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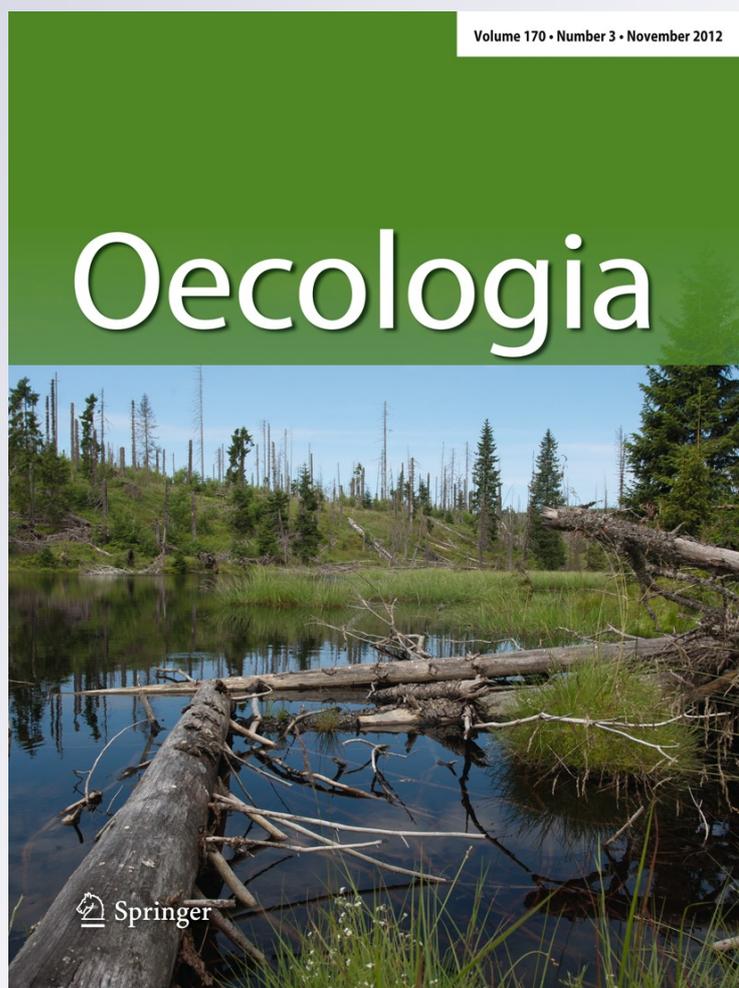
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Family matters: effect of host plant variation in chemical and mechanical defenses on a sequestering specialist herbivore

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Abstract Insect herbivores contend with various plant traits that are presumed to function as feeding deterrents. Paradoxically, some specialist insect herbivores might benefit from some of these plant traits, for example by sequestering plant chemical defenses that herbivores then use as their own defense against natural enemies. Larvae of the butterfly species *Battus philenor* (L.) (Papilionidae) sequester toxic alkaloids (aristolochic acids) from their *Aristolochia* host plants, rendering larvae and adults unpalatable to a broad range of predators. We studied the importance of two putative defensive traits in *Aristolochia erecta*: leaf toughness and aristolochic acid content, and we examined the effect of intra- and interplant chemical variation on the chemical phenotype of *B. philenor* larvae. It has been proposed that genetic variation for sequestration ability is “invisible to natural selection” because intra- and interindividual variation in host-plant chemistry will largely eliminate a role for herbivore genetic variation in determining an herbivore’s chemical phenotype. We found substantial intra- and interplant variation in leaf toughness and in the aristolochic acid chemistry in *A. erecta*. Based on field observations and laboratory experiments, we showed that first-instar larvae preferentially fed on less tough, younger leaves and avoided tougher, older leaves, and we found no

evidence that aristolochic acid content influenced first-instar larval foraging. We found that the majority of variation in the amount of aristolochic acid sequestered by larvae was explained by larval family, not by host-plant aristolochic acid content. Heritable variation for sequestration is the predominant determinant of larval, and likely adult, chemical phenotype. This study shows that for these highly specialized herbivores that sequester chemical defenses, traits that offer mechanical resistance, such as leaf toughness, might be more important determinants of early-instar larval foraging behavior and development compared to plant chemical defenses.

Keywords Aristolochic acid · Leaf toughness · *Battus philenor* · Heritable variation · Foraging behavior

Introduction

All plants encounter generalist and specialist insect herbivores, and they employ a number of strategies to deter them (Levin 1973; Karban and Myers 1989). Plants can invest in structures that impede herbivore foraging, such as trichomes (Hulley 1988; Ågren and Schemske 1993), or in defenses that directly compromise an herbivore’s ability to process food, such as latex or leaf toughness (Dussourd and Eisner 1987; Pérez-Harguindeguy et al. 2003). Plants can also employ chemical defenses that can function as herbivore deterrents. However, plants that invest in chemical defenses are presented with a particular challenge when, for example, insects sequester plant secondary compounds that provide the insect with a defense against natural enemies (Price et al. 1980; Malcolm and Zalucki 1996; Fordyce 2001). There can be substantial intraplant variation in the allocation of chemical defenses, and it is reasonable to

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assume that herbivore foraging behavior might be affected by the distribution of plant defensive chemistry (Rank 1992), as predicted by the optimal defense theory (McKey 1974). Although this theory has been developed to address how plant quality might affect herbivore foraging decisions (Zangerl and Bazzaz 1992), there is still only limited understanding of the relationship between intraplant defensive chemical variation and the foraging decisions of herbivores.

