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PLANT-MICROBE-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Host plant iridoid glycosides mediate herbivore interactions with natural enemies

Caitlin A. Kelly[1] · **M. Deane Bowers**[1]

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Abstract
Many insect herbivores are dietary specialists capable of sequestering the secondary metabolites produced by their host plants. These defensive compounds have important but complex implications for tritrophic interactions between plants, herbivores, and natural enemies. The sequestration of host plant secondary metabolites defends herbivores from attack by generalist predators, but may also compromise the immune response, making insect herbivores more vulnerable to parasitism. Here, we investigate the role of plant secondary metabolites in mediating interactions between a specialist herbivore and its natural enemies. The host plants are two *Penstemon* species, *Penstemon glaber* and *Penstemon virgatus*, which are chemically defended by iridoid glycosides (IGs). First, we examined how *Penstemon* iridoid glycoside content influences the sequestration of IGs by a specialist herbivore, *Euphydryas anicia*. Then, we performed ant bioassays to assess how host plant species influences larval susceptibility to predators and phenoloxidase assays to assess the immunocompetence and potential vulnerability to parasitoids and pathogens. We found that the concentration of IGs sequestered by *E. anicia* larvae varied with host plant diet. Larvae reared on *P. glaber* sequestered more IGs than larvae reared on *P. virgatus*. Yet, ant predators found larvae unpalatable regardless of host plant diet and were also repelled by sugar solutions containing isolated IGs. However, *E. anicia* larvae reared on *P. glaber* showed higher levels of phenoloxidase activity than larvae reared on *P. virgatus*. Our results suggest that the sequestration of some secondary metabolites can effectively protect herbivores from predation, yet may also increase vulnerability to parasitism via decreased immunocompetence.

Keywords Tritrophic interactions · Iridoid glycosides · Herbivory · Sequestration · Lepidoptera

Introduction

In tritrophic systems, the bottom–up effect of host plant variation can be important in determining the performance of an herbivore as well as its interactions with natural enemies (e.g., Awmack and Leather 2002; Teder and Tammaru 2002; Vogelweith et al. 2011). For instance, highly nutritional plants could improve herbivore performance and, consequently, increase the prey population available to their natural enemies (Awmack and Leather 2002; Coley et al. 2006). In contrast, higher host plant quality could strengthen herbivore defenses, and thus negatively affect the performance of natural enemies (e.g., Barbosa et al. 1986; Sime 2002). Although plant quality can refer to a multitude of factors, plant secondary metabolites are particularly important components of plant quality and play an important role in the evolution of plant–herbivore–natural enemy interactions (Turlings et al. 1990; Dyer 1995; Smilanich et al. 2009a, Smilanich et al. 2011). Plant secondary metabolites may improve plant fitness by acting as a defense against herbivores and pathogens; however, they may also attract specialized herbivores by providing oviposition and feeding cues (Nishida 2002; Optiz and Müller 2009). In addition, these compounds have been shown to attract the natural enemies of insect herbivores (Turlings et al. 1990; Dicke and van Loon 2000) and as such can mediate multi-trophic interactions.

Most insect herbivores are specialists, with a narrow diet breadth that is often limited to host plants containing specific

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✉ Caitlin A. Kelly
caitlin.a.kelly@colorado.edu

[1] Department of Ecology and Evolutionary Biology and University of Colorado Museum of Natural History, University of Colorado Boulder, UCB 334, Boulder, CO 80309, USA

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groups of plant secondary metabolites (Bernays and Chapman 1994; Bernays 2001). These compounds may serve as feeding and oviposition cues for specialists (Da Costa and Jones 1971; Raybould and Moyes 2001; Macel and Vrieling 2003; Nieminen et al. 2003), and some specialist species have the ability to sequester these compounds for their own defense (Nishida 2002; Optiz and Müller 2009). Bernays and Graham (1988) hypothesized that the prevalence of herbivore specialization is the result of predators preferring generalist prey, as they are less likely to be chemically defended. The sequestration of plant secondary metabolites provides an effective defense against many different generalist predators (e.g. Rayor and Munson 2002); however, feeding on highly defended plants can render some insect herbivores more susceptible to parasitoid attack (Dyer et al. 2004; Smilanich et al. 2009a). Parasitoid larvae may be better protected from their natural enemies if they develop within a chemically defended host; those sequestered compounds allow the insect host to provide enemy-free space for the parasitoid (Dyer and Gentry 1999; Gentry and Dyer 2002; Sznajder and Harvey 2003; Zvereva and Rank 2003; Murphy et al. 2014). Therefore, insect herbivores may experience an evolutionary trade-off between sequestering secondary metabolites for protection against predators, while becoming more vulnerable to parasitism.

