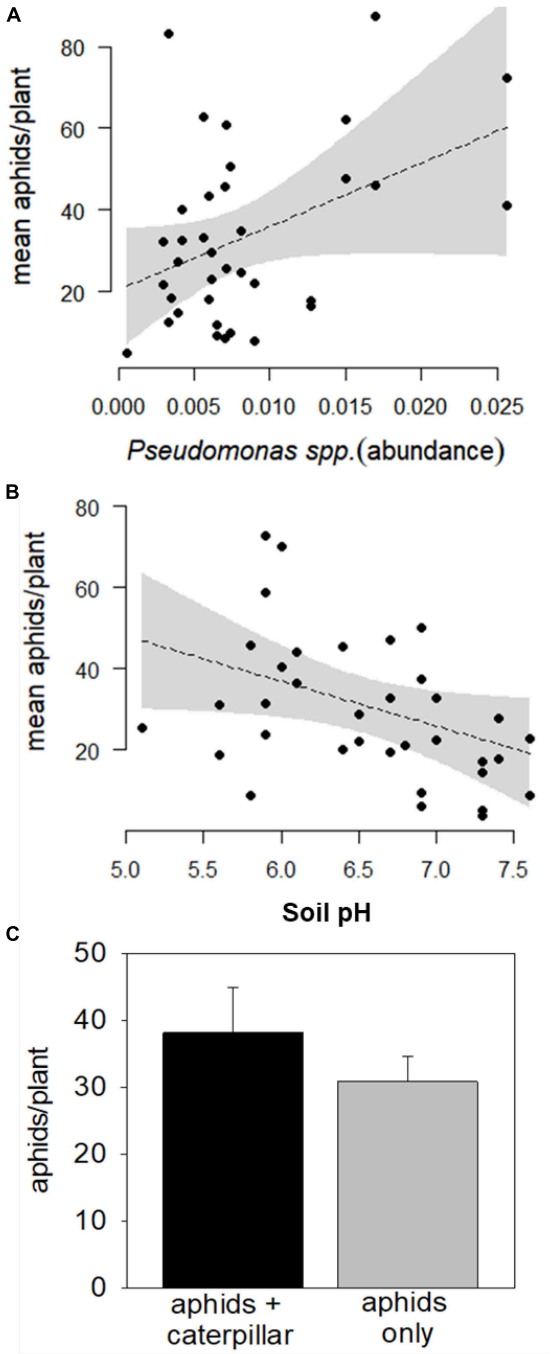


FIGURE 4 | Relationship between **(A)** *Pseudomonas* spp. relative abundance, **(B)** soil pH, and **(C)** caterpillar co-herbivory on colony growth of cabbage aphids (*B. brassicae*) in the common garden field assay across 20 organic farm soils. Scatterplots represent output from mixed-effects models and the shaded gray area indicates a 95% confidence interval. Error bars indicate standard error of the mean.

emphasizes the importance of herbivore community structure in determining the outcome of tritrophic interactions.

The best-fit model predicting aphid colony growth contained *Pseudomonas* spp., which associated with greater aphid increase

TABLE 4 | Output from the top model predicting aphid colony growth over 7 weeks across 20 organically managed farm soils.

Predictor	Coefficient	*SE*	*T*	*P*-value	
(Intercept)	159.51	165.76	0.96	0.348	
pH	−9.95	5.14	−1.93	0.0723	.
Pseudomonas spp.	2046.47	608.92	3.36	0.0043	*
Bacillus spp.	95.44	210.46	0.45	0.6567	
Bacterial community evenness	−88.41	191.06	−0.46	0.6502	

**Denotes significance at the 0.05 level of alpha.*

(**Figure 4A**), and soil pH, which marginally associated with decreased aphid colony growth (**Figure 4B**). Again, while *Pseudomonas* PGPRs have often been found to induce systemic resistance to chewing herbivores (Pineda et al., 2010; Hol et al., 2013), our results suggest that these well-known PGPRs may have facilitated growth of phloem feeders (e.g., Pineda et al., 2012). This putative case of induced susceptibility could have occurred because of a tradeoff in defense signaling pathways, as *Pseudomonas* may suppress salicylic acid-mediated defenses by priming induction of the JA pathway (e.g., Haney et al., 2017). However, defenses induced along the JA pathway are more likely to limit aphid growth rather than facilitate it Züst and Agrawal (2016). Instead, cabbage aphid growth may correlate with the relative abundance of JA-priming PGPRs in our study because specialist cabbage aphids (*B. brassicae*) use secondary metabolites as feeding stimulants and are often not limited by them (**Figure 3C**, Züst and Agrawal, 2016). Indeed, *B. brassicae* densities can increase in response to high glucosinolate concentration (Cole, 1997; Stafford et al., 2012), as they selectively sequester glucosinolates for their own defenses against predators (Kazana et al., 2007; Kos et al., 2012). For this reason, cabbage aphids notoriously perform better in organically managed soils (Staley et al., 2010; Stafford et al., 2012), which facilitate chemical defense induction, compared with conventionally managed soils that generally have lower levels of biological activity (Mäder et al., 2002).

The marginally negative effect of higher soil pH on cabbage aphid growth in our study (**Figure 3B**) may relate to historical soil inputs at our sites. Soil acidification can be a legacy of long-term ammoniacal fertilizer inputs in intensified conventional farming systems (Barak et al., 1997; Birkhofer et al., 2008), and many of our participating organic farms recently transitioned from such systems. Acidic soils can reduce plant vigor and may constrain anti-herbivore defenses (e.g., Birkhofer et al., 2008). Generally, the marginal effects of soil pH on aphid growth, along with other non-significant factors associated with soil fertility and host plant quality, suggest that microbial symbionts in our system (*Pseudomonas* spp. and *Bacillus* spp.) may be stronger drivers of herbivore performance than the nutritional components of chemical defense. Although the expense and challenge of collecting and sequencing soils from numerous sites limited the replication of our study, this work is biologically meaningful because there is still a dearth of evidence

from the field describing microbe-mediated interactions (Schreiter et al., 2018). As the affordability of microbial community sequencing improves, it is critical to evaluate tritrophic interactions in their realistic ecological contexts, accounting for diversity in microbial and herbivore community structure.

These results suggest a pattern of induced susceptibility to aphids by bacterial taxa that are usually considered PGPRs, ostensibly via direct and indirect (volatile and parasitoid-mediated) pathways. Because a correlative approach is necessary for evaluating ambient bacterial communities in the soil, we cannot determine causal links or provide clear mechanistic detail. However, our results from the field are consistent with experimental greenhouse work showing PGPR-induced susceptibility to phloem feeders (e.g., Pineda et al., 2012, 2013). This first step at evaluating the function and importance of known PGPRs in soil communities implies that there are costs as well as benefits of plant-colonizing soil microbes that prime plant defensive processes, and that these costs likely depend on the identity of herbivores (i.e., chewer or phloem-feeder) and the intensity of herbivore pressure. Next steps will (1) link these patterns in relative abundance of PGPRs with defensive plant chemistry, (2) identify environmental drivers of soil bacterial community structure, such as soil disturbance or fertility amendment materials, and 3) characterize interaction webs between naturally occurring soil microbes, naturally diverse herbivore communities, and volatile-mediated prey suppression. Importantly, neither herbivore growth nor top–down suppression associated with soil fertility in this study; this suggests that predictions made about plant investments in chemical defense based merely on resource availability are inadequate to describe the complexity of plant-mediated interactions in multi-trophic, real-world communities.

AUTHOR CONTRIBUTIONS

CB, WS, JR, and LC-B designed the experiment. CB performed experiment and wrote the paper. RS performed bioinformatics. All co-authors edited drafts of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01239/full#supplementary-material

TABLE S1 | Soil nutrient and microbial data, insect counts on broccoli plants, and site locations where soil samples were collected in April 2016 for a common garden experiment.

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Bringing Ecology Back: How Can the Chemistry of Indirect Plant Defenses Against Herbivory Be Manipulated to Improve Pest Management?

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Research on insect–plant interactions has highlighted the intricacies of constitutive and induced plant defenses. Of particular interest has been the relationship of natural enemies (especially parasitic hymenoptera) to herbivore induced changes to plants, especially their responses to herbivore induced plant volatiles (HIPVs). In recent decades this has been a fertile area for research, with elegant experiments showing that HIPVs are important in attracting natural enemies to plants. We critically appraise the application of work on HIPVs in plant–insect–natural enemy interactions. The promise of applications to improve pest management has not been forthcoming. We attribute this to a failure to include the multifaceted aspects of natural enemy–prey interactions – attraction, location, subjugation and experience. Attraction in an olfactometer by naïve parasitoids has not been translated to methodologically sound field-based estimates of higher parasitism rates. We highlight what needs to be done to better understand the information that HIPVs convey, how this is utilized by parasitoids and how a greater understanding of these interactions might lead to the development of new strategies so that this knowledge can be effectively deployed for improved pest management.

Keywords: herbivore induced plant volatiles, jasmonic acid, salicylic acid, indirect defense, parasitoid, chemical ecology, integrated pest management

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INTRODUCTION

A wide range of pathogens and sucking, chewing and boring herbivores assail all parts of plants in nature. Recent research has revealed the intricacies and sophistication of a Pandora's box of chemical interactions that mediate plant responses to this attack (see Kaplan, 2012; Schuman and Baldwin, 2016). Plants have a range of constitutive (e.g., Zalucki et al., 2001; Steppuhn et al., 2004) and induced chemical defenses (Kessler and Baldwin, 2002) that can act either directly or indirectly on herbivores. Induced defenses represent a sophisticated, layered set of responses that are modulated by a complex phytohormone system (Wu and Baldwin, 2010; Schuman and Baldwin, 2016; Howe et al., 2018). For example, necrotrophic microbes, some phloem-feeding insects and chewing herbivores induce the jasmonic acid (JA) pathway (Howe and Jander, 2008), whereas some phloem-feeding insects and biotrophic pathogens induce the salicylic acid (SA) pathway (Spoel et al., 2007). Cross-talk between these pathways, enables plants to regulate their defense responses according to the type of attacker (Koornneef and Pieterse, 2008; Thaler et al., 2012).

Following plant damage, induced direct defenses lead to localized and systemic elevation of toxic secondary compounds above constitutive levels, which affect the preference, performance and feeding behavior of herbivores (Wittstock and Gershenzon, 2002; Perkins et al., 2013; Zalucki et al., 2017). The increased release of volatiles from a plant after attack by a herbivore is considered to be an induced indirect defenze, communicating the location of herbivores on infested plants to parasitoids and predators of the attackers (Vet and Dicke, 1992; Dicke et al., 2003a,b; Turlings and Erb, 2018). Numerous studies have shown the importance of multi–trophic relationships in plant-insect-natural enemy "attack-defenze" systems. These may be as a result of aboveground (van Dam et al., 2003) or belowground herbivory (van Dam and Oomen, 2008; Qiu et al., 2009) and they can interact in complex ways (Soler et al., 2005, 2007; van Dam and Heil, 2011).

In natural environments it is very likely that plants suffer sequential and/ or simultaneous attack by numerous herbivores and pathogens. Plant pathogens can suppress the host plant defenses that they induce by leveraging SA-JA crosstalk using chemical toxins and the virulent effector proteins they secret (Xin and He, 2013; Kaloshian and Walling, 2016). In this context, the distinction between plant pathogens and insect symbionts can become blurred. For example, co-infection of tobacco plants with a begomovirus and its betasatellite represses JA-regulated defenses, allowing whitefly populations to increase (Zhang et al., 2012). In this system the compatibility of the tobacco–begomovirus interaction means that SA levels are unaffected, and the suppression of the JA-regulated defenses is independent of SA (Zhang et al., 2012). Similarly, herbivore manipulation of plant defenses has been associated with the chemical compounds and specific proteins in the saliva of chewing and phloem-feeding insect herbivores (Will et al., 2007; Elzinga et al., 2014; Villarroel et al., 2016), making the precise outcome of interactions difficult to predict. Thus, in any given system, simultaneous interactions, each of which can have positive or negative effects of varying degrees of magnitude at the individual and population level, likely manifest as an indeterminate orgy of multipartite interactions. This, coupled with an incomplete understanding of specific relationships, makes reliable predictions of outcomes problematic. This at least partly accounts for the near absence of successful field applications of technologies based on the deployment of herbivore induced plant volatiles (HIPVs) or the chemical manipulation of plants to suppress herbivore pest populations (Turlings and Erb, 2018).

