

Evolutionary ecology of plant resistance traits across the herbivore diet spectrum: a test in the model plant *Mimulus guttatus*

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ABSTRACT

Hypothesis: Based on the phytochemical coevolution hypothesis and related host specialization hypotheses, we predicted that chemical resistance traits would have greater detrimental effects on generalist herbivore performance than on specialist herbivore performance. In an extrapolation of the phytochemical coevolution hypothesis, we predicted that physical resistance traits may be more detrimental to specialist herbivores that do not regularly encounter a particular physical resistance trait, than to generalists.

Organisms: *Mimulus guttatus* (syn: *Erythranthe guttata*) plants, derived from natural populations chosen for variation in resistance traits, were fed to natural lepidopteran herbivores from the specialist to generalist diet spectra: *Euphydryas chalcedona*, *Junonia coenia*, *Grammia incorrupta*, *Trichoplusia ni*, and *Spodoptera exigua*. We also included the generalist slug species *Deroceras laeve*.

Methods: We used a series of performance or preference trials with a spectrum of specialist to generalist herbivores that feed on the model plant *Mimulus guttatus*. We analysed how specific constitutive resistance traits influenced the performance or preference of the organisms.

Results: We found more specialized herbivore performance tended to have positive associations with some phytochemicals while having a negative association with physical traits (i.e. trichome density). Performance of the generalist herbivores, with a few exceptions, had a negative relationship with phytochemical traits and neutral relationships with trichome density.

Conclusions: The patterns observed generally fit the predictions of the phytochemical coevolution theory and the related host specialization hypotheses, although there was one exception for each generalist species. Some of the results could also be explained by the arms race, pairwise coevolution, and/or synergism hypotheses of phytochemical evolution.

Keywords: diet breadth, herbivores, *Mimulus guttatus*, phenylpropanoid glycosides, phytochemical coevolution theory, plant resistance traits.

INTRODUCTION

Almost 18% of all terrestrial primary production is consumed by herbivores (Cyr and Pace, 1993). Plants defend themselves or limit the impacts of herbivores with a wide array of traits, including secondary chemical metabolites, life-history strategies, and physical structures. The high degree of dietary specialization (hereafter ‘specialization’) throughout many herbivore groups, particularly insects (Bernays and Graham, 1988), has long been of interest to evolutionary biologists (Brues, 1924; Ehrlich and Raven, 1964). Specialist herbivores feed on plants from a narrow phylogenetic breadth, such as on only a few closely related plant families or within a single genus. They are typically unable to utilize plants beyond a set diet breadth. Specialist herbivores are often able to avoid or mitigate (for example, through sequestration or detoxification) the effects of phylogenetically conserved plant secondary metabolites (Wink and Waterman, 1999), and may avoid direct competition from other herbivores (Schoonhoven *et al.*, 2005). Specialist herbivores may exert a stronger and/or divergent selection pressure on plant resistance traits than other herbivores because of specialists’ close and often positive association with specific plant traits (Becerra, 1997; Stinchcombe and Rausher, 2001; Cornell and Hawkins, 2003; Lankau, 2007; Mithöfer and Boland, 2008; Coley and Kursar, 2014). For example, within the plant family Apocynaceae there has been a gradual escalation of the concentration and diversity of pyrrolizidine alkaloids due to pressure from herbivores. In lineages of Apocynaceae that have greater selective pressures from specialist herbivores there is a de-escalation of total pyrrolizidine alkaloid concentrations due to this specialist pressure (Livshultz *et al.*, 2018).

In contrast to specialist herbivores, dietary generalist herbivores choose from a relatively broad phylogenetic range of plant species and families. Diet breadth varies considerably among generalists, from truly polyphagous herbivores [e.g. *Spodoptera exigua* (Greenberg *et al.*, 2001)] to locally adapted populations of herbivores [e.g. *Euphydryas editha* (Williams *et al.*, 1983; Bowers, 1986; Scott, 1986)] to herbivores that vary their diets based on instar or physiological needs [e.g. *Grammia incorrupta* (Singer and Bernays, 2009)]. Generalist herbivores thus exist on a gradient of specialization or lack thereof (Singer, 2008; Loxdale and Harvey, 2016). Hypotheses regarding the relationship between the diversity of plant resistance traits (both chemical and physical) and how they interact within the diet breadth of herbivores have been explored in a few systems as well as theoretically (Stamp, 2003; Orians and Ward, 2010). Much of this literature has focused on the phytochemical coevolution theory (Cornell and Hawkins, 2003).

Phytochemical coevolution theory [also termed the ‘escape and radiate hypothesis’ (Ehrlich and Raven, 1964; Berenbaum, 1983; Cornell and Hawkins, 2003)] and related hypotheses of host specialization (Jaenike, 1990; Ali and Agrawal, 2012) posit that specialist herbivores that regularly experience particular phytochemicals in their diets should evolve to be more tolerant of, or more efficient at detoxifying, these compounds than generalists, which must be able to succeed on a variety of plants with different defence traits (Dethier, 1954; Scriber, 1983, 1984). It has been shown that specialist and generalist herbivores exert opposing selection pressures on different resistance traits (Maceel *et al.*, 2005; Lankau, 2007; Iason *et al.*, 2011). There is more ambiguity in the literature regarding predictions for how generalist or specialist herbivores interact with physical resistance traits, with a very limited number of studies addressing this question (Levin, 1973; Traw and Dawson, 2002). Here, we consider physical resistance traits as an extrapolation of the phytochemical coevolution hypothesis, in that performance of generalists will be less negatively affected by widespread physical defence traits than specialist performance. Specialist performance is likely to be more negatively affected by defence traits that are not widespread, or commonly experienced, within their host species.