Natural enemy attack is often the highest source of mortality for immature herbivores (Cornell and Hawkins 1995). For insects, the innate immune response can be one of the most effective defenses against non-predator enemies, such as parasitoids and pathogens (Godfray 1994; Wilson et al. 2001; Smilanich et al. 2009b). An important component of insect immunity relies on rapidly activated cascades of enzymes, such as phenoloxidase (Cerenius and Söderhäll 2004; Siva-Jothy et al. 2005). Phenoloxidase (PO) catalyzes the initial steps in the production of melanin, which is part of the encapsulation response to foreign bodies in insect hemolymph (Sugumaran 2002; Siva-Jothy et al. 2005). Studies in other systems demonstrate that PO levels can be indicative of increased defense against parasitoids and certain pathogens (e.g., Chapman 1982: Wilson et al. 2001; Sugumaran 2002; Siva-Jothy et al. 2005; Schlenke et al. 2007). However, mounting an immune response, such as encapsulation, can be costly in terms of both growth and reproduction (Ahmed et al. 2002; Freitak et al. 2003; McKean et al. 2008; Honkavaara et al. 2009; Ardia et al. 2012). Furthermore, sequestration of plant secondary metabolites may weaken the immune response (e.g., Smilanich et al. 2009a). Thus, the sequestration of secondary metabolites by herbivores may result in a trade-off between defense from predators and protection from non-predator enemies, such as parasitoids.

This study examines how plant secondary metabolites mediate the interactions of a specialist herbivore with the third trophic level, including components of defense against both predators and enemies that elicit the insect immune response (parasitoids, fungi, bacteria, and viruses). We focus on the herbivore *Euphydryas anicia* Doubleday (Lepidoptera: Nymphalidae; the anicia checkerspot), which specializes on plants containing iridoid glycosides (IGs), including plants in the families Plantaginaceae, Scrophulariaceae, Orobanchaceae, and Caprifoliaceae (Cullenward et al. 1979; White 1979), and can sequester these compounds (Stermitz et al. 1986; Gardner and Stermitz 1988; L'Empereur and Stermitz 1990a). Iridoid glycosides are a group of monoterpene-derived compounds found in over 50 plant families (Bobbitt and Segebarth 1969; Jensen et al. 1975; El-Naggar and Beal 1980; Boros and Stermitz 1990; Bowers 1991) and these bitter compounds are known mediators of multitrophic interactions (e.g., Harvey et al. 2005; Lampert and Bowers 2010).

At our study site in Colorado, *Euphydryas anicia* primarily uses two host plant species, *Penstemon glaber* var. *alpinus* (Torr.) A. Gray (Plantaginaceae) and *Penstemon virgatus* A. Gray. To address how plant secondary metabolites may influence both herbivores and herbivore defenses against predators and other enemies, such as parasitoids and pathogens, we ask three questions: (1) How much variation in IGs do *E. anicia* herbivores encounter? (2) How does variation in *Penstemon* IGs influence *E. anicia* sequestration of IGs? (3) How does the IG content of *E. anicia* larvae affect defenses against the third trophic level (predators and non-predator enemies)? To address these questions, we: (a) compare the IG content of these two *Penstemon* species; (b) quantify sequestration of IGs by *E. anicia* larvae reared on either *P. glaber* or *P. virgatus*, (c) compare the ability of larvae reared on these two *Penstemon* species to repel predatory ants, and (d) examine how herbivore diet affects one component of larval defense against parasitoids and pathogens: the immune response as measured by PO activity.

Materials and methods

Study system

Plants

Penstemon glaber var. *alpinus* and *P. virgatus* are herbaceous, long-lived perennials native to Colorado, and the southwestern region of the United States of America (Shonle et al. 2004). These *Penstemons* grow in montane meadows and road cuts. Both species feature inflorescences with several small-to-medium flowers that bloom from June to August (Quintero and Bowers 2013). *Penstemon virgatus* (upright blue beardtongue) features narrow leaves, tall stems, and purple flowers (Crosswhite 1967). *Penstemon glaber* var. *alpinus* (alpine sawsepal penstemon) has broad

and occasionally puberulent leaves, thick stems, and blue or blue-purple flowers (Stubbendieck et al. 2017). The previous research found two major IGs in *P. virgatus,* catalpol and scutellarioside-II, and only one major IG in *P. glaber*, catalpol (L'Empereur and Stermitz 1990b; Kelly and Bowers 2016). Both species naturally co-occur at the field site (see below) where checkerspot eggs were collected. We used greenhouse grown plants to control for environmental differences. All plants used in experiments were obtained from a local nursery and were maintained in one-gallon pots outside of a greenhouse.