EXPLOITING HOST PLANT ODORS FOR PEST SUPPRESSION

This is not to suggest that host plant odors have not been successfully manipulated for improved pest management. They provide the mechanism underpinning push- pull strategies (Cook et al., 2007), which rely on plant odors to repel pest insects from crops and attract them to non-crop plants at the margins of fields. The most prominent example of the successful deployment of this strategy involves the companion cropping of maize with the forage grass *Melinis minutiflora* or the forage legume *Desmodium* sp. and the planting of other grasses, e.g., *Pennisetum purpureum* or *Sorghum vulgare sudanaense* at the field margins (Pickett et al., 2014; Pickett and Khan, 2016). Volatiles produced by *M. minutiflora* or *Desmodium* sp. repel ovipositing stem borers from the maize crop (Khan et al., 2000), thereby providing the "push," while other volatiles produced by the specific forage grasses planted on the margins attract the ovipositing females, thereby producing the "pull" away from the crop (Khan et al., 2000). This strategy, and well researched variants, has been adopted by approximately 120,000 small holders in sub-Saharan East Africa (Pickett and Khan, 2016). Despite this undoubted success, further claims that the attractive properties of volatiles emitted by *M. minutiflora* themselves result in meaningful greater parasitism of stem borers on maize (Khan et al., 1997; Midega et al., 2009) need to be treated with caution. These compounds clearly invoke strong behavioral responses in parasitoids in the laboratory (Khan et al., 1997; Tamiru et al., 2012), but their impact on pest mortality in the field requires further investigation. Reported parasitism rates are typically derived from small or unreported sample sizes and, even when statistically significant changes in parasitism rates are reported, increases are small and not contextualized with respect to other mortality factors (see Van Driesche et al., 1991). Similarly, although stemborer oviposition on maize has been demonstrated to increase attraction of egg and larval parasitoids in the laboratory (Tamiru et al., 2011), evidence demonstrating increased egg mortality in the field as a result is lacking. Further, the interpretation of increased larval parasitoid attraction as early recruitment of natural enemies in anticipation of egg hatching (Tamiru et al., 2011) is teleological. Indeed, *Cotesia sesamiae* (Cameron) (Hymenoptera: Braconidae) attacks late larvae of *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) (Chinwada et al., 2003) and naïve females of this species are attracted to, but do not discriminate between, maize plants infested by *C. partellus* and the non-host *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) (Ngi-Song et al., 1995, 1996).

PARASITOID RESPONSES TO HERBIVORE INDUCED PLANT VOLATILES

Although naïve parasitoids can be attracted to the volatiles produced by plants that are attacked by their host herbivores, our work shows that they can equally be attracted to volatiles emitted by those plants when they are attacked by non-host herbivores, or even when they are induced by a chemical elicitor, such as exogenous JA (**Figure 1A**). These responses are typically modulated by parasitoid experience, such that experienced parasitoids orientate preferentially toward plants upon which they have previously successfully located and parasitized a host (**Figure 1A**). Thus, although parasitoids might exhibit an innate preference for the volatiles produced by certain plants (Allison and Hare, 2009), such preferences can be overridden by experience (**Figure 1A**). Parasitoid orientation to a plant emitting volatiles that might be associated with the presence

of host larvae is merely the first step in host location. Upon alighting on the plant surface, parasitoids typically become arrested (**Figures 1B**, **2**), before increasing activity following the perception of post-alighting cues (which might include volatiles released as a result of herbivore feeding damage, non-volatile plant metabolites or herbivore frass), intensifying their search around sites of herbivore feeding damage before locating, sampling and then parasitizing their hosts (Wang and Keller, 2002; **Figures 1C**, **2**). The vast majority of studies that seek to manipulate the responses of plants to herbivory in order increase biological control of pests rely on the application of a plant defense response elicitor (e.g., JA, SA or their derivatives) with the express purpose of attracting parasitoids to treated plants. The importance of subsequent parasitoid behaviors (see **Figure 2** for description of post-alighting fraging behavior) is then not considered, ignoring factors that are critical to successful host location and subsequent attack. For example, although the diamondback moth parasitoid, *Diadegma semiclausum* (Hellén) (Hymenoptera: Ichneumonidae), orientates toward and alights on intact, host-free cabbage plants treated with exogenous applications of JA, its post-alighting behavior is inhibited. Our work shows that the residency time of individuals alighting on JA-treated plants is the same as that of individuals landing and foraging on plants infested with host larvae (**Figure 1B**). Furthermore, experienced individuals locate hosts much more quickly than naïve parasitoids, demonstrating the importance of post-alighting cues in successful host location (**Figure 1C**). The lack of consideration of the subtlety and sophistication of the post-alighting chemical ecology of host–parasitoid interactions likely contributes to the dearth of examples where these strategies have been successfully deployed in the field. Further research to better understand these behaviors in parasitoids that are targeted for field manipulation by exploiting host–plant interactions for improved pest management are encouraged.

Other examples of "field" experiments that demonstrate changes in parasitoid attack rates are small-scale and typically lack adequate controls. Invariably the design consists of a single field into which "test" plants are placed to assess parasitism, usually with both treatments juxtaposed (Lou et al., 2005, 2006; de Lange et al., 2018). Apart from the issues of pseudoreplication associated with such an approach, the key problem is that any change in parasitism rate reflects either a change in parasitoid searching efficiency or a change in number of parasitoids foraging due to a combination of short (within field) or longer-range attraction. If the mechanism responsible for increased parasitism is an increase in parasitoid foraging efficiency then the lower parasitism of non-treatment plants within the experimental field reflects the confounding influence of the more attractive plants, which results in decreased attacks on non-treatment plants within the field. What is required is the deployment of independent control blocks, without treatment, that allow background parasitism rates to be assessed and then compared with parasitism rates in independent treated blocks. Without this approach, the apparent changes in parasitism in treated plants cannot be quantified in appropriate context as such changes clearly do not necessarily reflect increased parasitism at the population scale, which must be the goal for improved pest management. The use of multiple fields (blocks) with treatments in a randomized design is a better approach but even when this is done, experimental units are small, e.g., 7×7 plants (Poelman et al., 2009) or 4–10 plants (Simpson et al., 2011) and not independent. Although such experiments can provide important and useful preliminary information, their relevance to actual farm fields is limited, except perhaps in situations where fields are very small, and they require larger scale follow-up studies.

UTILIZING HERBIVORE INDUCED PLANT VOLATILES FOR IMPROVED BIOLOGICAL CONTROL AND PEST MANAGEMENT

Herbivore induced plant volatiles can be used by parasitoids and predators to locate plants upon which their arthropod hosts or prey are feeding (e.g., Reddy, 2002; Sobhy et al., 2014). However, the role of these compounds in effecting arthropod herbivore population suppression at the field scale is still to be demonstrated. Synthetic volatiles have been deployed as lures within crops (Rodriguez-Saona et al., 2011), and although some success has been demonstrated with respect to the attraction of arthropod predators and parasitoids (James, 2003; James and Grasswitz, 2005), other studies show that HIPVs can have repellent effects on natural enemies (Braasch et al., 2012). The scale over which parasitoids and predators can be manipulated by synthetic HIPVs is typically small (<10 m) (Lee, 2010; Braasch and Kaplan, 2012), meaning that the major effect of their deployment is likely to be the localized redistribution of natural enemies within crops. This has been shown for some parasitic hymenoptera where the attraction to lures affects local abundances by depleting parasitoid numbers elsewhere in the crop (Braasch and Kaplan, 2012). The redistribution and retention of natural enemies in areas of the crop from which their prey might be absent (**Figure 1B**), and where their foraging behavior is further disrupted by the absence of appropriate post-alighting cues (**Figure 2**), is likely to disrupt rather than facilitate the conservation biological control strategies into which HIPVs are integrated. Given these effects perhaps the ways in which HIPVs can be best deployed for improved pest management should be re-evaluated; rather than attractants to support conservation biological control, they might be best utilized as arrestants to complement augmentative or even inundative releases of natural enemies (Kaplan, 2012).

The introduction of synthetic HIPVs into crops can induce natural defense responses in nearby plants (Kaplan, 2012) and the application of chemical elicitors (Sobhy et al., 2014) or the genetic manipulation of plants (Beale et al., 2006) to make then more attractive to natural enemies has been advocated. Various elicitors have been shown to change the volatile profiles of maize plants infested by *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), such that they become more attractive to parasitic hymenoptera (Rostas and Turlings, 2008; Sobhy et al., 2014) in laboratory assays. When the strategy was field tested, despite the elicitors changing plant volatile profiles in

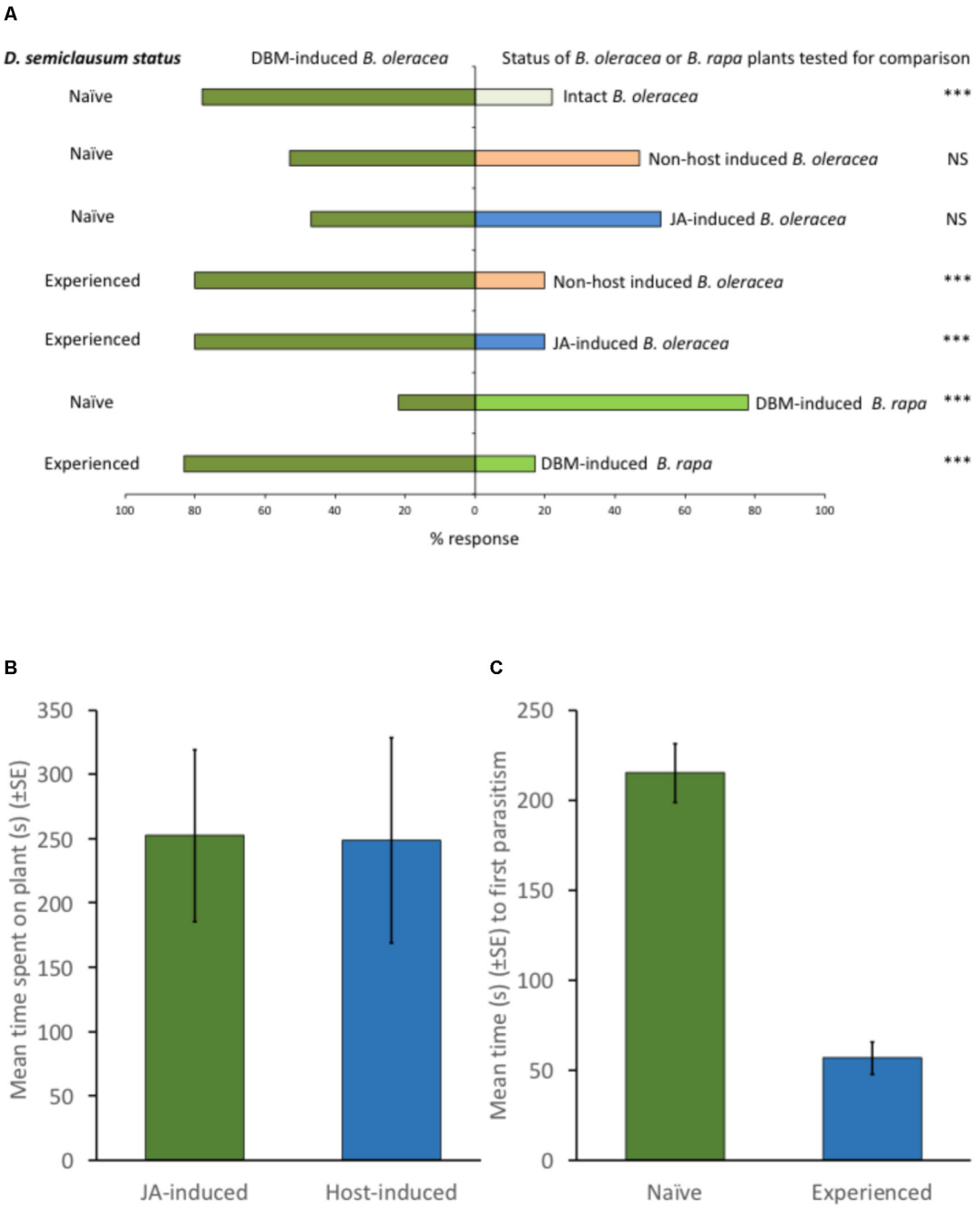

FIGURE 1 | (A) Effect of previous experience on the responses of *Diadegma semiclausum* Hellén (Hymenoptera: Ichneumonidae) to cabbage (*Brassica oleracea* capitata cv sugarloaf) and Chinese cabbage (*Brassica rapa* pekinensis cv Wombok) host plants (6-leaf stage) in olfactometer bioassays. *Methods:* The olfactometer consisted of a glass Y-tube (0.8 cm internal diameter, 7 cm stem) with two 9.5 cm arms at a 60° angle leading to sealed glass chambers, each containing a single plant. Clean air (filtered through activated charcoal filters before entry into the apparatus) was drawn through the system with a vacuum pump at a rate of 1 L min^{-1}. Test insects were introduced to the end of the Y-tube stem 6 cm away from the Y-split. A choice was considered made once a test insect breached the 2.5 cm mark up an arm of the Y-tube. Each test insect was given up to 10 min to respond, and each insect was used only once. For each pairwise combination of plant treatments, fresh female parasitoids were used until 30 individuals had responded. The Y-tube was rotated between each replicate, and between every five consecutive replicates, odor sources were replaced, and all glassware was washed in 95% ethanol, rinsed with distilled water and then dried at 75°C for 1 h. All tests were performed in a draught-free room at 24 ± 1°C under an artificial light. *Statistical tests:* Preferences of parasitoids in the various pairwise tests were analyzed using a X^2-test. $^{NS}P > 0.05$ ($X^2_{(df = 1)} < 3.841$); $^{***}P < 0.001$ ($X^2_{(df = 1)} > 10.828$). *Results:* Naïve parasitoids [= parasitoids (1–2 days post-eclosion)] that had not foraged for or oviposited into a host [*Plutella xylostella* L. (Lepidoptera: Plutellidae) (= diamondback moth, DBM)] larva on a cabbage plant were more attracted to host-damaged plants (feeding by 10 early 3rd instar DBM larvae for 24 h immediately prior to test, larvae removed prior to olfactometer bioassay) than to