Many tests of the phytochemical coevolution theory to date have involved the use of a single purified phytochemical fed in an artificial diet to a single specialist and a single generalist herbivore species (see Cornell and Hawkins, 2003 and references therein; but see also Kelly and Bowers, 2016). Although this approach isolates the effects of specific compounds, herbivores encounter not just a particular phytochemical, but a suite of phytochemicals and physical resistance traits in their host plants (Arnason *et al.*, 2004; Agrawal and Fishbein, 2006). In addition, the dichotomy of specialist and generalist herbivores is actually a gradient of dietary choices and ability to consume multiple plant species; thus use of more than two herbivore species to move beyond a specialist/generalist dichotomy can be informative (Ali and Agrawal, 2012).

Here, we conduct a series of no-choice performance trials and a preference (choice) trial with six herbivore species distributed across the spectrum of specific to broad diet breadth and all sharing the host plant *Mimulus guttatus* (Phrymaceae). We test the following predictions of phytochemical coevolution theory and related host specialization hypotheses: (1) the performance of specialist herbivores will often be less negatively associated with increased concentrations of specific chemical resistance traits than the performance of generalist herbivores; and (2) the performance of specialist herbivores will be negatively associated with increasing trichome density, a physical resistance trait that is not widespread in host plant populations of these specialists, while physical resistance traits will have little impact on generalist herbivore performance.

MATERIALS AND METHODS

Plants

Mimulus guttatus (syn. *Erythranthe guttata*; Phrymaceae) is native to western North America, ranging from Alaska to northern Mexico. It is a model organism in ecological and evolutionary genetics (Wu *et al.*, 2008) in part because of its phenotypic variability. This includes variation in life-history strategy (Galloway, 1995; Hall and Willis, 2006; Lowry and Willis, 2010; Kooyers *et al.*, 2015), corolla morphology (Carr and Fenster, 1994), leaf morphology (Wu *et al.*, 2008), and anti-herbivory defences (Holeski, 2007; Holeski *et al.*, 2013; Kooyers *et al.*, 2017). *Mimulus guttatus* produces a suite of phenylpropanoid glycosides (PPGs), phenolic compounds produced by the shikimic acid pathway (Keefover-Ring *et al.*, 2014). Variation in PPGs is genetically based and occurs both constitutively (in the absence of herbivory) and plastically [levels change in response to damage (Holeski *et al.*, 2013)]. Various PPGs can be found among many plant families but are particularly common and concentrated in families that were once found in the Scrophulariaceae (Jimenez and Riguera, 1994). Genetic variation also exists within and among natural populations of *M. guttatus* for a physical resistance trait, trichome density (Holeski, 2007; Holeski *et al.*, 2010), with plants ranging from glabrous to having dense glandular trichomes. Populations with glandular trichomes do not have PPGs present in the trichomes or trichome exudate (L.M. Holeski and K. Keefover-Ring, unpublished data).

We used plants derived from 11 natural populations (see Fig. 1; Table S1 at evolutionary-ecology.com/data/3151Appendix.pdf) of both annual and perennial ecotypes of *M. guttatus* with variable trichome density and constitutive PPG concentrations. Field-collected seed from multiple maternal plants per population was grown in the greenhouse for at least one generation, with crosses done to form multiple full-sibling families within each population. Plants were grown in Fafard 3B mix potting soil under 16 hour high-pressure sodium light

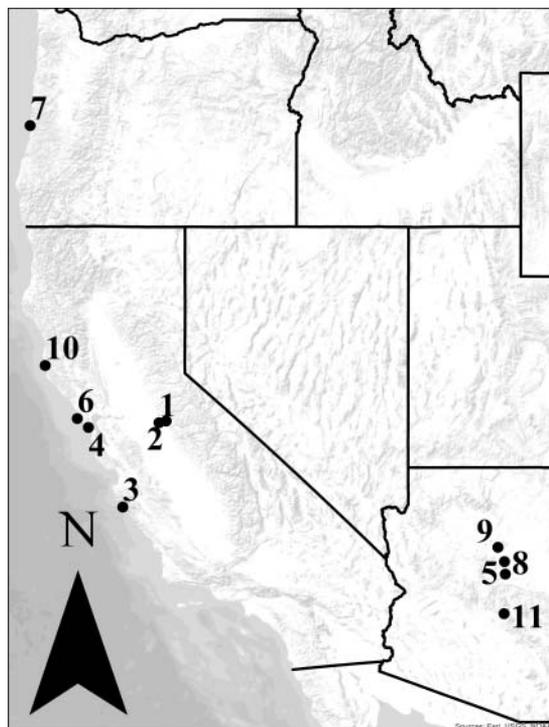


Fig. 1. Location of study populations used in the feeding trials. Numbers correspond with those in the text and tables (see Appendix Table S1).

and flood watered daily. A 10-30-20 fertilizer (Peters' Professional Base Formulation) was used once a week.