Insects

Euphydryas anicia occurs throughout the western half of the United States, including the mountains of Colorado (White 1979; Cullenward et al. 1979; Ferris and Brown 1981). Females lay large egg masses (50–200 eggs) on the underside of leaves. Larvae are gregarious in the early instars and form a web on their host plant. They enter diapause in the fourth instar, and then overwinter in this stage, emerging in spring to complete development. At our field site, Crescent Meadows (El Dorado State Park, Boulder County, Colorado, USA; 39° 55′51.60″ N 105°20′ 16.80″W, elevation 2258 m) adults are typically found in late June through mid July. *Euphydryas anicia* is known to sequester the IGs catalpol, macfadienoside, and aucubin (Gardner and Stermitz 1988; L'Empereur 1989; L'Empereur and Stermitz 1990a), but there is currently no evidence that they sequester scutellarioside. Sequestered IGs are retained through metamorphosis, and both larvae and adults are aposematically colored (Stermitz et al. 1986; Gardner and Stermitz 1988). Kelly and Bowers (2016) found that *E. anicia* larvae performed equally well when reared on *P. glaber* and *P. virgatus* in the laboratory. Larvae used in the experiments presented here were reared from eggs collected on *Penstemon* at Crescent Meadows and maintained as separate family groups in a growth chamber (Percival model LLVL, 25 °C day: 20 °C night, 14 h day length) at the University of Colorado.

Iridoid glycoside chemistry

Plants

Leaf samples were oven-dried at 50 °C and ground to a fine powder in a mortar. Each sample was then extracted overnight in 95% methanol. Sample preparation and quantification of IGs via gas chromatography followed previously described methods (see Kelly and Bowers 2016). An internal standard, phenyl β-D-glucopyranoside (0.500 mg), was added to each sample. The sample was partitioned between water and ether. The ether fraction, which contains lipophilic substances, was discarded, and the water fraction, containing primarily the IGs and sugars, was evaporated to dryness. An aliquot of this was derivatized using Tri-Sil Z (Thermo-Fisher Chemical Company, Waltham, Massachusetts), prior to injection onto an HP 7890A gas chromatograph (Agilent Technologies, Santa Clara, California) using an Agilent DB-1 column (30 m, 0.320, 0.25 mm particle size; Gardner and Stermitz 1988; Bowers and Collinge 1992; Fajer et al. 1992). The gas chromatograph was calibrated using the standards of purified catalpol and scutellarioside-II (hereafter scutellarioside). Concentrations of catalpol and scutellarioside were quantified using ChemStation B-03-01 software, and data were analyzed as percentage of dry mass.

Larvae

Upon reaching diapause, groups of *E. anicia* larvae were freeze killed and later processed for chemical analysis. Since larvae empty their gut contents before entering diapause, the analysis of iridoid glycoside content of these larvae did not inadvertently include any plant material. Sibling groups of whole caterpillars ($n = 5$ individuals in each family group) were ground inside 15 ml test tubes using sand and a glass rod. The mixture was extracted overnight in 95% methanol. The methanol extract was subsequently filtered to remove solid material, and the resulting residue was evaporated to dryness. Sample preparation for larvae was the same as for plant material and larval IG content was determined by gas chromatography in the same manner as the plant tissue (described above and in Kelly and Bowers 2016).

Predator bioassays

To test for possible repellence of catalpol and scutellarioside and their potential function as a defense against predators, we conducted bioassays with laboratory ant colonies of *Formica pallidefulva* Latreille. Worker ants were collected from six field colonies around the University of Colorado Boulder campus in September 2014. *Formica pallidefulva* also occurs at our study site and has been observed on the same *Penstemon* plants used by *E. anicia* (C. Kelly, personal observation). Ants were maintained as distinct colonies in the laboratory in rectangular plastic bins (16.5 × 31 × 9 cm) lined with Fluon to prevent the ants from escaping. Colonies were maintained on a diet consisting of water and a 20% sucrose solution. The sugar solution was removed for 18–24 h prior to bioassay trials. For two consecutive hours, ants were presented with the choice of two different solutions, the contents of which varied with the trial. All control and experimental offerings were prepared in a solution of 20% sucrose in water. These solutions were presented to ants as 100 µl drops on top of a two small Parafilm squares (5 cm^2), which were placed side by side approximately 2–3 cm apart. There was a minimum of 24 h between trials

for a given colony. Each trial was video recorded for 2 h with an HD camcorder (Sony HDR-CX405). Video recordings were then observed, and the number and length of individual feeding bouts by ants (i.e., total consecutive time spent at each drop by individual ants) were recorded.