(Continued)

FIGURE 1 | Continued
intact plants ($P < 0.001$), but did not discriminate between DBM-damaged plants and non-host [*Crocidolomia pavonana* (F) (Lepidoptera: Crambidae) (= large cabbage moth, LCM)] damaged plants (feeding by 10 2nd instar LCM larvae for 24 h immediately prior to test, larvae removed prior to olfactometer bioassay) ($P > 0.05$) or DBM-damaged plants and plants treated with jasmonic acid (JA) (aqueous solution of 0.1 μmolL^{-1} JA applied to plants 24 h prior to test; see Lu et al., 2004 for methods). Experienced parasitoids (= parasitoids that had oviposited into a host DBM larva on a cabbage plant) were more attracted to DBM-damaged plants than LCM-damaged plants ($P < 0.001$) or plants treated with JA ($P < 0.001$). Although naïve parasitoids were more attracted to DBM-damaged Chinese cabbage plants than DBM-damaged cabbage plants ($P < 0.001$), parasitoids previously experienced on DBM on cabbage plants were more attracted to DBM-damaged cabbage plants than DBM-damaged Chinese cabbage plants in subsequent olfactometer assays ($P < 0.001$). **(B)** Effect of DBM larvae (damage and presence) and jasmonic acid (JA) treatment on *D. semiclausum* post-alighting residency time on cabbage plants. *Methods:* Experiments were conducted in a wind tunnel with a Perspex flight chamber (160 cm × 65 cm × 65 cm). A fan circulated clean air (passed over an activated charcoal filter) through the chamber at 0.7 ms^{-1}. Laminar airflow was obtained by passing air through a honeycomb of soda straws and a fine stainless-steel screen (1.25 mm aperture) before it entered the chamber. A single potted cabbage test plant (6-leaf stage) was placed in the centre of the chamber 45 cm from the screen and a single female *D. semiclausum* released 50 cm downwind. The responses of parasitoids to JA-treated plants (aqueous solution of 0.1 μmolL^{-1} JA applied to plants 24 h prior to test; see Lu et al., 2004 for methods) and to DBM-infested (10 early 3rd instar larvae for 24 h) plants were compared by alternating JA-treated and DBM-infested plants in the chamber and recording the *D. semiclausum* response; each parasitoid was given up to 5 min to respond, and each insect was used only once. When a parasitoid alighted on the test plant it was watched carefully and the time it spent on the plant (= residency time) recorded. The responses of single parasitoids to 9 plants of each treatment were recorded. *Statistical tests:* Data were tested for normality (D'Agostino and Pearson test) and then subject to an unpaired *t*-test (Prism 7, Graphpad Software, Inc., 2017). *Results:* Parasitoids spent as long on JA-treated plants ($n = 9$) as they did on DBM-infested (10 early 3rd instar larvae) plants ($n = 9$) [$t = 0.036$ ($df = 16$), $P = 0.972$]. **(C)** Effect of host experience on post-alighting parasitoid foraging efficiency. *Methods:* Single *D. semiclausum* cocoons were transferred to clean glass tubes (0.8 cm × 4 cm). Upon eclosion female parasitoids were fed (10% honey solution) and some parasitoids were then experienced by allowing a single wasp to forage on cabbage plants (6-leaf stage) infested with third instar DBM larvae in a mesh cage (45 cm × 45 cm × 45 cm). Once a parasitoid attacked a host larva it was removed and held in a labeled glass tube. Cabbage plants (6-leaf stage; $n = 20$) were infested with 20 late instar larvae and transferred singly to mesh cages for 24 h. A single naïve or a single experienced *D. semclausum* was then introduced to each cage, carefully observed and the time taken to attack the first host larva was recorded. *Statistical tests:* Data were tested for normality (D'Agostino and Pearson test) and then subject to an unpaired *t*-test (Prism 7, Graphpad Software, Inc., 2017). *Results:* Naïve parasitoids ($n = 10$) took significantly longer to locate and parasitize a larva after alighting on a DBM-infested cabbage plant than previously experienced parasitoids ($n = 10$) [$t = 7.834$ ($df = 18$), $P < 0.0001$].

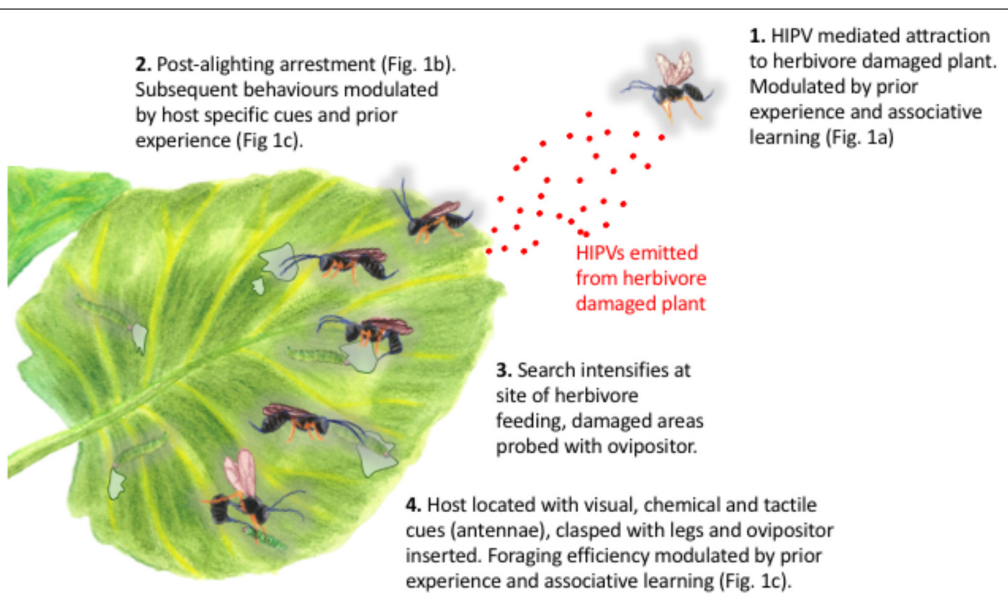

FIGURE 2 | Attraction, orientation and post-alighting behavior of parasitoid wasps in response to host-herbivore infested plants. Post-alighting behavioral sequence based on *Diadegma semiclausum* Hellén (Hymenoptera: Ichneumonidae) as described by Wang and Keller (2002).

ways consistent with previous work in the laboratory, reliable increases in parasitism of *Spodoptera frugiperda* (J.E. Smith) could not be demonstrated (von Mérey et al., 2012). Similarly, genetic manipulation of *Arabidopsis thaliana* and wheat plants so that they constitutively produced aphid alarm pheromone was successful at repelling aphids and attracting aphid parasitoids in the laboratory (Beale et al., 2006; Bruce et al., 2015). However, when tested in the field wheat plants became infested with aphids and these showed no increased levels of parasitism (Bruce et al., 2015). In addition to changing the responses of parasitoids to induced plants, HIPVs also affect herbivore–plant interactions. In the *Brassica oleracea–Plutella xylostella* interaction, herbivore and JA-induced plants are more attractive to ovipositing *P. xylostella* (Lu et al., 2004), *D. semiclausum* (**Figure 1**) and predatory lacewing larvae (Furlong, unpublished data). The increased attraction is temporary, and plants revert to their original level of attractiveness 3–4 days after the application of JA or a feeding event (Ang et al., 2016). Selective application of JA to *B. oleracea* plants in the field can manipulate the spatial patterns of *P. xylostella* oviposition (Ang, 2018), raising the possibility of

utilizing JA to temporarily induce parts of the crop as a "temporal trap-crop," where *P. xylostella* eggs can be concentrated for destruction.

CONCLUSION

Understanding of the molecular basis of plant responses to herbivore attack has increased markedly in recent years, but the technologies that have been developed have not led to more effective use of natural enemies for pest management. This is at least in part due to an under appreciation of how natural enemies utilize and respond to the cues they perceive from herbivore damaged plants under field conditions. Simply deploying HIPVS to attract natural enemies has not worked, however, developing a better understanding of the ecology of these interactions will inform how HIPVs and other components of plant–herbivore–natural enemy interactions might be more effectively used in as yet untested ways.

AUTHOR CONTRIBUTIONS

MF and MZ conceived and designed the overall study. GA, RS, and MF designed the experiments. GA and RS conducted the experiments. MF and MZ wrote the paper. GA did the artwork for **Figure 2**.

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PERSPECTIVE
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The Ecology of Plant Chemistry and Multi-Species Interactions in Diversified Agroecosystems

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Over the past few years, our knowledge of how ecological interactions shape the structure and dynamics of natural communities has rapidly advanced. Plant chemical traits play key roles in these processes because they mediate a diverse range of direct and indirect interactions in a community-wide context. Many chemically mediated interactions have been extensively studied in industrial cropping systems, and thus have focused on simplified, pairwise and linear interactions that rarely incorporate a community perspective. A contrasting approach considers the agroecosystem as a functioning whole, in which food production occurs. It offers an opportunity to better understand how plant chemical traits mediate complex interactions which can enhance or hinder ecosystem functions. In this paper, we argue that studying chemically mediated interactions in agroecosystems is essential to comprehend how agroecosystem services emerge and how they can be guaranteed through ecosystem management. First, we discuss how plant chemical traits affect and are affected by ecological interactions. We then explore research questions and future directions on how studying chemical mediation in complex agroecosystems can help us understand the emergence and management of ecosystem services, specifically biological control and pollination.

Keywords: volatile organic compounds, indirect interactions, ecosystem services, biological control, pollination, secondary plant chemistry, induced plant responses, agroecosystems

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INTRODUCTION

It is well known that plant mediated interactions strongly influence the structure of natural communities (Connell and Slatyer, 1977; Roughgarden and Diamond, 1986). Resource competition (Connell, 1983; Schoener, 1983), allelopathy (Rice, 1984; Williamson, 1990) and facilitation (Hunter and Aarssen, 1988; Callaway, 1995) affect plant community organization, while plant mutualistic and antagonistic interactions with other organisms may structure communities (Van Zandt and Agrawal, 2004; Lewinsohn et al., 2006; Vizentin-Bugoni et al., 2014; Bergamo et al., 2017). Such interactions occur either by direct effects, when a plant trait affects the physiology or behavior of another organism; or by indirect effects, when an interaction with the plant has knock-on effects on a third organism not initially involved in this interaction (Ohgushi et al., 2007). Therefore, plant

mediated interactions potentially link organisms of different trophic levels and add complexity within community interactions (Utsumi et al., 2010).

Plant-mediated interactions have been studied in agricultural systems due to the impact that herbivores, pathogens and pollinators have on plant production, and the mechanisms behind plant resistance, tritrophic interactions and pollinator attraction are well known (Agrawal and Rutter, 1998; Gatehouse, 2002; Klein et al., 2007; Schiestl, 2015). However, most studies have focused on commodity crops produced in large-scale monocultures. In Neotropical systems, there is surprisingly little focus on smallholder production systems (Pinto-Zevallos et al., 2016; Pinto-Zevallos et al., 2018), while soybean, maize and sugarcane are extensively studied (e.g., Peñaflor et al., 2011, 2017; Michereff et al., 2015). However, staple and horticultural crops are produced overwhelmingly by smallholders (Food and Agriculture Organization [FAO] et al., 2015) who often use intercropping and diversification practices (Altieri et al., 2012). These alternative practices are applied to manage agroecosystems based on ecological knowledge for ecosystem function improvement(Lewis et al., 1997; Vandermeer et al., 2010).