Herbivores

We used five species of Lepidoptera ranging in their degree of specialization on *Mimulus guttatus* for no-choice growth performance trials, and a slug species for a preference (choice) trial (Table 1). All species used feed on *M. guttatus* in the field (Rotter and Holeski, 2017; M.C. Rotter, personal observation). The specialist with the narrowest host breadth was *Euphydryas chalcedona* (variable checkerspot, Nymphalidae); this species occurs throughout the range of *M. guttatus*. The feeding habits of *E. chalcedona* are highly localized, with individual populations typically focusing on one host plant species even if several other acceptable hosts are available to that population (Williams *et al.*, 1983; Bowers, 1986; Scott, 1986). Additionally, we used a specialist herbivore with a wider host breadth, *Junonia coenia* (buckeye, Nymphalidae). *Junonia coenia* feeds on members of the Scrophulariaceae and families formerly included in this group (e.g. Phrymaceae and Plantaginaceae). Buckeyes are found commonly in the southern range of *M. guttatus* (Scott, 1986). Third, we performed trials with *Grammia incorrupta* (woolly bear, Erebididae), a 'selective generalist'. *Grammia incorrupta* often selectively graze within a diverse plant community based on their immediate nutritional or defensive needs (Singer and Bernays, 2009). *Grammia incorrupta* is native to the

southwestern USA. Fourth, the generalist *Trichoplusia ni* (cabbage looper, Noctuidae) was used; this species has a wide-ranging diet but some predilection for members of the Brassicaceae. *Trichoplusia ni* naturally occurs throughout the range of *M. guttatus* (Powell and Opler, 2009). Fifth, we used *Spodoptera exigua* (beet armyworm, Noctuidae), a generalist with an extremely wide host breadth (Smits *et al.*, 1987). This species has been recorded to feed from over 200 different plant species. It also occurs throughout the native range of *M. guttatus* (Greenberg *et al.*, 2001). Finally, we completed a choice (preference) feeding trial with the slug species *Deroceras laeve* (Agriolimacidae). Originally native to the Palearctic, *D. laeve* has been introduced throughout the native range of *M. guttatus* and consumes a large variety of dead and live plants (Wiktor, 2000). This species has been observed feeding on *M. guttatus* in the greenhouse as well as in the field (M.C. Rotter, personal observation).

Table 1. Study organisms and diet breadth (Scott, 1986; Wiktor, 2000; Powell and Opler, 2009)

Organism	Family	Geographic range	Breadth of host diet
<i>Euphydryas chalcedona</i>	Nymphalidae	Throughout the range of <i>M. guttatus</i> in western North America	Locally variable with populations utilizing perennial members of Scrophulariaceae <i>sensu lato</i>
<i>Junonia coenia</i>	Nymphalidae	Southern areas of North America, as far north as Oregon within the range of <i>M. guttatus</i>	Plants containing iridoid glycosides and those related to Scrophulariaceae <i>sensu lato</i>
<i>Grammia incorrupta</i>	Erebidae	Southwest North America from Arizona to western Texas and south to Durango, Mexico	A generalist species that has been recorded to feed from over 50 plant families, mainly herbaceous plants
<i>Trichoplusia ni</i>	Noctuidae	Across North America. Within the range of <i>M. guttatus</i> in all parts except the most northern populations	Feeds on almost any herbaceous plant but prefers members of the Brassicacea
<i>Spodoptera exigua</i>	Noctuidae	Non-native to North America. Populations established throughout the continent in non-freezing areas, although expands northward seasonally. Regularly reaches northern California within the range of <i>M. guttatus</i>	An extreme generalist with records of over 33 families, mostly herbaceous species and crop plants
<i>Deroceras laeve</i>	Agriolimacidae	Present throughout the range of <i>M. guttatus</i>	Broad feeder on living and dead plant material

All Lepidoptera species were obtained from commercial growing facilities with the exception of *E. chalconotus* and *G. inornata*. We collected *E. chalconotus* as gravid females from wild populations in Maricopa County, Arizona, where they feed locally on *Keckiella antirrhinoides* (Plantaginaceae). In the experiment we used neonates from eggs that these females oviposited after collection. *Grammia inornata* neonates were from a captive colony originally collected from southeastern Arizona (M.S. Singer, personal communication). Slugs were collected from the Northern Arizona University greenhouse. The previous generation of all of the larval species were raised on artificial diets with the exception of *E. chalconotus*; we fed the slugs a diet of lettuce prior to the start of the preference trials.

Herbivore growth performance and preference trials

We conducted no-choice growth performance trials with neonate larvae of each lepidopteran herbivore species. No-choice tests limit the herbivores to feeding from one individual plant (or portion of a plant), thus masking some of the potential ecological cues that may contribute to finding, selecting, and consuming a plant in the wild. Unique plants were seeded and used for each trial. For each trial, we took a pair of opposite leaves from each experimental plant. One leaf was placed in an envelope and treated as described below for analysis of PPGs. We assessed trichome density on the second leaf, as described in Holeski (2007). The leaf scored for trichomes was then placed into a water pic and placed in a petri dish. In each petri dish (100 × 15 mm diameter), for all herbivores except *E. chalconotus*, we placed a single recently hatched first instar caterpillar. For *E. chalconotus*, a pilot feeding trial indicated that they would not feed unless in their natural, gregarious state (M.C. Rotter, personal observation). We thus placed three neonates of *E. chalconotus* on each experimental leaf. Leaves were immediately replaced with leaves from the same plant (with the opposite leaf harvested for phytochemical analysis) if/when the caterpillar consumed the entire leaf or if the leaf wilted. After larvae had fed for 10 days, we ended each trial, froze the caterpillars, and then dried and weighed them to determine caterpillar final dry mass. Larval initial (wet) weights were all within 0.001 µg of each other for a particular species, so we assumed that initial dry mass was identical across larvae within each species. Dry mass for *E. chalconotus* larvae was calculated as the average across the larvae within a single petri dish. Higher caterpillar mass and growth rates are important indicators of greater larval survival and pupal size rates as well as increased adult fitness (Haukioja and Neuvonen, 1985; Awmack and Leather, 2002). Additionally, a more rapid growth rate may allow higher pupal survival rates when faced with pressure from predators and parasitoids (Feeny, 1976; Benrey and Denno, 1997; Uesugi, 2015).