To investigate the chemical defenses of *E. anicia* larvae, ant bioassays with the same colonies were conducted comparing solutions containing pulverized, fresh mealworm larvae (no IGs), or *E. anicia* larvae reared on either *P. glaber* or *P. virgatus*. We used pulverized larvae instead of live caterpillars to specifically test the effects of the chemical (IG) content and to avoid potentially confounding variables, such as differences in behavior. The mealworm (control) solution contained ground mealworm larvae (35 mg/ml). The experimental solutions consisted of sibling groups of freshly ground *E. anicia* larvae that were reared on either *P. glaber* (10 larvae/ml = 36 mg/ml) or *P. virgatus* (10 larvae/ml = 34 mg/ml). All larvae were in the third instar and ground in the sucrose solution with a glass rod. Ant colonies were given the following pairwise comparisons: (a) control solution and *P. glaber*-reared caterpillar solution; (b) control solution and *P. virgatus*-reared caterpillar solution; (c) *P. glaber*-reared caterpillar solution and *P. virgatus*-reared caterpillar solution.

To investigate the effects of specific IGs, assays were conducted with solutions containing isolated IGs with no insect tissue. These bioassays allowed us to compare the effects of the IGs alone, with no influence of nutritional differences or other factors. For these trials, the control solution (20% sucrose in water) did not contain a protein source. The IG-containing solutions contained either catalpol (4 mg/ml) or catalpol + scutellarioside (4 mg/ml combined) in a 20% sucrose solution. Ant colonies were then presented with the following choices: (d) control solution and catalpol solution, and (e) catalpol solution and catalpol + scutellarioside solution.

Phenoloxidase assays

A parasitoid specific bioassay was not feasible, since *E. anicia* larvae are attacked only by specialist parasitoids, which are difficult to locate in the field. Therefore, we investigated the effects of host plant and the resulting chemical variation in sequestering caterpillars on the immune response by comparing the phenoloxidase activity of caterpillars reared on *P. virgatus* or on *P. glaber*. The *E. anicia* larvae used in this study had reached diapause and were in their fourth instar, a time when parasitoids may attack larvae. To rear the two groups of larvae, groups of full siblings were divided into two groups upon hatching: one group was reared exclusively on *P. virgatus* and the other on *P. glaber*. The small size of these larvae precluded extracting sufficient hemolymph from an individual larva for the immune assay (e.g. Vogelweith et al. 2011); therefore, each sample consisted of four, previously frozen, full-sibling larvae. The small size of these larvae also prevented our being able to conduct other measures of the immune response.

The activity of naturally activated PO enzymes was measured with a spectrophotometer using the methods described in Vogelweith et al. (2011). For sample preparation, groups of frozen, whole caterpillars were ground on ice in microcentrifuge tubes with 20 µl of phosphate buffer saline (PBS) and then centrifuged (4000*g*, 15 min, 4 °C). Ten microliters of supernatant were added to a microplate well containing 20 µl of PBS and either 140 µl of distilled water to measure PO activity only or 140 µl of chymotrypsin solution (Sigma C-7762, 0.07 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) to measure total PO activity. Finally, 20 µl of L-Dopa solution (Sigma D-9628, 4 mg/ml) was added to each well. The reaction was allowed to proceed at 30 °C in a microplate reader (Synergy HTX, BioTek, Sunnyvale, VT, USA) for 40 min. Readings were taken every 30 s at 490 nm and analyzed using the software Gen5 1.11 (BioTek Instruments Inc.). Enzyme activity was measured as the Vmax (change in absorbance unit per minute) during the linear phase of the reaction and reported per milligram of caterpillar.

Statistical analyses

Non-parametric tests were used, because all data failed to meet the assumptions of normality, as determined by Shapiro–Wilk tests. Wilcoxon–Mann–Whitney tests were used to compare plant IG concentrations, larval IG concentrations, and feeding times in the ant predator bioassays. Chi-square analyses compared the number of individual ant visits for each predator bioassay trial. The PO assay data were analyzed with a Wilcoxon–Mann–Whitney test. All statistical analyses were performed in R version 3.1.2.