The integration of knowledge from chemical ecology into agroecology has been slow in the last decades, with a few notable exceptions (Cook et al., 2007; Khan et al., 2008, 2014). Chemical ecology can provide mechanistic knowledge of plant mediated interactions, which is essential due to the ecological complexity of agroecosystems. Chemical traits are important in mediating plant interactions because: (1) the high diversity of chemical compounds may confer unique combinations of primary and secondary compounds to species and individual plants (Krischik and Denno, 1989; Hartmann, 1996); (2) their expression is dynamic, changing with plant ontogeny and environmental cues (Barton and Koricheva, 2010; Quintero and Bowers, 2012); (3) they are subject to significant spatial and temporal variation (Feeny, 1970; Edwards et al., 1990; Okolie and Obasi, 1993); and (4) chemical perception is the dominant sensory mode in arthropods and microorganisms; these are the most abundant groups of organisms that interact with plants (de Bruyne and Baker, 2008; Bruce and Pickett, 2011; Junker and Tholl, 2013). Therefore, the plant chemical profile is a dynamic and multifunctional trait involved in a great diversity of interactions (Strauss and Agrawal, 1999; Arnason et al., 2004; Jones and Agrawal, 2016).

When secondary metabolites produced by plants are perceived by other organisms they acquire ecological functions and become subjected to selective pressures (Parachnowitsch and Manson, 2015; Jones and Agrawal, 2016; Petschenka and Agrawal, 2016). Some of these ecological functions are frequently explored in the literature, such as resistance against herbivory and mutualistic interactions (Strauss and Agrawal, 1999; Pichersky and Gershenzon, 2002; Heil and Karban, 2010; Jamieson et al., 2017). Following plant stress, there can be induced changes in plant traits like extrafloral nectar and secondary metabolites, which are tightly linked to other interactions established by plants (Agrawal and Rutter, 1998; Kessler and Baldwin, 2004; Heil, 2008). In particular, herbivore-induced volatile organic compounds (HI-VOCs) are involved in many interactions due to their ease of dispersal and perception by other organisms (Baldwin, 2010; Bruce and Pickett, 2011; McCormick et al., 2012). VOCs are detected by the emitter plants themselves, herbivores, predators, pathogens, parasitoids, hyperparasitoids, soil microorganisms, other plants and pollinators (Kessler and Halitschke, 2009; Junker and Tholl, 2013; Heil, 2014; Jamieson et al., 2017).

The fact that plant chemical traits such as VOCs have many functional roles makes them candidates for research and technology for agriculture. However, we still lack knowledge about the ecological consequences of the manipulation of plant chemical traits in a multitrophic and evolutionary context. Experimental manipulation of HI-VOCs emission by plants in the field, for example, is usually planned considering predators and parasitoids of herbivores as target receivers of these signals (Kaplan, 2012). However, such signals can also be perceived by other organisms in the community that interact with the focal plant, such as pollinators and other herbivores (Halitschke et al., 2008; Kessler and Halitschke, 2009; Jamieson et al., 2017). These interactions are considered non-target effects of HI-VOC manipulation and could result in positive or negative impacts on plant fitness. Due to the complex interactions mediated by volatiles (Dicke and van Loon, 2000; Dicke and Baldwin, 2010), non-target effects should be considered when manipulating volatiles in an agricultural context. Integrating this perspective in agroecosystems may help managing plant chemical traits more efficiently and responsibly.

In this paper, we propose that studying chemically mediated interactions structured by VOCs and phytochemical diversity in agroecosystems is essential to understand how ecosystem functions emerge and can be enhanced in these systems. We explore broad research questions and future directions on how chemical ecology can be studied in diversified agroecosystems and discuss its applications in two of the most studied agroecosystem services: pollination and biological control. By highlighting these perspectives, we hope to help comprehend how chemical ecology can be integrated in agroecosystem management.

INTEGRATION OF CHEMICAL ECOLOGY IN DIVERSIFIED AGROECOSYSTEMS

Ecological interactions are responsible for providing important agroecosystem services. A goal of chemical ecology should be to help us understand these interactions so that agroecosystems can be managed more efficiently. To achieve this goal, a rigorous application of ecological theory is necessary. It is also fundamental to investigate traditional diversified agroecosystems that have been developed over thousands of years. Traditional cropping systems hold substantial amounts of information that detailed ecological study can make available more generally. For this purpose, we highlight two general interlinked questions that we think will be relevant in guiding future research.

(1) What are the mechanisms that generate phytochemical diversity in agroecosystems? Agroecosystem diversification can affect phytochemical diversity through three major paths: (1) planned taxonomic diversity, i.e., the plants chosen for inclusion in the agroecosystem; (2) associated diversity, which comprises the organisms that spontaneously appear in the agroecosystem; and (3) changes in plant chemistry resulting from induced responses to environmental cues such as biotic interactions and abiotic stress. Understanding the relevance of each of these processes for chemically mediated interactions is fundamental if we want to comprehend the ecological processes supporting agroecosystem function. VOC-mediated plant-plant interactions are promising candidates for these studies since it is known that they affect plant growth and defense (Ninkovic et al., 2002, 2003). It is possible that future research might reveal that specific plant combinations are effective choices for intercropping due to the characteristics of their VOCs-mediated communication and the consequences these VOCs have on multitrophic interactions.

(2) How does plant chemistry help modulate the interaction web in diversified agroecosystems? Research that focuses on pairwise interactions and/or linear tritrophic chains could lead to underestimation of the impacts of plant chemistry on interaction webs (Ohgushi et al., 2007). Agroecosystems are complex communities, and their management may alter the structure of interaction webs through modifications in plant diversity and, as a consequence, changes in plant chemical traits available to mediate direct and indirect interactions. By incorporating a whole-system approach (Lewis et al., 1997) that explicitly treats the agroecosystem as a complex of interactions, we may begin to understand the web of chemically mediated information superimposed upon the web of ecological interactions. Therefore, we can comprehand how plant chemistry affects the entire web, not only individual interactions (Larue et al., 2016).

The two topics highlighted above emphasize the mechanisms that generate phytochemical diversity and how plant chemistry affects the entire web of interactions. An integration of these two aspects is required to comprehend the emergence of agroecosystem services. This approach could provide knowledge to improve the quality of agroecosystem services through more efficient ecosystem management (e.g., use of plants with attractive compounds for natural enemies and/or pollinators). We can therefore analyze how different agroecosystem management practices affect the community structure and the plant chemical traits specifically related to ecological processes that guarantee such services (Lewis et al., 1997). In the following sections, we apply these general questions to two relevant ecosystem services for food production: pollination and biological control (**Box 1**).

Pollination

Agroecosystem diversification, through management of flower strips, for example, has shown that increasing plant diversity is positively associated with the richness and abundance of pollinators, pollination, and fruit production (Nicholls and Altieri, 2013; Pereira et al., 2015; Isbell et al., 2017). Moreover, in agroecosystems, flowers are not presented against uniform, monospecific backgrounds, but against variable mosaics of plant odors. Therefore, identifying the role of different processes generating phytochemical variation is fundamental to understand chemical mediation of pollination in agroecosystems.

The landscape of VOCs can be influenced by plant diversity and also by biotic interactions. For example, herbivore-induced changes in plant physiology can affect flower odor (Pareja et al., 2012) and pollinator abundance and behavior (Lucas-Barbosa et al., 2011; Lucas-Barbosa, 2016; Glaum and Kessler, 2017). This happens because the volatile signaling of flower rewards is the most reliable trait pollinators use in selecting flowers, so that when floral volatiles are more distinctive and less variable, pollinators learn to associate them with floral rewards more efficiently (Wright and Schiestl, 2009). Thus, if plants in highly diverse systems suffer lower herbivory (Randlkofer et al., 2010), and damaged plants attract fewer pollinators (Lehtilä and Strauss, 1997; Kessler et al., 2011) plants grown in polyculture may benefit from a more effective attraction of their pollinators (Orford et al., 2016; Isbell et al., 2017).

Floral volatiles are also important in plant-plant communication. They are produced and emitted at higher rates than vegetative VOCs, with over than 1700 known compounds. These compounds can vary quantitatively and qualitatively between species or between floral states within a species (i.e., pollinated vs. unpollinated flowers emit different signals; Knudsen et al., 2006; Raguso, 2008; Ibrahim et al., 2010). A plant receiving VOC signals gains valuable information about its reproductive environment, including signals regarding whether neighboring plants are in anthesis, whether they are hetero- or conspecific, and whether pollinators are present. Detecting the flowering state of neighboring plants can affect the floral traits of receiver plants such as anthesis, floral rewards and floral volatiles (Caruso and Parachnowitsch, 2016). Plant-plant VOC-based interactions require further study and will likely improve the efficiency of plant combinations for pollination management in agroecosystems.

It is also important to ask how phytochemically diverse environments affect the structure of plant-pollinator interaction webs. Each plant has specific floral traits that promote pollinator visitation while limiting non-pollinating visitors, a mechanism known as floral filtering (Junker et al., 2010). VOCs play an important role in floral filtering and present signals associated with flower presence and rewards recognized by flower visitors (Raguso and Willis, 2002; Cunningham et al., 2004; Milet-Pinheiro et al., 2012). Floral odors also act as guides with spatial orientation gradients, depending on the distribution of emitting sources on the vertical stratification of the vegetation (Baldwin et al., 2006; Randlkofer et al., 2010). Thus, choosing plants with diverse architecture in terms of vegetative and reproductive structure creates heterogeneous signals that can be efficiently exploited by multiple pollinators (Farré-Armengol et al., 2013).

BOX 1 | Future directions for studies of chemical ecology in diversified agroecosystems.
The perspectives discussed on how plant chemistry can mediate ecological interactions in a community-wide agroecosystem context lead us to propose a series of research questions to comprehend the emergence of agroecosystem services. We focus on the application of chemical ecology to the study of pollination and biological control.
(1) What are the relative roles of taxonomic diversity, biotic interactions and abiotic stress in generating phytochemical diversity in agroecosystems?
(2) How does phytochemical diversity of VOCs affect pollinator diversity, flower-pollinator interaction webs and plant reproductive output?
(3) Do VOC-mediated plant-plant interactions have an effect on floral biology and agricultural production?
(4) How does phytochemical variation resulting from taxonomic plant diversity, biotic interactions and abiotic stress affect the natural enemies of herbivores in agroecosystems?
(5) How do VOC-mediated plant-plant interactions make plants more resistant or susceptible to herbivores and affect the structure of interaction webs?
(6) Are there trade-offs between cascading effects of induced plant responses on biological control and pollination at the agroecosystem level?
(7) Can knowledge of phytochemical traits be applied to improve crop combinations?

Autonomous Biological Control

Researchers have applied chemical ecology primarily in the study of individual pest species focused on pairwise interactions (Rosenheim and Coll, 2008). However, work in diversified agroecosystems shows that top-down and bottom-up ecological interactions can limit the population growth of species that may become pests (Letourneau et al., 2011). This ecosystem service has been termed Autonomous Biological Control (Vandermeer, 2011), Ecological Pest Management (Shennan et al., 2005) and Environmental Pest Management (Coll and Wajnberg, 2017). Chemical ecology has enormous potential for understanding ecological interactions in diversified agroecosystem management and ensure effective biological control.

Plant chemistry influences pest incidence by affecting plant localization and selection by herbivores (Webster et al., 2008) or through indirect defense, by the attraction of predators and parasitoids that may limit herbivory from the top-down (Aljbory and Chen, 2018; Furlong et al., 2018). Damaged plants emit a blend of VOCs that can signal herbivore presence and recruit predators, parasitoids or entomopathogenic nematodes (Rasmann et al., 2005; Filgueiras et al., 2016). Besides VOCs, other plant chemical traits also function to increase predator permanence on plants, like sticky compounds present in glandular trichomes that facilitate the capture of herbivores by immobilizing them (Romero et al., 2008) and extrafloral nectar that serves as an alternative food resource and enhances the presence of predators in the community (Heil, 2014; Rezende et al., 2014).

Despite research focused on the behavior of individual predators and parasitoids in response to volatiles in the laboratory, their response to volatiles in diverse odor backgrounds remains to be studied (Aartsma et al., 2017). VOC signals influence predator and parasitoid search behavior by relaying information about the presence of herbivores, the existence of other food sources (e.g., nectar and pollen), and the presence of plants that provide natural enemy-free space (Rossbach et al., 2005; Meiners, 2016). VOCs thereby lead different predator and parasitoid species to ignore, avoid or prefer particular odor mixtures (Perfecto and Vet, 2003; Wäschke et al., 2013). Therefore, VOC diversity resulting from agroecosystem management can influence the structure of trophic webs and help regulate herbivory by affecting herbivore localization by predators and parasitoids.

Plant-plant communication mediated by volatiles has rarely been included in biocontrol studies (Glinwood et al., 2011). It is known that signals emitted by plants can be detected by neighboring plants and can activate defense production in the receiving plants (Kost and Heil, 2006; Heil, 2008; Ninkovic et al., 2013). Perception of HI-VOCs by plants can lead to changes in their own VOC profiles, increase extrafloral nectar production, and enhance the attraction of natural enemies, leading to a reduction in herbivore pressure on the receiving plant (Glinwood et al., 2011; Heil, 2014). Undamaged plants also emit chemical signals that change the growth patterns, biomass accumulation, and defense mechanisms in neighboring plants (Glinwood et al., 2011). This communication affects multitrophic interactions as plants responding to volatile signals emit compounds repellent to herbivores and/or attractive to the natural enemies of herbivores (Ninkovic et al., 2013; Vucetic et al., 2014).