For the *D. laevis* preference trial, one slug (starved for 48 hours) was placed into a petri-dish arena with three 19.63 mm² hole punches of leaf tissue from three different *M. guttatus* populations. Leaf discs were made by hole-punching intact leaves and were used to make food choices less dependent on leaf shape, size, or other aspects of plant appearance. Because the hole punching disturbed and sometimes smashed trichomes, we did not characterize trichome density in these slug preference trials. We used one plant from each of five different populations of *M. guttatus*. Each slug was offered each one of all possible combinations of three from these five populations, with three replicate petri dishes (and slugs) per combination. The slug was placed in the centre of the petri dish equidistant from each of the leaf discs. When a slug consumed a whole leaf disc, the plant population was noted and the slug/petri dish removed from the trial, as all choices were no longer available to the slug. If the slug partially consumed a disc, we estimated the percentage consumed but continued the trial until one disc was completely consumed.

Resistance traits

For phytochemical analysis, the leaf opposite the leaf used to feed the caterpillar/slug was cut at the base of the petiole with scissors and immediately flash frozen in liquid nitrogen before being transferred to a -20°C freezer. Tissue was then lyophilized using a pre-chilled FreeZone triad freeze dry system (Labconco; Kansas City, KS). We finely ground the freeze-dried tissue in a small capacity ball mill (dental amalgamator with steel bearings). Samples were stored and extracted as described in Holeski *et al.* (2013). We quantified the PPG content of each sample using high-performance liquid chromatography [HPLC; Agilent 1260 HPLC with a diode array detector and Poroshell 120 EC-C18 analytical column (4.6 · 250 mm, 2.7 μm particle size); Agilent Technologies, Santa Clara, CA] maintained at 30°C , as described in Kooyers *et al.* (2017). The seven PPGs analysed here represent the PPGs present in detectable levels in the populations used in this study.

Trichome density was measured by counting all the trichomes at the basal section of the adaxial side of each leaf within the field of view of a dissecting microscope at $25\times$ magnification. This count was converted to number of trichomes per cm^2 .

Statistical analyses

For each Lepidoptera performance trial, we assessed genetic variation in constitutive resistance traits within and among *M. guttatus* populations via nested analysis of variance (ANOVA; plant family nested within population, and population as a factor). For each lepidopteran herbivore species, we assessed whether there were differences in larval performance within and among *M. guttatus* populations using nested ANOVAs.

We used partial least square regression (PLSR) to quantify the relationship between the concentrations of each PPG compound, trichome density, and caterpillar performance (final dry caterpillar mass or survival). Within each trial, resistance traits and caterpillar growth performance were averaged within populations of plants. Partial least square regression has been widely used within analytical chemistry and increasingly used in ecological studies because of its ability to deal with multicollinearity as well as when there are more independent variables than sample size would permit in a normal regression (Carrascal *et al.*, 2009). Partial least square regression takes independent (predictor) variables and reduces them to uncorrelated latent variables. A select number of latent variables are then chosen from an independent data matrix and used to capture independent variable (predictor) variation that is highly correlated with the dependent (response) variable (Geladi and Kowalski, 1986). Individual independent variables can be evaluated by regression coefficients. To generate confidence intervals, we used a Monte Carlo simulation to run 80% of the data points used to predict the remaining 20% of the data set. We considered a variable to be significant if the standard deviation did not overlap zero, suggesting a consistent negative or positive association.

To assess *M. guttatus* population preferences of the slug *D. laeve*, we used a contingency analysis. Within the populations used in the preference trial, we also examined the PPG concentrations of the plants used. All statistics were performed in R (R Development Core Team, 2016).

RESULTS

Herbivore resistance traits vary among monkeyflower populations

Summed (total) and most, but not all, individual constitutive PPG concentrations differed among plant populations used for each trial (Appendix Table S2). Total PPGs varied 1.5- to 3-fold and individual PPG concentrations varied 2- to 4-fold among populations within each trial (Appendix Tables S4–11). Constitutive trichome density also varied substantially among populations for all trials (Appendix Tables S2, S3), with 2-fold to 10-fold differences across populations.

Performance differs among monkeyflower populations

Growth performance of some, but not all, herbivore species differed among populations (Fig. 2; Appendix Tables S2, S12). Many of the specialist *Euphydryas chalcedona* larvae died before completion of the trial, so we assessed patterns of larval survival for this species in addition to final caterpillar mass. Total larval survival differed significantly across *E. chalcedona* feeding on different populations ($F_{7,134} = 5.20$, $P < 0.001$; Fig. 2A). There were no survivors on plants from populations 1 and 10. Survival on population 3 was the highest with almost twice as many survivors than on the population with the next highest survival (population 9). There was no significant difference in performance (i.e. mass gain) across populations for surviving *E. chalcedona* ($F_{7,134} = 1.83$, $P = 0.087$; Fig. 2B). The specialists *Junonia coenia* and generalists *Grammia incorrupta* and *Trichoplusia ni* exhibited performance differences across populations (*J. coenia*: $F_{6,99} = 6.70$, $P = 0.001$; *G. incorrupta*: $F_{7,192} = 4.68$, $P = 0.001$; *T. ni*: $F_{7,106} = 4.15$, $P < 0.001$; Fig. 2C, D, E; Appendix Table S2). Like *E. chalcedona*, *J. coenia* performed best on population 3. A similar pattern was not observed between performance results on particular populations between the *G. incorrupta* and *T. ni* trials. For instance, *G. incorrupta* grew the most, while *T. ni* grew the least on population 9 leaves. There was no significant difference in performance across populations for the generalist *Spodoptera exigua* ($F_{7,80} = 1.23$, $P = 0.294$; Fig. 2F).