Herbivore foraging decisions in response to plant chemical variation can be considered in various ways. If herbivores sequester plant defensive chemicals, they might preferentially forage on parts of the plant with the highest concentration of secondary metabolites in order to maximize their defense against natural enemies (Rank 1992; Martinsen et al. 1998; Van Alstyne et al. 1999). On the other hand, if sequestration is costly, herbivores might forage on parts of the host plant that are less toxic (Murakami 1998). Alternatively, herbivore foraging behavior might not be directly determined by plant chemistry; it may be more strongly influenced by structural or mechanical defenses (e.g., trichomes or leaf toughness). Beyond plant defensive traits, herbivore foraging decisions might also be influenced by the nutritional quality of plant tissues, the presence of herbivore natural enemies, or the microclimate.

Little is known about the evolutionary dynamics of herbivore chemical sequestration. In the laboratory, Müller et al. (2003) studied three populations of the sawfly *Athalia rosae ruficornis* that harbor glucosinolates, a defensive compound derived from sequestered bioactive host-plant metabolites (Boevé and Schaffner 2003). Concentrations of sequestered chemical compounds in the insect were highly correlated with chemical concentrations in the host plant such that host-plant chemical variation was a more important determinate of variation in the glucosinolate defenses of the sawfly compared to the heritable variation for glucosinolate sequestration. However, the relationship between sequestration ability and survivorship of herbivores has scarcely been studied in the field. In a rare study of variation in sequestration, Fordyce and Nice (2008) found that the probability of larval survivorship through the first instar for *Battus philenor* (L.) (Papilionidae) was positively correlated with larval sequestration ability. Thus, natural selection might operate on the sequestration ability in wild populations.

The pipevine swallowtail, *B. philenor*, is a specialist herbivore on plants in the genus *Aristolochia* (Aristolochiaceae) (Racheli and Pariset 1992; Fordyce et al. 2010). Plants in the family Aristolochiaceae are known to possess toxic alkaloids: nitrophenanthrene carboxylic acids, commonly called aristolochic acids. The primary aristolochic acid (AA) constituents of North American *Aristolochia* spp. are AA-I and AA-II (Fordyce 2000; Sime et al. 2000). Aristolochic acids are bitter and highly toxic, inducing vomiting

in vertebrates when consumed, are nephrotoxic and hepatotoxic, and are known mutagens (Chen and Zhu 1987). Larvae of *B. philenor* sequester these metabolites, meaning that both larvae and adults are chemically defended against many invertebrate and vertebrate predators (Brower 1958; Rothschild et al. 1970; Codella and Lederhouse 1989; Fordyce 2001).

Beyond chemical defenses, leaf toughness is a physical barrier that is especially important to first-instar larvae (Farrow et al. 1994; Casher 1996; Zalucki et al. 2002). From natural observations and field experiments, it is known that larvae of *B. philenor*, especially neonate and first-instar larvae, preferentially feed on growing tips of their *Aristolochia* host plants (Fordyce and Agrawal 2001). Leaf toughness and leaf secondary compounds are characteristics of leaf tissue that might explain *B. philenor* larval feeding behavior. Larvae of *B. philenor* might forage on the growing tips of their *Aristolochia* host plants because young, growing leaves are less tough than older leaves. Alternatively, they might be feeding on the tip leaves because these leaves contain higher concentrations of aristolochic acids, thereby providing more resources for larval chemical defense.

In this study, we examined the role of two plant traits, leaf toughness and leaf chemistry, that serve as putative defenses against *B. philenor* larvae, and examined the importance of plant chemical variation in determining the chemical phenotype (chemotype) of larvae. Specifically, we addressed the following questions. (1) Is there intraplant variation in aristolochic acid content and/or leaf toughness? (2) Where on the plant (tip, middle, or bottom leaves) do first-instar larvae tend to feed? (3) Does larval performance vary depending on larval feeding location on the plant and, if so, is this best explained by aristolochic acid content or leaf toughness? (4) Does larval family or host-plant chemistry best predict the aristolochic acid content of larvae?