Push-pull technology is an example of how chemical ecology has been integrated to small-scale farming to improve pest and weed management (Khan et al., 2016). An example of successful push-pull system is the planting of *Desmodium* spp. among crop plants to act as 'push' components by emitting VOCs repellent to stemborers. Simultaneously, Napier grass is planted surrounding the crop as 'pull' components to chemically attract egg laying stemborers. Additionally, allelopathic compounds emitted by *Desmodium* spp. prevent the emergence of *Striga* ssp., a parasitic plant of maize and sorghum (Khan et al., 2008). The success of push-pull systems demonstrates how knowledge-intensive agricultural management can increase crop production and promote ecological interactions. Approaches like these involve the integration of chemical ecology in agriculture and collaborations between farmers and scientists (Khan et al., 2016).

Push-pull studies have broken new ground on communication between plants and plants and the third trophic level in diversified agroecosystems. A major goal should be to understand whether specific plant chemical traits have consistent effects in enhancing biological control across systems. This could lead to predictions of the effects of plant combinations on pest incidence based on VOC profiles. This information can be used to plan taxonomic and interaction diversity in agroecosystems.

CONCLUSION

Chemical ecology has long focused on providing technological products for industrial agriculture, but it is now beginning to be recognized that small-scale agroecological systems have great potential to sustainably guarantee food security, particularly to

the most vulnerable populations (Chappell and LaValle, 2011; Kremen and Miles, 2012; Food and Agriculture Organization [FAO], 2015). Such small-scale agroecological systems can greatly benefit from the perspectives discussed here: chemical ecology can shed light in the mechanisms of ecological interactions in these agroecosystems and thus build a collective base of transferable knowledge. In the few cases in which this approach has been implemented, it has shown enormous success for food security (Khan et al., 2016). We believe that further work on the chemical ecology of local production systems could contribute both to increase knowledge of chemical mediation of multi-species interactions in complex systems and to the development of more resilient and equitable crop production systems.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the conception, literature search, and writing of the manuscript.

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frontiers in Plant Science

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Unraveling the Host Plant Alternation of *Cacopsylla pruni* – Adults but Not Nymphs Can Survive on Conifers Due to Phloem/Xylem Composition

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Plant sap feeding insects like psyllids are known to be vectors of phloem dwelling bacteria ('*Candidatus* Phytoplasma' and '*Ca*. Liberibacter'), plant pathogens which cause severe diseases and economically important crop damage. Some univoltine psyllid species have a particular life cycle, within one generation they alternate two times between different host plant species. The plum psyllid *Cacopsylla pruni*, the vector of European Stone Fruit Yellows (ESFY), one of the most serious pests in European fruit production, migrates to stone fruit orchards (*Prunus* spp.) for mating and oviposition in early spring. The young adults of the new generation leave the *Prunus* trees in summer and emigrate to their overwintering hosts like spruce and other conifers. Very little is known about the factors responsible for the regulation of migration, reasons for host alternation, and the behavior of psyllids during their phase of life on conifers. Because insect feeding behavior and host acceptance is driven by different biotic factors, such as olfactory and gustatory cues as well as mechanical barriers, we carried out electrical penetration graph (EPG) recordings and survival bioassays with *C. pruni* on different conifer species as potential overwintering hosts and analyzed the chemical composition of the respective plant saps. We are the first to show that migrating psyllids do feed on overwintering hosts and that nymphs are able to ingest phloem and xylem sap of coniferous trees, but cannot develop on conifer diet. Analyses of plant saps reveal qualitative differences in the chemical composition between coniferous trees and *Prunus* as well as within conifer species. These differences are discussed with regard to nutritional needs of psyllid nymphs for proper development, overwintering needs of adults and restriction of '*Ca.* P. prunorum' to *Prunus* phloem.

Keywords: phloem, chemical composition, psyllid, development, overwintering, host alternation, migration, conifer

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INTRODUCTION

Phloem and xylem tissue enables plants to allocate their resources from sources to sinks and distribute phytohormones to regulate physiological processes. Especially the phloem is rich in nutrients (Douglas, 2006), making it a suitable food source for sap-sucking insects. Although mechanical barriers like sclerenchymatous fibrous rings are able to hinder phloem-feeders from

reaching the vascular bundles (George et al., 2017), the phloem is poorly chemically defended (Douglas, 2006). Since decades studies focused on the chemical composition of phloem sap and the nutrition of phloem-feeding insects. Most work was done in the field of crops, such as rice (Fukumorita and Chino, 1982), broad bean, clover, and peas (Sandström and Pettersson, 1994; Wilkinson and Douglas, 2003) and their pests (especially aphids), because of the economic importance and the role of aphids as model organisms. Information about the composition of phloem and xylem sap of coniferous plants is rare. Ziegler and Mittler (1959) extracted phloem sap from *Picea abies* by stylectomy and found sucrose as the only sugar in paper chromatography analysis. Later studies focused on induced defense mechanisms in bark phloem after bark beetle attack (Rohde et al., 1996), food quality of needles (Schopf et al., 1982; Fisher and Fisher, 1987) and impact of air pollution on nutrition of conifers (Zedler et al., 1986; Kainulainen et al., 1993). These studies give an impression of which metabolites could be found in plant sap of coniferous trees, but compounds were extracted from whole plant tissue (bark resp. needles). More explicit knowledge about plant sap composition is important for a better understanding of the biology of phloem-feeding insects that migrate between two different host plant species, e.g., psyllids (Hemiptera: Psyllidae).

Psyllids or jumping plant lice are plant sap feeding insects encompassing more than 3000 species. Most of them are oligophagous and use perennial dicotyledonous angiosperms as host plants for reproduction (Hodkinson, 2009; Mayer et al., 2009, 2011). In the genus *Cacopsylla* two different strategies can be observed: There are polyvoltine species reproducing and feeding exclusively on the same host plant and univoltine species with an obligate alternation of two host plants (Ossiannilsson, 1992; Hodkinson, 2009). The latter migrate between their reproduction host plants (respective fruit crops) and their overwintering host plants (conifers) (Mayer and Gross, 2007; Mayer et al., 2011). For identifying their particular host plants for feeding and reproduction, volatile signals are used in many species during migration (Soroker et al., 2004; Gross and Mekonen, 2005; Mayer et al., 2008a,b, 2009; Weintraub and Gross, 2013).

The plum psyllid, *Cacopsylla pruni* is the only known vector of one of the most serious pests in European fruit production, the cell wall lacking bacterium '*Candidatus* Phytoplasma prunorum' (Carraro et al., 1998). The phloem dwelling bacterium induces the European Stone Fruit Yellows (ESFY) (Seemüller and Schneider, 2004). Because infected trees yield poorly and die quickly, this plant disease causes high economic losses in European fruit production every year. So far no curative approach was found against this disease. Unfortunately, it is not possible to cultivate this obligate cell parasite outside of the host plant or vector, which hampers research toward a cure. Therefore, the only measure to inhibit infection of stone fruit orchards is to prevent invasion of the vector insect, as *C. pruni* alternates between *Prunus* spp. and coniferous trees during its life cycle. After reproduction and development on *Prunus* spp., the young adults (emigrant stage) emigrate and spend the rest of the year on spruce and other conifers (Thébaud et al., 2009; Jarausch and Jarausch, 2016). In early spring they return to *Prunus* spp. for reproduction (remigrant stage). Very little is known about the reason for migration and feeding behavior of psyllids during their life on conifers (Thébaud et al., 2009). To date it remains unclear whether overwintering psyllids actually feed on conifers. Former experiments with the closely related hawthorn psyllid *Cacopsylla melanoneura* failed, although the maintenance of body condition and level of hydration suggested feeding (Jackson et al., 1990). Because it was shown that adult *C. pruni* did not survive the winter on one of their reproduction hosts *Prunus spinosa* (Carraro et al., 2002; Thébaud et al., 2009), and that some migrating species including *C. pruni* already start migration to their overwintering host during summer (Mayer and Gross, 2007; Mayer et al., 2009; Jarausch and Jarausch, 2016), we hypothesize that *C. pruni* needs to feed on overwintering host plants during this long period and therefore needs to leave deciduous *Prunus* trees to migrate to evergreen conifers, which show yearlong photosynthesis and phloem activity. On the other hand, reproduction on coniferous trees could be impossible for *C. pruni*, forcing them to migrate back to *Prunus*. A better knowledge of the vector biology is needed to develop new control strategies against vector insects and bacterial pathogens (Gross and Gündermann, 2016; Perilla-Henao and Casteel, 2016).

Here, we studied the feeding behavior of adults and nymphs on several conifer species as well as *Prunus domestica*, and conducted bioassays to unveil *C. pruni*'s ability to survive and develop on plant sap of overwintering hosts. Furthermore, we extracted the phloem/xylem sap of both *Prunus* spp. and conifers and analyzed sugars and organic acids including amino acids.

MATERIALS AND METHODS

Insects

Cacopsylla pruni remigrants (overwintered adults) were caught by beating tray method from *Prunus domestica* trees located at the experimental field of the Julius Kühn-Institut in Dossenheim, Germany and at an experimental orchard of Dienstleistungszentrum Ländlicher Raum Rheinpfalz, Neustadt an der Weinstrasse, Germany in spring 2017. Psyllids were maintained on *Prunus* trees (cv. GF655/2 and *Prunus spinosa*) in cages housed in a climate chamber at 20°C during photophase and 16°C during scotophase (L16:D8). After mating and oviposition the field captured adults were transferred to cages with fresh plants. For survival experiments about 200 fifth instar nymphs were gently transferred to a new *P. domestica* (cv. Wavit) tree and emerged adults (emigrants) were collected daily.

Plants

Four conifer species, *Abies alba* (Silver fir), *Larix decidua* (European larch), *Picea abies* (Norway spruce), and *Pinus sylvestris* (Scots pine), and the *P. domestica* cultivar Wavit were used for experiments. Plants were grown under natural conditions in an insect safe environment. Hexythiazox (Ordoval, BASF, Ludwigshafen am Rhein, Germany) and Fenpyroximate (Kiron, Cheminova Deutschland GmbH & Co.

KG, Stade, Germany) were applied once to *P. domestica* plants in April 2017 to prevent infestation with spider mites.

EPG-Recordings

To investigate whether *C. pruni* adults and nymphs feed on coniferous trees in general, the electrical penetration graph technique (EPG) was applied. EPGs were recorded using an 8 channel amplifier (model Giga-8d, EPG-Systems, Wageningen, Netherlands). Data acquisition and analysis was performed with Stylet+ software (EPG-Systems). To connect the psyllids to a copper electrode, a piece of fine gold wire (18 µm) was attached to the pronotum with a small droplet of water based silver glue (EPG-Systems). The electrode was attached to an EPG probe and the reference electrodes were placed in the soil of the test plants. Feeding behavior of *C. pruni* male and female emigrants (minimum age 6 weeks) was recorded in a climate chamber at 10°C with 60–65% RH for 16 h and of fifth instar nymphs (about 6 weeks old) at 20°C under the same conditions. Plants and insects were housed in a grounded self-constructed Faraday cage during recordings. Recordings were replicated 10 times for nymphs on each *P. abies*, *A. alba,* and *P. domestica* (cv. Wavit). Feeding behavior of emigrants was recorded on *P. sylvestris* (4 males and 6 females), *P. abies* (6 males and 4 females), *A. alba* (5 males and 5 females), and *L. decudia* (6 males and 4 females). To ensure that emigrants used for EPG recordings were not repelled by conifers (due to their developmental stage), *C. pruni* adults were caged with *P. abies* and *A. alba* twigs one day prior recordings and only emigrants which were found on conifer twigs were chosen for the experiment. Recordings were examined for occurrence of stylet penetration and waveforms indicating phloem and xylem uptake according to Bonani et al. (2010) and Civolani et al. (2011).

Bioassays

Survival

Survival of emigrants was studied on *P. abies*, *A. alba*, and *P. domestica* cv. Wavit plants. Transparent plastic cups (0.5 l capacity) were used as cages. The bottom of each cup was replaced by gauze for venting. A hole was punched into the lids to attach the cups on twigs of living plants. The lid was sealed with self-made modeling clay (composed of 42.6% water, 42.6% flour, 3.2% sunflower oil, 10.6% salt, and 1.1% citric acid) and five newly emerged emigrants (<24 h) were released in each cup. Living individuals were recorded daily over a period of 40 days. Additionally, the mortality of emigrants in the same type of cups, but without food supply (control), was observed. The experiment was replicated eight times for every plant species and five times without plants (control) under rearing conditions.