Resistance traits and herbivore performance

Partial least square regression (PLSR) models (Fig. 3A, B, C) for the two most specialized herbivores had moderate overall explanatory power of variation (*E. chalcedona* survival = 54.3%, *E. chalcedona* mass = 73.1%, and *J. coenia* mass = 50.4%). This suggests that additional factors beyond the measured resistance traits may contribute to the variation in survival and growth of these species. In *E. chalcedona*, both survival and growth were positively associated with some PPGs (one for survival, two for mass), and negatively (or neutrally) associated with others (Fig. 3A, B). *Euphydryas chalcedona* performance was neutral with respect to total PPG levels. Trichome density did not substantially affect *E. chalcedona* survival but was negatively associated with its mass (Fig. 3A, B).

Growth performance of *J. coenia*, the specialist with a slightly broader diet breadth, was positively associated with levels of four PPGs, negatively associated with levels of two PPGs, and neutral with respect to total PPGs (Fig. 3C). Trichome density was negatively associated with *J. coenia* performance.

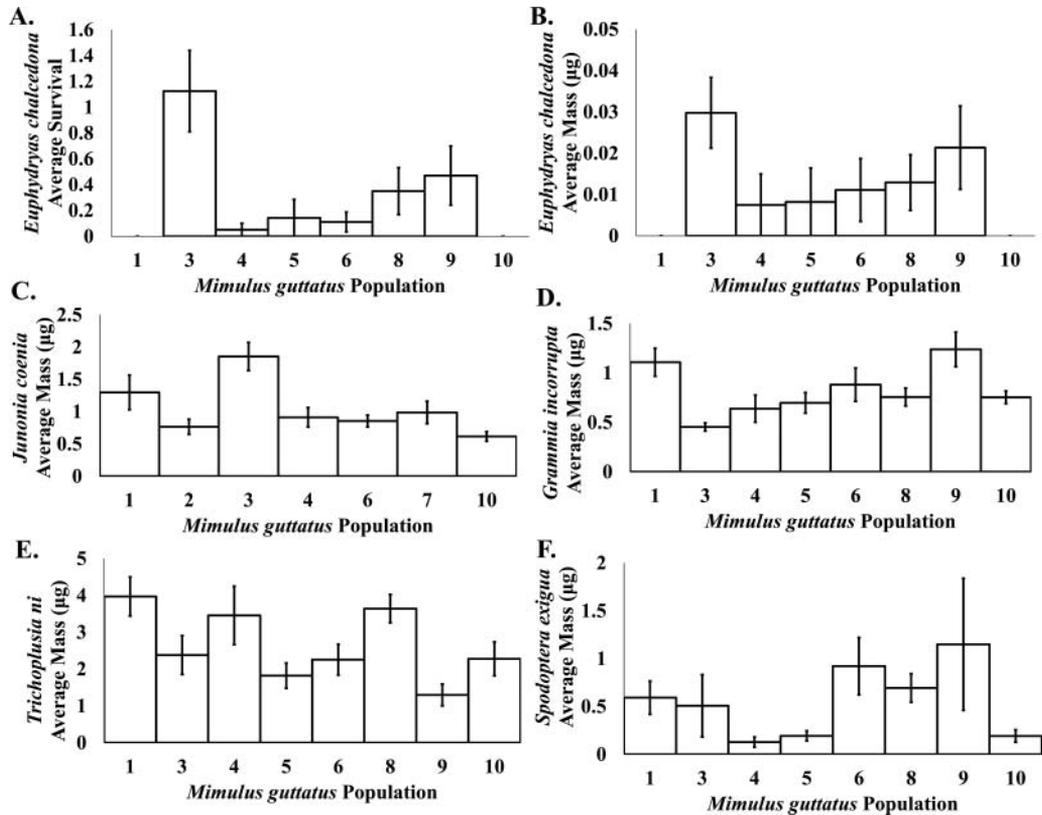


Fig. 2. Performance of caterpillar larvae on populations of *Mimulus guttatus*. (A) *Euphydryas chalcedona* average number of larvae surviving until the end of the trial. Panels (B–F) show average final larval weights of: (B) *Euphydryas chalcedona*, (C) *Junonia coenia*, (D) *Grammia incorrupta*, (E) *Trichoplusia ni*, and (F) *Spodoptera exigua*. Error bars represent ± 1 standard error.

For the generalist herbivores, our PLSR models had a relatively high degree of explanatory power (*G. incorrupta* = 96.3%, *T. ni* = 81.2%, and *S. exigua* = 85.9%). The performance of each generalist was positively associated with levels of one PPG (Fig. 3D, E, F). For each generalist, there were also multiple negative associations between PPG levels and performance. The performance of two of these generalists, *G. incorrupta* and *S. exigua*, had relatively strong negative relationships with total PPG levels. The performance of each generalist was neutral with respect to trichome density (Fig. 3D, E, F).