Results

Penstemon chemistry and herbivore sequestration

Penstemon glaber leaves contained only catalpol, whereas *P. virgatus* leaves contained both catalpol and scutellarioside (Fig. 1a). *Penstemon glaber* leaves contained significantly more catalpol than *P. virgatus* leaves (Wilcoxon $p < 0.001$), with an average of 5.33% dry weight. This is noticeably less catalpol than that measured in *P. glaber* in a recent study (18.12% dry weight; Kelly and Bowers 2016). *Penstemon virgatus* leaves had more IGs overall (Wilcoxon $p < 0.001$, Fig. 1a), due to the high concentrations of scutellarioside (average of 25.05% dry weight). The IG concentrations found in *P. virgatus* were similar to those found in the previous research (Kelly and Bowers 2016).

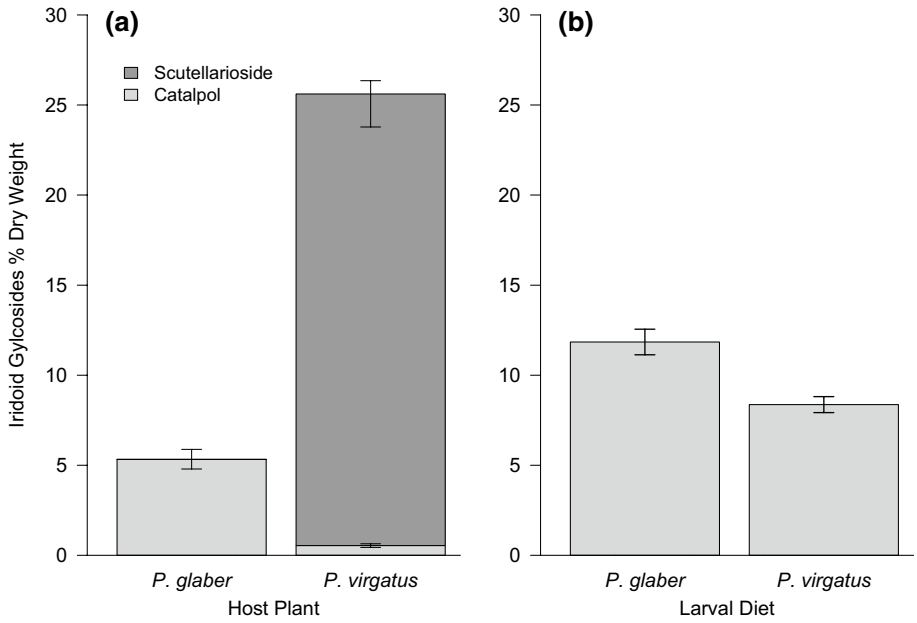

Fig. 1 Iridoid glycoside (IG) content of **a** *Penstemon glaber* ($n=25$) and *P. virgatus* ($n=30$) leaves and **b** *Euphydryas anicia* larvae reared on either *P. glaber* ($n=30$ groups of five siblings) or *P. virgatus* ($n=35$ groups of five siblings). Mean ± SE

Larvae reared on either *P. virgatus* or *P. glaber* only sequestered catalpol. Larvae reared on *P. glaber* sequestered more catalpol than those reared on *P. virgatus* (Wilcoxon $p<0.001$, Fig. 1b).

Predator bioassays

The number of ant visits showed that ants had a significant preference for the control solution ($n=110$ visits) over the *P. glaber*-reared caterpillar solution ($n=16$ visits; $\chi^2=68.64$, $p<0.0001$). The mean feeding times for that same comparison indicated a marginally significant preference for the control solutions (Wilcoxon $p=0.0504$, Fig. 2a). Ants demonstrated a clear preference for the control solution ($n=78$ visits) over the *P. virgatus*-reared caterpillar solution ($n=24$ visits; Visits: $\chi^2=27.54$, $p<0.0001$; Feeding time: Wilcoxon $p<0.001$, Fig. 2b). Ants showed no preference when given a choice between the *P. glaber*-reared caterpillar solution ($n=4$ visits) and the *P. virgatus*-reared caterpillar solution ($n=6$ visits) and both solutions had low visitation (Visits: $\chi^2=0.1$, $p=0.75$; Feeding time: Wilcoxon $p=0.625$, Fig. 2c).