Development

For developmental experiments *C. pruni* nymphs of second and third instar were gently transferred with a fine brush from rearing plants to twigs with young flush of *P. abies*, *A. alba*, or *P. domestica* cv. Wavit, respectively. On each plant, five nymphs were caged in insect rearing sleeves (40 cm × 20 cm, 100 × 80 mesh/square inch, MegaView, Taiwan). The experiment was replicated seven times on each conifer species and five times on cv. Wavit. Experimental plants were housed under rearing conditions in a climate chamber. After 21 days cages were controlled consistently once a week for hatched *C. pruni* adults (emigrants). After 56 days all cages were opened and checked for living nymphs.

Xylem and Phloem Sap Sampling

Phloem and xylem saps were collected in June 2017 using modified centrifugation technique according to Hijaz and Killiny (2014). The twigs from young flush from *P. domestica* (cv. Wavit) and conifer species *P. abies, A. alba, L. deciduas,* and *P. sylvestris* were sliced into 2–3 cm pieces with a clean scalpel. The bottom of a 0.5 ml Eppendorf tube was removed with a razor blade and twig pieces were placed into the tube. The tube was immersed in a 1.5 ml tube. For collecting the phloem and xylem sap, the tubes were centrifuged at 12.000 rpm at 4°C for 10 min. The collected samples were stored at −80°C up to analysis. In the following, this collected mixture of phloem and xylem sap is referred as plant sap.

Plant Sap Derivatization

The sap samples were derivatized with methyl chloroformate (MCF) to focus the GC-MS analysis on amino and other organic acids (Smart et al., 2010). An aliquot of 20 µl plant sap was mixed with 180 µl sodium hydroxide (1 M) in a silanized glass vial. Then 167 µl methanol and 34 µl pyridine were added, followed by 20 µl MCF. The sample was vortexed for exactly 30 s, additionally 20 µl MCF were added and the sample was mixed again for 30 s. To extract the alkylated derivatives 150 µl chloroform were added to each sample and mixed for another 10 s. A 200 µl aliquot of sodium bicarbonate solution (50 mM) was added and mixed for 10 s again. After a double meniscus was formed, the aqueous phase was discarded and a few milligrams of anhydrous sodium sulfate were added to the organic layer to bind the remaining water. The supernatant was transferred to a GC-MS vial with a glass insert.

For the derivatization with trimethylsilyl (TMS) 5 µl aliquots of the sap samples were added to 60 µl of an internal standard solution (Ribitol in ultrapure water) and dried under nitrogen stream (Reacti-Vap, Thermo Fisher Scientific Inc., Waltham, MA, United States). Samples were derivatized by adding 70 µl methoxyamine hydrochloride solution (MOX) in pyridine (2%) and allow to incubate for 90 min at 37°C stirring at adjustment of 7 (Reacti-Therm, Thermo Fisher Scientific Inc.). 90 µl of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) were added and the silylation was allowed to react for 60 min at 37°C stirring at adjustment of 7 (Reacti-Therm, Thermo Fisher Scientific Inc.). The supernatant was transferred to a GC-MS vial with a glass insert.

Chemical Analysis

Derivatized samples were analyzed by gas chromatography coupled with mass spectrometry (GC-MS) using a PerkinElmer Clarus R 680 GC system coupled to a PerkinElmer quadrupole inert mass selective detector for molecular structure analysis.

A non-polar Elite-5MS (Crossbond 5% diphenyl-95% dimethyl polysiloxane, PerkinElmer) capillary column (30 × 0.25 mm id × 0.25 µm film thickness) was used for GC separation. Carrier gas flow rate (Helium, Linde, Germany) was about 5 ml/min (column head pressure 150 kPa). Injection of 1 µl of the samples derivatized with MCF was done at 290°C injector temperature with a split flow of 1 ml/min. The initial oven temperature of 70°C was held for 3 min, followed by a temperature increase of 20°C/min up to 240°C held for 3.5 min and a further increase to 300°C at a rate of 20°C/min. The final temperature of 300°C was held for 2 min. The GC temperature program to analyze samples after silylation was as follows: the initial oven temperature of 80°C was held for 3 min, followed by an increase of 5°C/min up to 320°C. The final temperature of 320°C was held for 4 min. One microliter of each sample was injected at 220°C with a split flow of 5 ml/min. Transfer line and ion source temperatures were set to 250°C and 180°C, respectively. The quadrupole mass detector was operated in electron-impact (EI) mode at 70 eV. All data were obtained by collecting the full-scan mass spectra within the range of 35–550 m/z. Blank samples, reference standards and mixtures of alkanes (C8–C20 and C10–C40) were analyzed additionally according to both methods.

Identification and Quantification With AMDIS

GC-MS chromatograms were analyzed using "Automated Mass spectral Deconvolution and Identification System" (AMDIS, V. 2.71; National Institute of Standards and Technology NIST, Boulder, CO, United States). Detected compounds were identified by comparing characteristic ion fragmentation patterns, retention times and retention indices with standard compounds according to Weintraub and Gross (2013). For quantification, the peak areas were integrated after deconvolution with AMDIS. Identification criteria were applied as follows: match factor had to be ≥80% and the relative retention index deviation ≤5% from reference value. The settings for deconvolution were: component width: 32; adjacent peak subtraction: one; resolution: medium; sensitivity: medium; shape requirements: high; level: strong; maximum penalty: 20, and

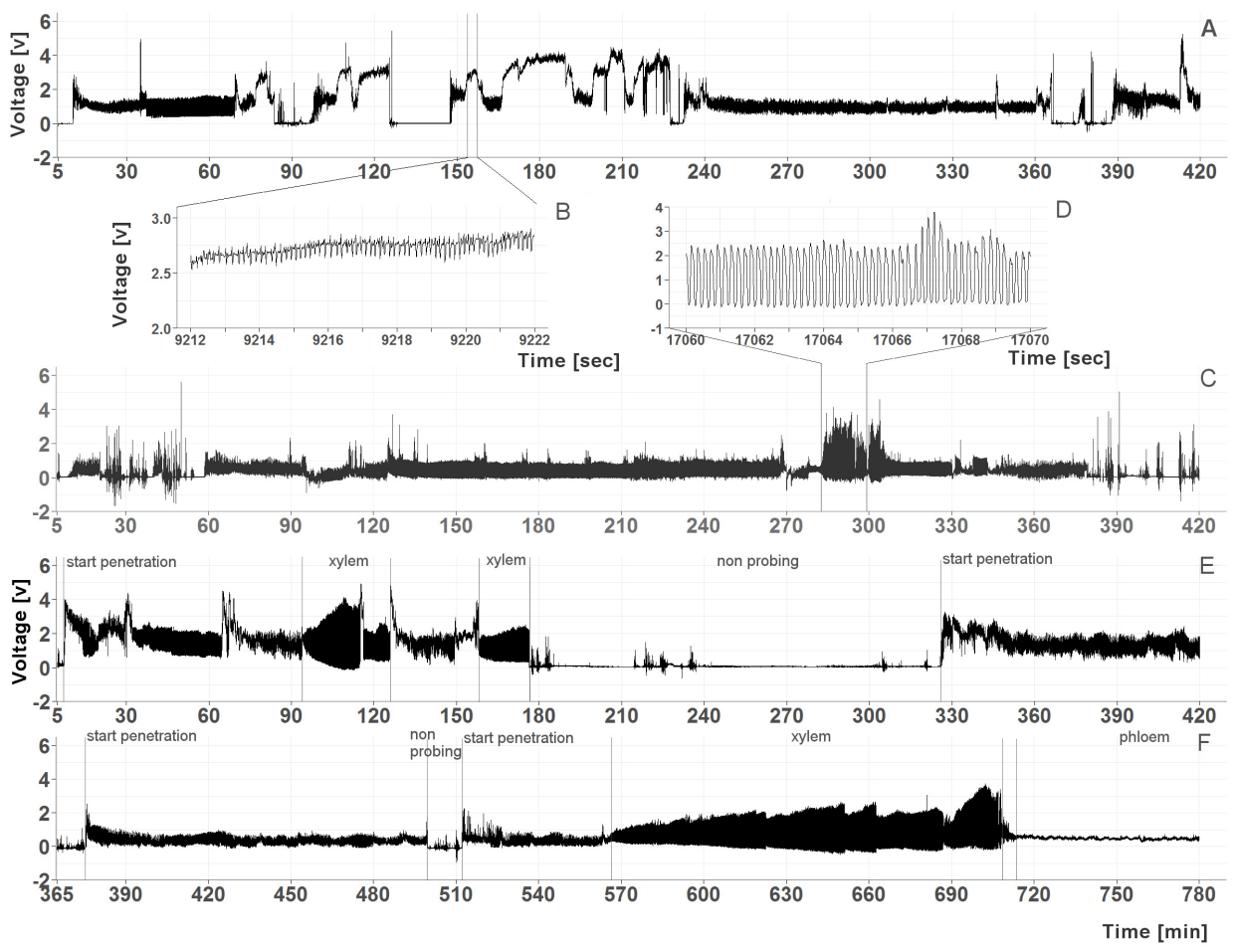

FIGURE 1 | (A–D) Examples of EPG recordings from *C. pruni* nymphs (5th instar) on spruce **(A)** with a detailed magnification of phloem phase waveform **(B)** and on fir **(C)** with a detailed magnification of the waveform of xylem feeding **(D)**. **(E,F)** Examples of recordings from a female *C. pruni* emigrant on larch **(E)** and a male emigrant on fir **(F)** with marked penetration and feeding phases.

'no RI in library': 20. Methionine, threonine, and serin were only found in traces (match < 80) and were therefore excluded from the analysis. Relative proportions of amino and organic acids were calculated by setting the sum of the selected compounds as 100%. Proportions of detected compounds after TMS derivatization were normalized to internal standard.

Chemicals and Standards

Alanine, aspartic acid, cysteine, glutamic acid, histidine, leucine, lysine, proline, threonine, tryptophan, valine, salicylic acid, pyridine, methanol, chloroform, methyl chloroformate (MCF), sodium bicarbonate, sodium sulfate, methoxyamine, ribitol, myo-inositol, xylose, pinitol, and iso-leucine were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). Arginine and phenylalanine were purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany). Glycine, methionine, serine, malic acid, caffeic acid, succinic acid, arabinose, and saccharose from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Asparagine, mannitol, glucose, and galactose from Merck KGaA (Darmstadt, Germany). Sorbitol and glutamine from AppliChem GmbH (Darmstadt, Germany). MSTFA from Macherey-Nagel GmbH & Co. KG (Düren, Germany). Citric acid was purchased from Acros Organics (Thermo Fisher Scientific, Geel, Belgium).

Statistical Analysis

Statistical analysis was done in R version 3.4.2 "Short Summer" (R Core Team, 2017). Visualizations were conducted with the ggplot2 package (Wickham, 2009). Death hazard from *C. pruni* emigrants on different host plants were compared by Cox's proportional hazard regression through likelihood ratio test. Efron approximation was used for tie handling. The proportional hazards assumption for Cox regression model fit was confirmed using the *cox.zph* function of the survival package (Therneau, 2017). Non-metric multidimensional scaling (NMDS) plots were used to visualize Bray–Curtis dissimilarities of the chemical composition of xylem and phloem between plant species. NMDS was performed using the *metaMDS* function from vegan package (Oksanen et al., 2017). Scaling was standardized by Wisconsin double standardization. Significantly ($p < 0.01$, $N = 10000$) influential factors (chemical compounds) were plotted as arrows in NMDS plots. Dissimilarity matrix was calculated to test for discrimination of plant species by Permutational Multivariate Analysis of Variance (PERMANOVA). Additionally, the dispersion of groups was tested for multivariate homogeneity (PERMDISP).

RESULTS

EPG-Recordings

To determine if *C. pruni* feeds on overwintering hosts (conifers), feeding behavior of emigrants was recorded on potential host plants. The recordings revealed that both male and female emigrants fed on plant saps of all four offered conifers: *P. abies*, *A. alba, P. sylvestris,* and *L. decudia*. Recordings from nymphs of *C. pruni* showed that they were also able to feed on *P. abies* and *A. alba* (**Figure 1**).

Bioassays

Survival

Newly emerged *C. pruni* emigrants survived on *P. abies* and *A. alba* as long as on *P. domestica* cv. Wavit (**Figure 2**). The Cox regression model showed that death hazard differed significantly between host plants and controls without food supply (likelihood ratio = 81.76, $df = 3$, $R^2 = 0.431$, $p < 0.001$). Death hazard for emigrants fed on *P. domestica* cv. Wavit did not differ from

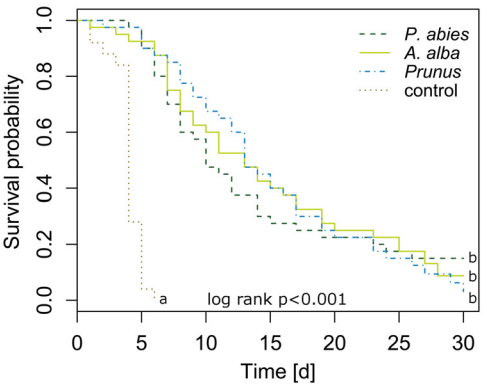

FIGURE 2 | Kaplan–Meier curves visualizing the survival of newly emerged emigrants caged on *P. abies* ($n = 40$), *A. alba* ($n = 40$), *P. domestica* cv. Wavit ($n = 40$), or in cages without a plant (control, $n = 25$). Letters indicate significant differences between survival curves (likelihood ratio = 81.76, $df = 3$, $R^2 = 0.431$, $p < 0.001$).