In the *D. laeve* slug preference trial, slugs preferred leaf discs from a single *M. guttatus* population (population 6) almost exclusively in the choice tests when comparing petri dishes with a completely consumed leaf disc ($\chi^2 = 55.255$, $df = 4$, $P < 0.001$; Table 2). Occasionally, *D. laeve* would consume a leaf disc from population 8 or 11, but these were chosen almost six times less than a leaf disc from population 6. Populations 3 and 10 were never chosen in our preference trials. These preferences may have been influenced by secondary compounds in the populations used (Appendix Table S14). For instance, the population overwhelmingly chosen the most, population 6, had the lowest overall concentration of total (summed) PPGs.

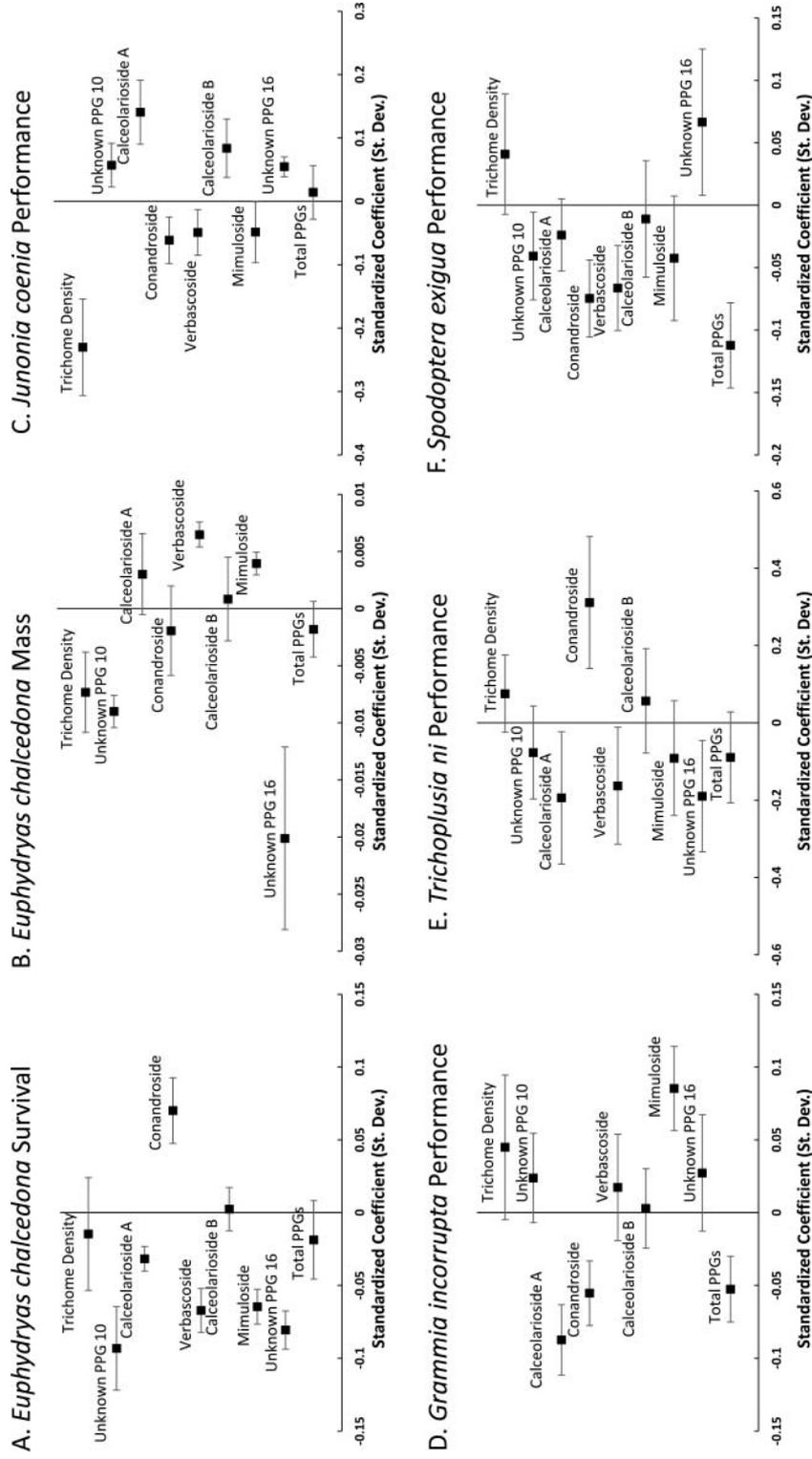


Fig. 3. Partial least square regression model standardized coefficients. Black dots are the mean coefficient and the error bars represent one standard deviation from the Monte Carlo randomization. Standard deviation bars are considered significant if they do not overlap zero.