In the assays comparing the control sugar solution to the sugar–catalpol solution, ants were indeed repelled by the presence of catalpol, as ants spent significantly more time feeding at the control solution (Wilcoxon $p<0.001$, Fig. 3a)

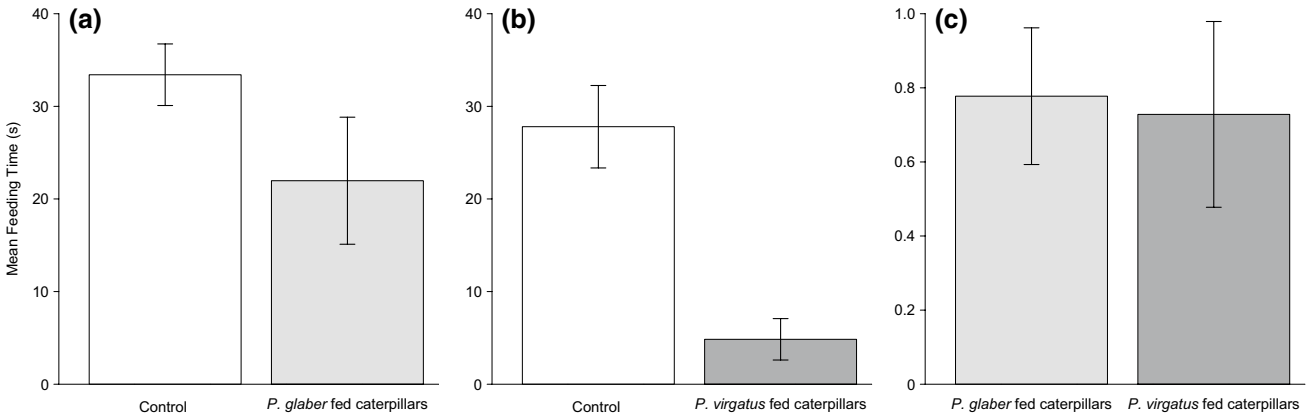

Fig. 2 Ant bioassays of the following pairwise comparisons: **a** control solution and *Penstemon glaber*-reared caterpillar solution (36 mg/ml), **b** control solution and *P. virgatus*-reared caterpillar solution (34 mg/ml), and **c** *P. glaber*-reared caterpillar solution and *P. virgatus*-reared caterpillar solution. All solutions were 20% sucrose in water and the control solution additionally contained ground mealworm larvae (35 mg/ml). Please note the different scale for the *y*-axis in **c**. Bars are mean ± SE

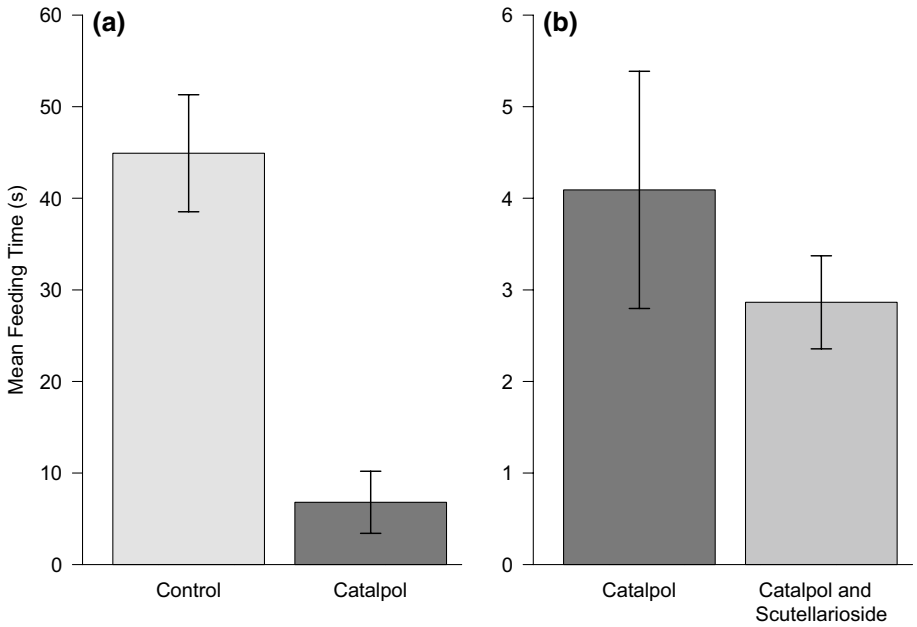

Fig. 3 Ant bioassays comparing **a** catalpol and **b** catalpol and scutellarioside. The control solution was 20% sucrose in water, and the treatment solutions were 20% sucrose plus either catalpol (4 mg/ml) or catalpol + scutellarioside (4 mg/ml combined) in water. Bars are mean ± SE time spent feeding (in seconds) per feeding bout by foraging individuals of *Formica pallidefulva*