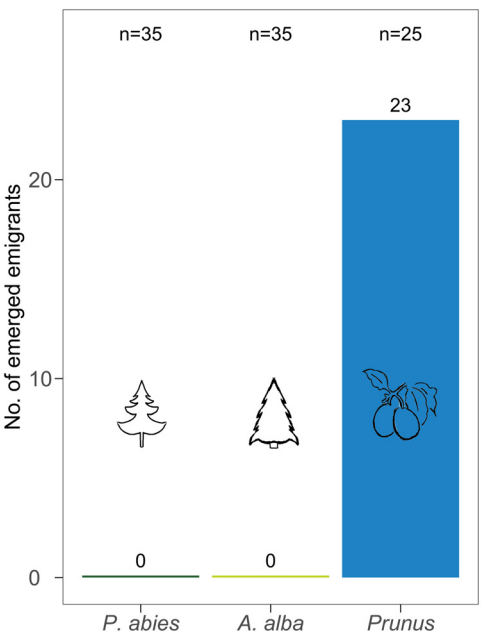

FIGURE 3 | Number of emerged *C. pruni* emigrants from nymphs (2nd instar) on *P. abies*, *A. alba*, and *P. domestica* cv. Wavit.

P. abies (likelihood ratio = 81.76, df = 3, R^2 = 0.431, p = 0.803) and *A. alba* (likelihood ratio = 81.76, df = 3, R^2 = 0.431, p = 0.846). Emigrants on all three potential host plant species had a significant lower death hazard than psyllids without food (control). The hazard ratio was reduced by 97, 97, and 96% if *C. pruni* was allowed to feed on *P. abies*, *A. alba*, or *P. domestica* cv. Wavit, respectively.

Development

After 56 days 92% of the *C. pruni* nymphs on *P. domestica* cv. Wavit emerged while none of the nymphs developed neither on *P. abies* nor *A. alba* (**Figure 3**). As no living nymphs could be found on the coniferous trees, we conclude that they all died in nymphal stage.

Chemical Composition of Phloem and Xylem Content

Plant species differed significantly in the chemical composition of sugars and other compounds detected by GC-MS analysis after TMS derivatization of plant sap (PERMANOVA, df = 4, R^2 = 60.83, N = 10000, $P < 0.001$). The dispersions differed not significantly between the groups (PERMDISP, df = 4, F = 0.42, N = 10000, $P > 0.05$), confirming that separation of species was due to their location. The NMDS plot illustrates the differences of chemical profiles (**Figure 4**).

Plant saps from *P. domestica* trees contained a high amount of sorbitol. This sugar alcohol constituted about 58% of the plant sap from *P. domestica* cv. Wavit but was not detected in samples from coniferous trees (**Figure 5**). In contrast, pinitol was exclusively found in plant sap from conifers. However, the most abundant component was quinic acid in all conifer samples (**Figure 5**). The relative abundance of quinic acid ranged from 30% in pine to 56% in spruce. Sap samples of *P. domestica* were composed of 80% sugars and sugar alcohols and 18% acids, whereas spruce, fir, pine, and larch samples consisted of 29, 41, 50, and 36% sugars and sugar alcohols and 69, 53, 43, and 61% acids, respectively.

The composition of amino acids and other organic acids differed significantly between the plant species (PERMANOVA, df = 4, R^2 = 46.85, N = 10000, $P < 0.001$). The dispersions between the groups also differed significantly (PERMDISP, df = 4, F = 3.96, N = 10000, $P < 0.01$), indicating that the separation of the plant species could be effected by different variation within species (**Figure 6**). The NMDS plot shows caffeic acid and asparagine contributing to the separation of *P. domestica* cv. Wavit from coniferous trees (**Figure 6**). Caffeic acid was exclusively found in *P. domestica* cv. Wavit, while asparagine was more abundant in *P. domestica* cv. Wavit as in *P. abies* and *A. alba* (**Figure 7**).

The main organic acid component in the plant sap of all tested plant species was malic acid (29–48%). Aspartic acid was the second most abundant component in all plants, except in larch which contained more glutamic acid. Differences between the plant species were detected concerning the relative amounts of lysine in the plant sap composition. Lysine represented about 17% of the sap samples of spruce trees and was the third most abundant component in those trees, as glutamic acid was in fir

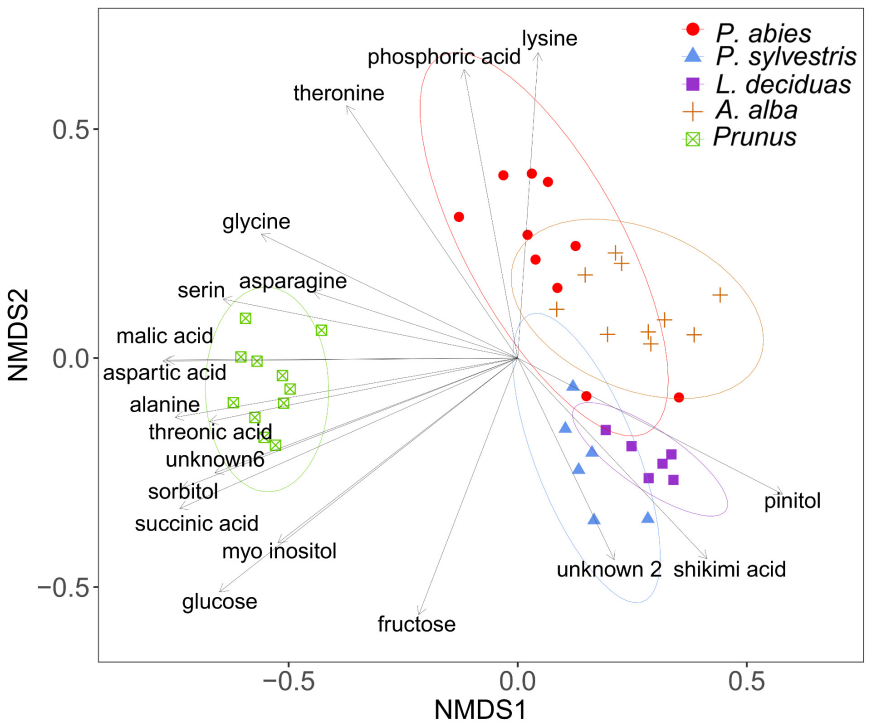

FIGURE 4 | Visualization of Bray–Curtis dissimilarities with non-metric multidimensional scaling (NMDS) plots (stress = 0.14) of plant sap samples from spruce (n = 10), pine (n = 6), larch (n = 6), fir (n = 10), and *P. domestica* cv. Wavit (n = 11) after methoximation followed by trimethylsilylation.

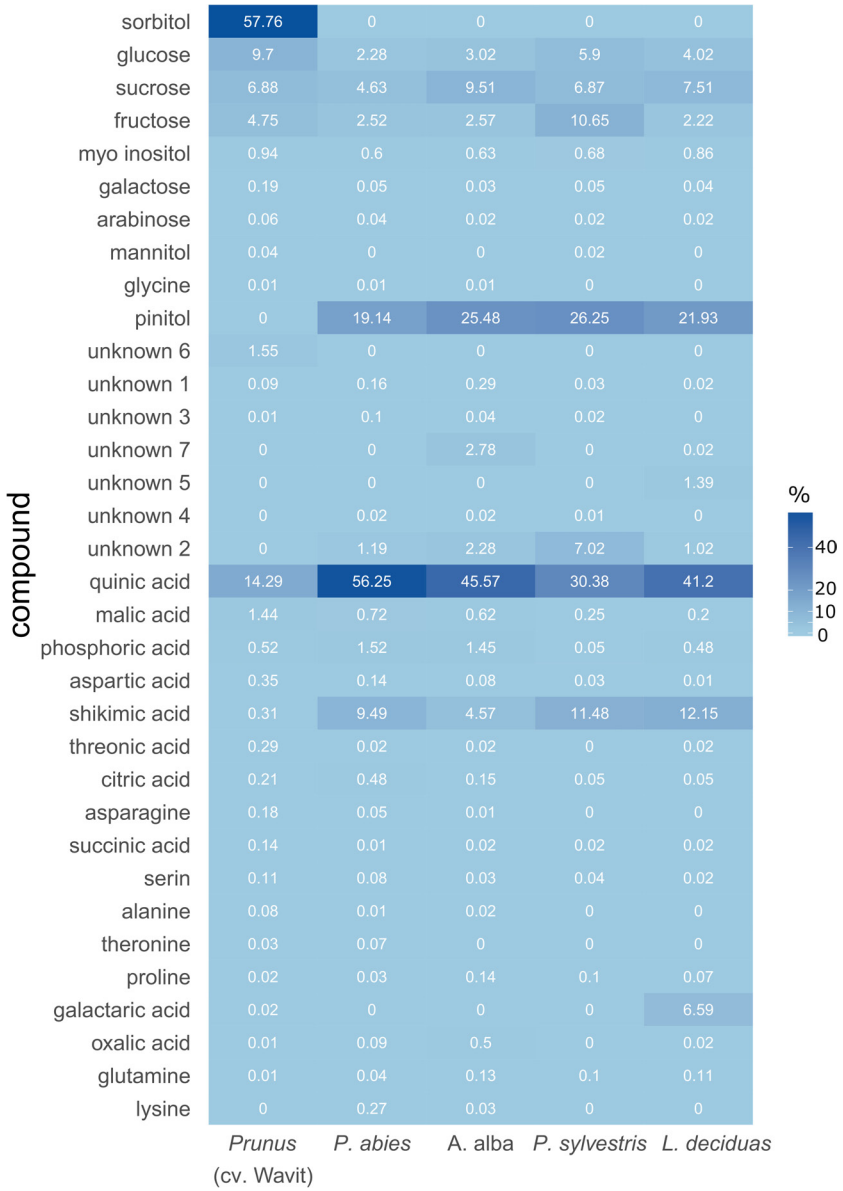

FIGURE 5 | Composition of sugars and acids in vascular bundle content of *P. domestica* cv. Wavit (n = 11), spruce (n = 10), fir (n = 10), pine (n = 6), and larch (n = 6). Plant sap was collected by centrifugation and derivatized by trimethylsilylation after methoximation. Dark blue indicates a high relative abundance of the components, light blue a low abundance. Numbers are mean values of relative abundance.

(10%), pine (15%), and *P. domestica* cv. Wavit (12%) (**Figure 7**). Cysteine, methionine, and threonine were under detection limits in all samples. The NMDS plots indicate the responsibility of the essential amino acids tyrosine, tryptophan, lysine, and histidine on the separation of spruce and fir from *P. domestica* cv. Wavit (**Figure 6**).