Table 2. Contingency table from the χ^2 analysis for preference of populations in the slug *Deroceras laeve* ($\chi^2 = 55.255$, $df = 4$, $P < 0.001$)

Population	Rejected choice	Accepted choice	Total
3			
Count	11	0	11
Expected	7.33	3.66	
Deviation from expected	3.66	-3.66	
Cell χ^2	1.83	3.67	
11			
Count	10	3	13
Expected	8.66	4.33	
Deviation from expected	1.33	-1.33	
Cell χ^2	0.21	0.41	
6			
Count	1	18	19
Expected	12.66	6.33	
Deviation from expected	-11.67	11.66	
Cell χ^2	10.75	21.49	
8			
Count	11	1	12
Expected	8.00	4.00	
Deviation from expected	3.00	-3.00	
Cell χ^2	1.25	2.25	
10			
Count	11	0	11
Expected	7.33	3.66	
Deviation from expected	3.66	-3.66	
Cell χ^2	1.83	3.66	
Total	44	22	66

DISCUSSION

Each of the six herbivore species used in our study had different performance or preference responses to particular resistance traits, when the significant performance/resistance trait relationships were considered. For specialist herbivores, the association between performance and resistance traits follows the general predictions of the phytochemical coevolution hypothesis and related host specialization hypotheses. The performance/preference patterns of the more generalist herbivores are partially but not completely consistent with phytochemical coevolution theory/host specialization hypotheses and also fit predictions of alternative hypotheses for diversity in plant resistance traits. The relationship between both specialist and generalist species and trichome density is consistent with our extrapolations of plant phytochemical coevolution theory and related host specialization hypotheses.

Concordant with the predictions of phytochemical coevolution theory and related host specialization hypotheses, that specialist herbivores may adapt to feeding on particular phytochemicals, the performance of both specialist species (*E. chalcona* and *J. coenia*) in our study was positively associated with concentration of several PPGs. Support for the phytochemical coevolution theory has been shown previously in other systems (Cornell and Hawkins, 2003). For example, when comparing a specialist and a generalist lepidopteran, both of which utilize *Penstemon* spp. as food sources, Kelly and Bowers (2016) note that the specialist had greater growth and survival on plants with higher levels of an iridoid glycoside, while the generalist had limited growth and survival on the more heavily defended plants. We also found negative associations between several phytochemical compounds and specialist performance. The narrow specialist *E. chalcona* had a negative relationship between performance and levels of two PPGs (unknown PPG 16 and 10). These PPGs are not found in all populations of *M. guttatus* and when found they are in low concentrations. As an extreme specialist (specializing on specific populations), *E. chalcona* may not have the predicted evolutionary relationship with these PPGs, as it may have been in contact with *M. guttatus* populations or other host plants that do not have these particular PPGs.

Our results demonstrate that specialist performance is negatively correlated with trichome density, while generalist performance is neutral with respect to trichome density. Those results are consistent with an extrapolation of phytochemical coevolution theory and host specialization hypotheses, which predict that generalists will be less affected by widespread defence traits than specialists that do not commonly encounter such defences in their host plants (Jaenike, 1990; Ali and Agrawal, 2012). Previous work comparing generalist/specialist performance with physical defence traits showed that *Brassica nigra* induced trichomes at a higher rate when attacked by the specialist lepidopteran herbivore *Pieris rapae* than by the generalist lepidopteran herbivore *T. ni* (Traw and Dawson, 2002). Early instars of *J. coenia* have been observed consuming trichomes on a section of the leaf surface prior to consuming the 'mowed' foliar material (L. Holeski, personal observation). This mowing (although without the consumption of the mowed material) has also been shown in other specialist herbivores such as *Pardasena diversipes* and *Danaus plexippus* (Hulley, 1988; Agrawal *et al.*, 2009). If trichomes provide less nutritional value than foliar tissue, this behaviour is one potential explanation for the negative relationship between *J. coenia* performance and trichome density as it reduces foraging efficiency.

The performance of generalist Lepidoptera was either negatively or not significantly correlated with individual or total PPG concentrations, with one exception for each of the three generalist lepidopteran species (*G. incorrupta* and mimuloside, *T. ni* and conandroside, and *S. exigua* and unknown PPG 16). When feeding broadly within a plant community, the generalist may frequently come across unfamiliar phytochemicals. In contrast to specialists, the generalist must cope with these unfamiliar phytochemicals using adaptations that have broad utility. Various generalists (including *T. ni*) have adopted both behavioural and physiological ways to deal with phytochemicals, for example cutting the vascular tissue before eating to avoid the transfer of induced phytochemistry of the plant to the attacked area (Dussourd and Eisner, 1987; Dussourd and Denno, 1991) or with a generalized detoxifying method (Li *et al.*, 2007; Ali and Agrawal, 2012). Dietary switching is another behavioural trait employed by generalists such as *G. incorrupta*, which often selectively graze within a diverse plant community. This grazing behaviour can help herbivores avoid toxic levels of particular compounds from their diet (Freeland and Janzen, 1974; Singer and Bernays, 2009). Consistent with our

results, there is growing evidence in the literature that generalist herbivores may have positive responses to some phytochemicals (see meta-analysis by Smilanich *et al.*, 2016).

The generalist slug *D. laeve* showed a strong preference for foliar tissue with low concentrations of individual and total PPGs. Most work on mollusc herbivory has focused on the impacts to the plant communities instead of the impacts that plants have on molluscs (Allan and Crawley, 2011; Hanley *et al.*, 1996; but see Hägele *et al.*, 1998). Slugs tend to react to phytochemicals in a much different manner than insect herbivores, which generally base feeding choices on plant morphology, condition, and ‘coarse’ resistance traits. These ‘coarse’ resistance traits include traits such as total phytochemical defences or simple presence/absence of a resistance trait (Moshgani *et al.*, 2016). Previous work has shown that a PPG, verbascoside, acts as a mollusc feeding deterrent (Molgaard, 1986).