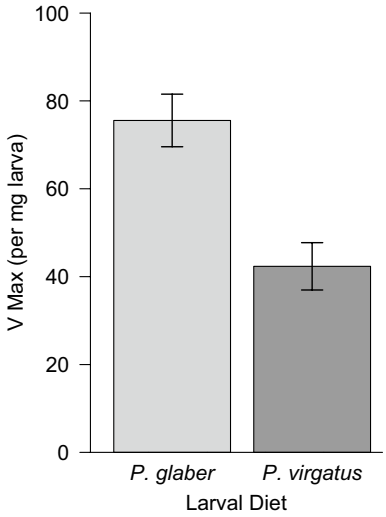

Fig. 4 Phenoloxidase (PO) activities of larvae reared on either *Penstemon glaber* ($n = 27$) or *P. virgatus* ($n = 27$). Mean ± SE

and the control solution received more visits ($n = 67$) than the catalpol solution ($n = 23$; $\chi^2 = 20.54$, $p < 0.0001$). However, ants were equally repelled by the catalpol solution ($n = 7$ visits) and the catalpol + scutellarioside solution ($n = 5$ visits; feeding time: Wilcoxon $p = 0.533$, Fig. 3b; visits: $\chi^2 = 0.8$, $p = 0.533$).

Phenoloxidase assays

Penstemon glaber-reared larvae had significantly higher PO activity than larvae reared on *P. virgatus* (Wilcoxon $p = 0.00281$; Fig. 4).

Discussion

In this study, we found that host plant diet indeed affected the IG sequestration of herbivore larvae, which, in turn, had consequences for some herbivore interactions with the third trophic level. Larvae of the IG specialist *Euphydryas anicia* sequestered 50% more catalpol when reared on *P. glaber* than larvae reared on *P. virgatus,* and catalpol was the only IG detected in these larvae. Yet, larvae reared on these two *Penstemon* species were equally unpalatable to invertebrate predators. These results suggest that there may be a threshold effect, such that levels of sequestered IGs above a certain level are equally repellent (e.g., De La Fuente et al. 1994/1995). Supporting this hypothesis, ants did not distinguish between the solutions containing *P. glaber*-reared larvae and those containing *P. virgatus*-reared larvae. Bioassays with pure compounds indicated that ants were repelled by the presence of catalpol but were similarly repelled by the solution containing both catalpol and scutellarioside. This suggests that no synergistic interaction exists between catalpol and scutellarioside, since the combination of these compounds did not produce an antiherbivore effect that was noticeably greater than their expected additive effects (Berenbaum et al. 1991; Nelson and Kursar 1999). Parasitoids and pathogens, however, may be affected by the plant diet on which their host larvae have fed. Larvae reared on *P. glaber* had higher phenoloxidase activity than those reared on *P. virgatus*, which indicates that *E. anicia* larvae reared on *P. glaber* may be capable of mounting a stronger immune response.

Euphydryas anicia can sequester catalpol but not scutellarioside. Larvae reared on *P. glaber,* the more catalpol-rich

host plant, sequestered more catalpol. However, we detected much higher concentrations of catalpol in larvae reared on either *Penstemon* diet than was found in either host plant (*P. glaber* diet: 11.84% dry caterpillar weight and 5.33% dry plant weight; *P. virgatus* diet: 8.37% dry caterpillar weight and 0.54% dry plant weight). Likewise, Stermitz et al. (1986) found that *E. anicia* contained higher concentrations of catalpol than was in the host plant *Besseya plantaginea* (James) Rydb (Plantaginaceae). *Besseya plantaginea* contains five other IGs in addition to catalpol, although three of them are catalpol esters. *Euphydryas anicia* may be selectively sequestering and concentrating catalpol, or they may be metabolizing catalpol esters into catalpol, as suggested by the previous study of Gardner and Stermitz (1988). Gardner and Stermitz (1988) found evidence that *E. anicia* is capable of such conversion, demonstrating that larvae metabolized 6-isovanillylcatalpol into catalpol and then excreted the remaining isovanillic acid. Therefore, it is possible that *E. anicia* larvae metabolically convert scutellarioside into catalpol by hydrolyzing the side chain.

Iridoid glycosides, including catalpol, have been shown to play an important defensive role against both vertebrate and invertebrate predators (Bowers 1991; De La Fuente et al. 1994/1995; Dyer and Bowers 1996; Camara 1997). Although host plant diet resulted in differences in the amount of catalpol sequestered by *E. anicia*, larvae reared on these two plant species appeared equally defended from invertebrate predators; the *Formica pallidefulva* ants used in the predator bioassays did not distinguish between sugar solutions containing *P. glaber*-reared larvae and *P. virgatus*-reared larvae. These data suggest that relatively small amounts of catalpol may be sufficient to successfully prevent predation by invertebrates. The results from the bioassays with isolated IGs confirmed that ant predators found catalpol unpalatable. The bioassay trials comparing a catalpol solution to a combined catalpol and scutellarioside solution showed no indication of a synergistic interaction of these two IGs. Ants were repelled by both solutions, as demonstrated by the low number of visits to each solution and the short duration of each feeding bout. Although there was a downward trend in foraging bouts to the solution that contained both catalpol and scutellarioside, the difference in feeding time was only one second and this difference was not statistically significant.