DISCUSSION

Electrical penetration graph recordings showed that *C. pruni* emigrants and nymphs are able to feed on the plant saps of spruce, pine, larch, and fir. EPGs recorded from 5th instar nymphs prove that nymphs are not repelled by metabolites of coniferous plants and able to reach the phloem and xylem tissue with their stylet. The question arises why *C. pruni* migrates to *Prunus* for reproduction when their progeny is able to ingest food from conifers. We suggest that there is no change in host acceptance of nymphs between different instars, but nutritional needs could change between nymphal development stages. Therefore we investigated the emergence of adults starting from the earliest possible instar (2nd). Because the impact of low food quality or inhibitory components may accumulate and negative influence raise over time, 5th instar

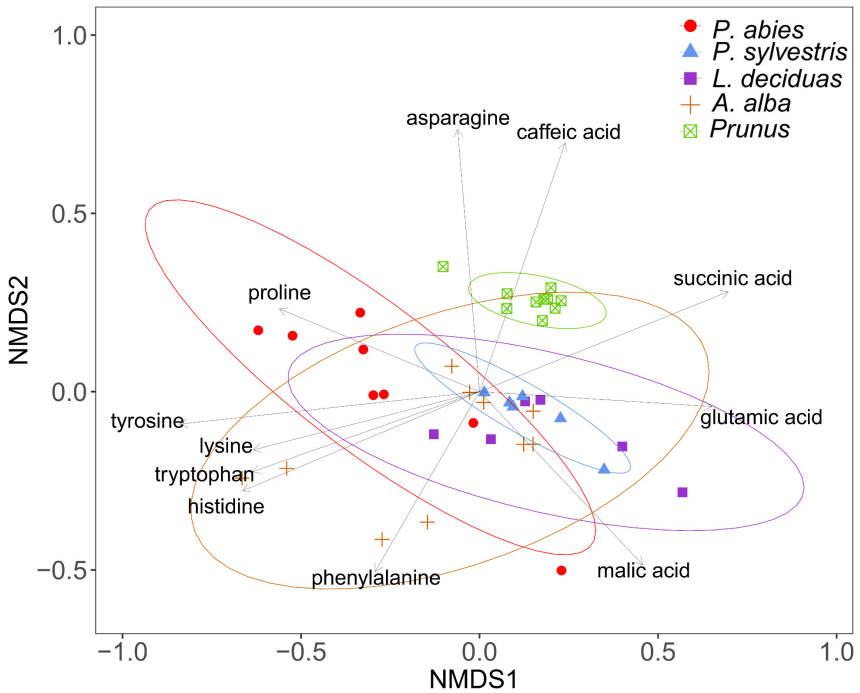

FIGURE 6 | Visualization of Bray–Curtis dissimilarities with NMDS plots (Stress: 0.13) of plant sap samples from spruce (n = 8), pine (n = 6), larch (n = 6), fir (n = 10), and *P. domestica* cv. Wavit (n = 10) derivatization with methyl chloroformate.

nymphs may able to compensate a short period on a non-optimal diet while early instars would suffer more from low food quality than later ones. But it is of crucial importance, whether *C. pruni* is able to fully develop from egg to adult stage on coniferous trees. Bioassays revealed that adult psyllids survived on coniferous trees, while nymphs did not develop and died, although they were able to ingest plant sap from conifer needles. Thus, the chemical composition of the respective conifer saps influences the nymphal survival and development. Therefore the plant saps of overwintering hosts were subsequently analyzed and compared to sap content of their reproduction host plant (*P. domestica*).

The GC-MS analysis revealed enormous differences in the chemical compositions of plant sap of the Rosacea species *P. domestica* cv. Wavit and the four studied conifer species. Especially the lack of sorbitol in all four conifers as well as the high amount of quinic acid and pinitol (which was not detected in *Prunus* trees) could be challenging for phloem feeding insects, which alternate between Rosacea and conifers during their life cycle. Even though it was known that spruce needles contain quinic acid, shikimic acid, fructose, glucose, sucrose, and pinitol (Schopf et al., 1982), to date it was unclear, in which proportions they occur in the phloem and xylem sap of coniferous trees, and how their proportions differ between tree species.

Until today it was a widespread belief that conifers are used by migrating *Cacopsylla* species like *C. pruni, C. picta*, and *C. melanoneura* for shelter during winter time, exclusively (Burckhardt et al., 2014; Jarausch and Jarausch, 2016). In the presented study we were able to show for the first time, that conifers are not only shelter plants for migrating species belonging to the genus *Cacopsylla*, but also an important food resource enabling their overwintering. Thus, the term "shelter plant" should hereafter be replaced by "overwintering host" or just "alternate host" plant.

Due to the lack of knowledge that psyllids feed on conifers, the effect of coniferous phloem constituents like quinic acid, shikimic acid, and pinitol on psyllid feeding behavior and development was not studied before. Pinitol is a cyclic polyol, which serves as osmoprotectant and is involved in a broad spectrum of physiological processes in plants (Chiera et al., 2006; Kordan et al., 2011; Saxena et al., 2013). It is found in conifers, legumes (Fabaceae) and Caryophyllales such as *Simmondsia chinensis* (Angyal and Macdonald, 1952; Dittrich and Korak, 1984; Guo and Oosterhuis, 1995; Chiera et al., 2006). D-pinitol induces oviposition of the Grass Yellow Butterfly *Eurema mandarina* (Mukae et al., 2016). However, an influence of pinitol from the phloem of alfalfa on phloem-feeding pea aphid could not be found (Campbell and Binder, 1984).

There is evidence, that psyllid adults and nymphs are tolerant to high osmotic pressures of their diets (Hall et al., 2010; Russell and Pelz-Stelinski, 2015). Therefore, we hypothesize no negative influence of pinitol on *C. pruni*, even if it occurs in high amounts in overwintering hosts. Quite the contrary, pinitol could act as mechanism of protection against freezing stress, as shown for other polyols (Bale, 2002). The freezing temperature of the green spruce aphid is reduced in the presence

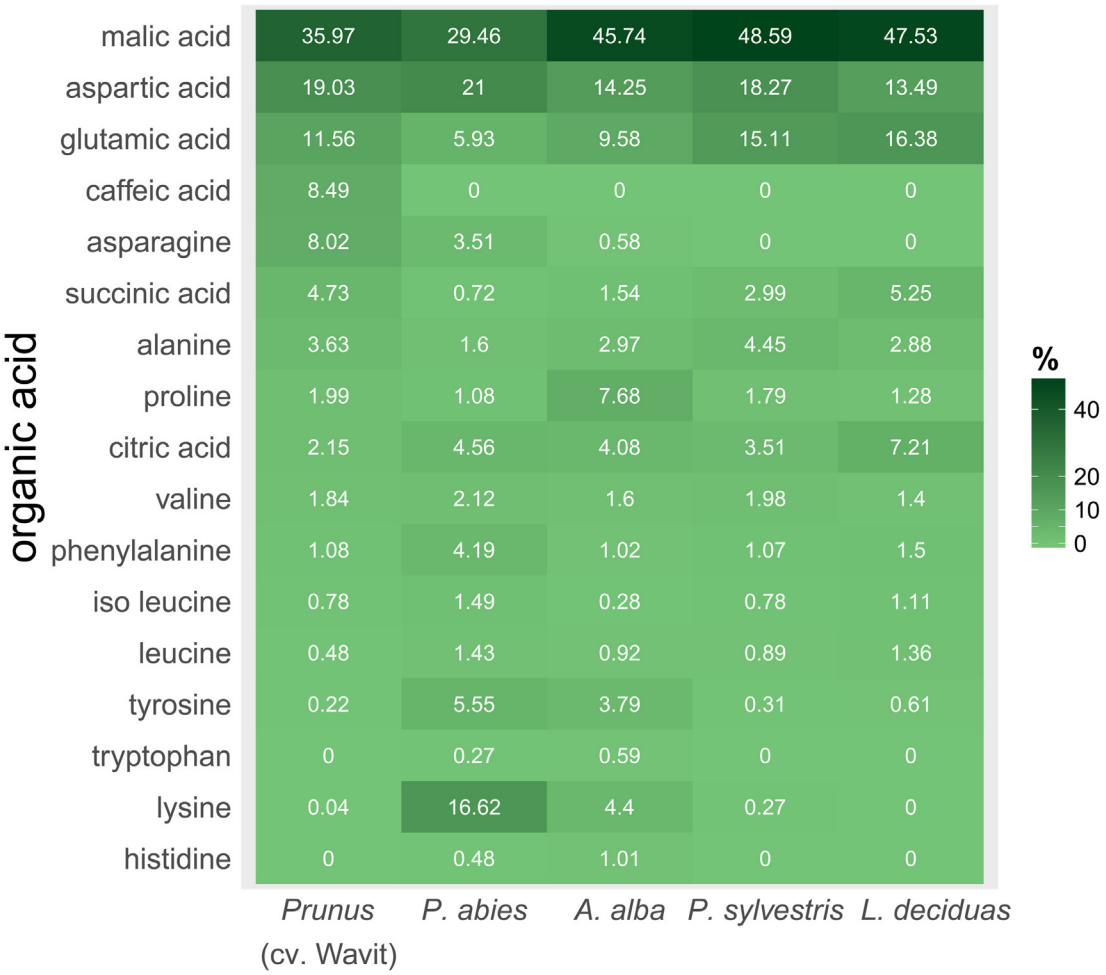

FIGURE 7 | Composition of organic acids in vascular bundle content of *P. domestica* cv. Wavit (n = 10), spruce (n = 8), fir (n = 10), pine (n = 6), and larch (n = 6). Plant sap was collected by centrifugation and derivatization with methyl chloroformate. Dark green indicates a high relative abundance of a respective organic acid, light green a low abundance. Numbers are mean values of relative abundance.

of mannitol in aphid hemolymph (Parry, 1979). Whiteflies accumulate sorbitol for thermo- and osmoprotection (Hendrix and Salvucci, 1998). Sømme (1965) found an accumulation of sorbitol in overwintering eggs of European red mite (*Panonychus ulmi*).

We found that sorbitol is the most abundant component in sap samples of *P. domestica* cv. Wavit, which is in accordance with the fact that sorbitol is most often found in Rosacea (Loescher, 1987). Sorbitol is also known to be accumulated in the phloem of apple trees (Bieleski, 1969) and is the most abundant soluble sugar in the phloem of pear and apple fruits (Zhang et al., 2004, 2014). Nevertheless, adult *C. pruni* can tolerate high amounts of sorbitol or pinitol in their diet. EPG recordings suggest that *C. pruni* (both adults and nymphs) also ingest xylem content (unpublished results), which could be a regulatory reaction to reduce the phloem's high osmotic pressure by dilution. Pompon et al. (2011) showed that aphids ingest more xylem sap after feeding on high concentrated sucrose diets to compensate osmotic unbalance. Moreover, for nymphal development the availability of amino acids (especially essential amino acids) could be of higher importance, as nitrogen content of food is an important limiting growth factor for phytophagous insects (Douglas, 2006). In accordance with Douglas (1993) we found asparagine besides aspartic acid and glutamic acid as one of the most abundant amino acids in young leaves of *Prunus*, while we found only low concentrations of glutamine in *Prunus* flush leaves. All plant species contained only low concentrations of the essential amino acids histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. To compensate for low quality of nitrogen in plant saps phloem feeders harbor microsymbionts (Douglas, 2006). Many psyllid species harbor the bacterial endosymbiont *Carsonella ruddii,* which provides its host with essential amino acids (Thao et al., 2000). Also representatives of the genus *Wolbachia*, *Arsenophonus* and other *Enterobacteriaceae* were found in psyllids (Baumann, 2005). Although the microsymbionts harbored by *C. pruni* are unidentified, differences in the symbiont community in adults

and nymphs were not expected, because vertical transmission of endosymbionts was shown for many species. Furthermore, recent studies indicated the transovarial transmission of *Arsenophonus* in *Cacopsylla pyricola* (Cooper et al., 2017).

We suggest that the inability of *C. pruni* nymphs to develop on coniferous trees is due to differences in organic acid availability. The caffeic acid, which is exclusively found in cv. Wavit, could play a key role in host acceptance of *C. pruni* and maybe act as a phagostimulant. Caffeic acid was found in several stone fruits like peaches and plums, which are typical host plants of *C. pruni* (Carbonaro et al., 2002; Lombardi-Boccia et al., 2004). However, not all of the components responsible for the separation of cv. Wavit from the coniferous species need to be of biological relevance. To unravel which components are actually important for proper development or which ones may inhibit nymphal growth, feeding experiments with nymphs on artificial diets are crucial. The analysis of excreted honeydew could suggest important information on how psyllids process plant nutrients. This study also revealed differences between the plant saps of the investigated coniferous trees. Therefore, a detailed analysis of EPG recordings from nymphs on the different tree species could be needful to identify feeding stimulants or deterrents and will be investigated in future. This knowledge could be used for development of an artificial diet system for rearing of *C. pruni* and screening for potential toxins against psyllids (Jancovich et al., 1997; Hall et al., 2010). Interestingly, although some of the migrating psyllids like *C. pruni* harbor phloem-limited plant pathogenic bacteria ('*Ca.* Phytoplasma' or '*Ca.* Liberibacter') and feed on conifers, the phytopathogens seem to be restricted to vector insects and their reproduction host plants (Gross, 2016). Because the genomes of Phytoplasma spp. lack metabolic genes but contain a lot of transporter systems, it is suggested that they depend strongly on the nutrition of their hosts (Oshima et al., 2004; Kube et al., 2008). Insight on the chemical composition of the phloem sap of host plants could support developing a culture media for phytoplasmas and may advance the research on phytoplasma diseases (Trivedi et al., 2016).

CONCLUSION

No mechanical nor chemical border prevents *C. pruni* adults and nymphs from feeding on conifers. Emigrants feed and survive on their overwintering hosts. Nymphs can feed on, but are not able to develop on spruce and fir. This is likely due to strong differences in the compositions of organic acids and sugars between plant saps of conifers and *P. domestica*. Furthermore, feeding experiments with nymphs on artificial diets should reveal which components are responsible for successful development of *C. pruni*. Additionally, more insight on phloem sap composition could open up new possibilities for phytoplasma cultivation and pathogen research.

AUTHOR CONTRIBUTIONS

JGa and JGr designed the study, contributed to the interpretation of the data, approved the final version of the manuscript, and ensured the accuracy and integrity of the work. JGa conducted the experiments and analysis and wrote the first draft of the manuscript, which was revisited and edited by JGr. JGr supervised the project.

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