In general, much of the coevolution between herbivores and host plants is likely diffuse coevolution due to multiple herbivore species selecting against variable and differing resistance traits (Iwao and Rausher, 1997). While our results are generally consistent with the phytochemical coevolution theory and related host specialization hypotheses, alternative, non-mutually exclusive hypotheses regarding the generation and maintenance of plant defense diversity may also be applicable. The results of the *J. coenia* trial are also consistent with the predictions of the arms race hypothesis, which posits that insect specialization and the observed diversity in plant resistance traits are a result of evolutionary escalation, whereby herbivores specialize on particular resistance traits and the plant subsequently produces novel compounds. Evidence for the arms race hypothesis would include, for example, compounds with slight differences in structures acting as kairomones vs. allomones for specialist herbivores (Berenbaum and Feeny, 1981). In our study, *J. coenia* had a positive association with two PPGs (calceolarioside A and B) that are monosaccharides (Keefover-Ring *et al.*, 2014). However, the disaccharides conandroside, verbascoside, and mimuloside had a negative association with *J. coenia*.

Our finding that no single phytochemical had consistent significant effects on performance across the six species tested fits the predictions of hypotheses predicting pairwise coevolution between a host plant and multiple herbivores. Under this scenario, a particular phytochemical should have different effects on different herbivore species (Macel *et al.*, 2005; Iason *et al.*, 2011). Phytochemical compounds used as feeding stimulants by specialist herbivores likely still deter many generalist herbivores. These differences in host plant preference and diet breadth between specialist and generalist herbivores can result in them exerting opposing selection pressures on plant defences. For example, specialists of *Brassica nigra* (Brassicaceae) prefer plants that are more chemically defended (higher concentrations of glucosinolates), while generalists prefer plants with lower concentrations of glucosinolates. In an experiment where specialists were excluded from a *B. nigra* population, the population levels of phytochemical defences increased across generations and vice versa when generalists were excluded (Lankau, 2007).

Finally, in at least some plant species, different phytochemicals can work synergistically to deter herbivores. For example, within the genus *Piper* a variety of amides (nitrogen-containing carboxylic acid derivatives) defend against herbivores (Dyer *et al.*, 2003). Generalist herbivores perform worse when fed a diet with a variety of these compounds than a diet with the same concentration of a single compound, while specialist herbivores are not affected differently by being fed a singular or multiple different amides (Richards *et al.*, 2016). These synergisms may also be important in avoiding herbivore adaptation to specific resistance traits (Steppuhn and Baldwin, 2007). The overall trend of generalists being inhibited more

from synergistic chemical resistance than from individual compounds and specialists being fairly resistant to synergistic effects has been shown in a meta-analysis (Richards *et al.*, 2016), although there are exceptions to this overall pattern. For example, mixed pyrrolizidine alkaloid diets did not have a synergistic effect on resistance to generalist western flower thrips (*Frankliniella occidentalis*), relative to diets with individual pyrrolizidine alkaloids added (Liu *et al.*, 2017). In our study, the selective generalist *G. incorrupta* and extreme generalist *S. exigua* had relatively strong negative relationships with total PPGs. This may be a result of various PPGs working synergistically or additively to inhibit feeding or conversion efficiency. Similarly, a synergistic impact of multiple defence compounds, but not individual compounds, inhibited *S. exigua* in a test using pyrrolizidine alkaloids (Macel *et al.*, 2005). As an extreme generalist, *S. exigua* may be able to avoid the build-up of certain phytochemicals by simply switching its diet in the wild. We do not know whether PPGs act additively or synergistically in our system.

We measured the effects of only specific constitutive plant traits on growth in our study. Herbivores interact with their plant hosts in multi-dimensional ways. Alternative plant traits affecting their nutritional value to herbivores were not measured in our study, and likely influenced specialist herbivore performance and/or choice, given the moderate variation in larval performance explained in the PLSR models for *E. chalcona* and *J. coenia* (Dudt and Shure, 1994; Berenbaum, 1995). In a field common garden experiment with *M. guttatus*, Colicchio (2017) found that trichomes did not affect the probability of field damage, but did affect the extent of damage in plants that received at least some herbivory. This could be due to many factors, including herbivore preference for plants with lower trichome densities, unmeasured differences in PPG or nutrient levels, or trichomes decreasing the rate at which herbivores could consume foliar material. Additionally, inbreeding within plants can also affect plant tolerance and in turn herbivore fitness (Ivey *et al.*, 2004). There are also ecological pressures that might play into herbivore choice. For example, several of the caterpillars in this study are known to sequester (*J. coenia*) phytochemicals for defence against their own predators (Bowers and Collinge, 1992), or to selectively seek out (*G. incorrupta*) phytochemicals in order to ‘medicate’ themselves if they have a parasitoid infection (Singer *et al.*, 2009). These ecological interactions could cause a ‘switch’ from phytochemical resistance traits being detrimental to them being beneficial to herbivore fitness. Finally, we did not assess the effects of plant induction in this study. It is possible that *M. guttatus* induce defences differently in response to different herbivores; this differential induction might differentially affect herbivore performance.

Herbivores are diverse and contend with their plant hosts in a variety of complicated ecological and evolutionary interactions. Finding broad, general patterns to explain interactions of herbivores and host plants, as well as general patterns in the diversity of plant resistance traits, has long been a goal of evolutionary and chemical ecology. Although our study showed some support for the phytochemical coevolution theory and related host specialization hypotheses, our results for generalist herbivores were not entirely consistent with that theory, and the predictions of several other hypotheses fit with at least some of our results. Future work directed at understanding the behavioural and physiological mechanism utilized by herbivores spanning the continuum of host specialization will help elucidate the ability of herbivores to feed on few or many host plants.

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