The results of the PO assays suggest that *E. anicia* larvae reared on *P. virgatus* may be less well defended against attack from parasitoids and pathogens. One possible mechanism underlying this difference may be the cost of metabolizing scutellarioside. This metabolic process may weaken the immune response of *P. virgatus*-reared caterpillars. A previous study with *Junonia coenia* (Nymphalidae), an IG specialist caterpillar (Bowers and Stamp 1997), found a significantly lower melanization response when larvae sequestered high concentrations of IGs (Smilanich et al. 2009a). Thus, *P. glaber*-reared larvae may be less immunocompromised, despite sequestering higher concentrations of catalpol overall. Though mounting an immune response can sometimes decrease fitness in other ways (e.g. growth rates, reproduction, and parental effort; Råberg et al. 2000; Zuk and Stoehr 2002), Kelly and Bowers (2016) found that *E. anicia* larvae performed equally well when reared on *P. glaber* and *P. virgatus* in a laboratory. These results suggest that any metabolic costs incurred by *P. virgatus*-reared larvae are only detected in the context of immune defense.

We found significantly higher PO activity in larvae reared on *P. glaber,* suggesting that larvae reared on *P. glaber* may be capable of mounting a stronger melanization response to non-predator natural enemies. The higher PO levels could be an indication that larvae reared on *P. glaber* are in a better condition than individuals reared on *P. virgatus*, as suggested by a relatively recent review (González-Santoyo and Córdoba-Aguilar 2012). We decreased the likelihood of prior infection by pathogens or parasitoids affecting our results in several ways: we collected egg masses in the field, reared larvae in the laboratory, and used sibling groups divided between the two host plant diets to control for genotypic differences; we used greenhouse grown plants to control for environmental differences between the two host plants. Furthermore, it is possible that PO activity levels may not differentially affect internal natural enemies and that even low amounts of PO activity could sufficiently protect *E. anicia* larvae. While some studies include additional metrics for immune response (e.g., Satterfield et al. 2013; McKay et al. 2016a, 2016b), the small size of our caterpillars did now allow us to use other measures of innate immunity. In addition, inferences are limited because we were not able to conduct a bioassay using parasitoids. This was not possible, since checkerspot caterpillars are attacked by specialist parasitoids, which are difficult to locate and collect in the field. However, PO assays indicate the strength of the larval response to multiple types of natural enemies, including pathogens, and are not limited to particular species of parasitoids.

Plant secondary metabolites can have important bottom–up effects on both insect herbivores and higher trophic levels. Our results indicate that certain host plant diets may result in a trade-off between defense against predators and protection from non-predator enemies. Recent work has demonstrated that *E. anicia* females preferred to oviposit on *P. glaber* in the field, but larvae showed no preference for either *P. glaber* or *P. virgatus* in the laboratory (Kelly and Bowers 2016). The results of the present study suggest that, when defense against natural enemies is considered, females are likely choosing the host plant that is better for their offspring. While larvae reared on either host plant appear to be unpalatable to invertebrate predators, larvae reared on *P.*

glaber may be less susceptible to parasitoids and pathogens. Gentry and Dyer (2002) suggest that larval sequestration is driven more by predators than parasites, since well-defended hosts provide enemy-free space for parasitoids. However, there is contrary evidence, demonstrating that sequestering defensive compounds protects insects from parasitoids (e.g., Sime 2002) and also helps fight microbial infections. We found that the immunocompetence (as assessed by one measure of the immune response, PO activity) of specialist larvae varied with host plant species and that feeding on plants containing compounds that larvae cannot directly sequester, but metabolize before sequestration, may result in a weakened melanization response. This suggests that, in the *Penstemon–Euphydryas* system and perhaps other systems in which herbivores can sequester plant secondary metabolites, the selective advantage of sequestration may be driven more by predators than other types of enemies. Our study highlights the importance of plant secondary metabolites in mediating herbivore vulnerability to natural enemies and the role of sequestration in shaping the evolution of complex multi-trophic interactions.

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Author contribution statement CAK and MDB conceived and designed the experiments, CAK performed the experiments, conducted the chemical analyses, and analyzed the data, and CAK and MDB wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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