Materials and methods

Laboratory and field experiments

Intraplant variation in aristolochic acid content and leaf toughness

To assess plant variation at the individual level in aristolochic acid content and leaf toughness, we collected bottom, intermediate, and tip leaves (a total of five leaves per individual) of *Aristolochia erecta*, a commonly used host-plant species of *B. philenor* in central Texas. We rank ordered the leaves from bottom to top, with leaf number 1 indicating the bottom leaf and leaf number 5 indicating the tip leaf. We sampled leaves from 20 plants in Hays County, TX,

USA. We measured leaf toughness (g/cm^2) by averaging three measurements with a force gauge penetrometer (type 516; Chatillon, Largo, FL, USA) on fresh leaf material. The leaf penetrometer measures the mass (g) needed to puncture a leaf using a 3 mm diameter rod. Tip leaves are always the youngest leaves for this *Aristolochia* species, and leaves become progressively older as you proceed down the stem. Comparisons of toughness and aristolochic acid content (see below) among leaves (tip, three intermediate, and bottom leaves) were performed using a mixed model ANOVA implemented in JMP (v. 9.02) software (SAS Institute 2010). Individual plant was considered a random effect, with leaf position a fixed effect. Variance components for the random effect were estimated using restricted maximum likelihood (REML). We also examined the correlation between leaf toughness and aristolochic acid content for all *A. erecta* individuals combined.

Larval feeding position on A. erecta

To assess and quantify the preference of *B. philenor* larvae for leaves of varying ages and toughness, we conducted a choice test using neonate larvae at Freeman Ranch in south-central Texas (Hays County), a field station operated by Texas State University. At this site, *A. erecta* was the only naturally occurring host plant available for *B. philenor*. Wild-caught females were induced to lay eggs in the laboratory. We removed eggs from plant material and allowed them to hatch. On the day of hatching, larvae were transported to the Freeman Ranch study site. In the field, we placed each neonate larva ($N = 30$) on an individual stem of *A. erecta* for 24 h. Neonate larvae were randomly assigned to plants and leaf positions. After placing larvae on the stem next to their designated starting leaf, the larvae were allowed to move and begin feeding. After 24 h we recorded the position on the plant (i.e., leaf number) where larvae were feeding. We grouped the position data into two groups: feeding on the tip leaf (leaf 6) and feeding on any other leaf (leaves 1–5). We tested the differences in numbers of larvae in each group using a Wilcoxon signed-rank test under the null expectation that the larvae would be equally distributed among the two groups.

Effects of aristolochic acid content and leaf toughness on larval performance

We examined larval performance in response to leaf chemistry and leaf toughness using tip (less tough) and middle (more tough; see “Results”) leaves of *A. erecta*, with or without aristolochic acid supplementation. This design allowed us to simultaneously assess the effect of chemistry while controlling for leaf toughness and vice versa. We supplemented leaves with aristolochic acid using a

saturated solution (100 μg AA-I and AA-II/100 ml ethanol), resulting in a near-threefold increase in concentration compared to concentrations that naturally occur in leaf tissue in the field (Fordyce 2001; Fordyce and Nice 2008). The saturated solution was applied to leaves by spraying each leaf three times with three standardized applications. We sprayed control leaves in the same manner with ethanol only. Neonate larvae were permitted to feed on leaves for 48 h, after which two measures of larval performance were made: leaf area consumed and larval dry weight. We quantified leaf area consumed by digitizing the leaves, and the area missing relative to the total leaf area was assessed using Image J software (Rasband 2003). Each larva was dried under reduced pressure and weighed to the nearest microgram. We assessed differences in larval performance (dry weight) using an analysis of variance (ANOVA), with aristolochic acid supplementation and leaf toughness as factors and leaf area consumed as a covariate.

Heritable variation in larval sequestration versus variation in host-plant chemistry

To examine the relative importance of larval family vs. variation in host-plant chemistry for aristolochic acid sequestration, we conducted a field study at Freeman Ranch (Hays Co., TX, USA). We placed sibling neonate larvae obtained from 12 wild-caught females in the field in groups of five, the average clutch size in this population (Fordyce and Nice 2004). We permitted larvae to feed for three days, after which larvae and plant material were collected for chemical analyses. Larvae from each individual plant were pooled, providing a single response variable of larval chemotype for each plant. We estimated variation in aristolochic acid content explained among families (broad sense heritability) and plant chemistry using a mixed model ANCOVA, where family was considered a random effect. Because the number of replicate groups varied among females, we used restricted maximum likelihood (REML) implemented in the JMP (v.9.02) software package (SAS Institute 2010) to estimate the among-female variance component.

Chemical analysis

Larvae and leaves were dried under reduced pressure prior to extraction. We weighed larvae to the nearest 0.1 μg , and leaves were weighed to the nearest milligram. We extracted aristolochic acids from larvae twice in 0.4 mL of 100 % ethanol, sonicated for 20 min at 50 °C, and the resultant extract was dried under reduced pressure. Leaf aristolochic acids were extracted twice in 5 mL of 100 % ethanol and sonicated for 20 min at 50 °C. These extracts were similarly dried under reduced pressure. The ethanol extracts

from larval and leaf samples left a yellow residue. Larval extracts were resuspended in 0.04 mL of 100 % methanol and placed into total recovery autosampler vials for HPLC analysis. Leaf extracts were resuspended in 1 mL of 100% methanol and passed through a 0.45 μm filter into an autosampler vial for HPLC analysis.

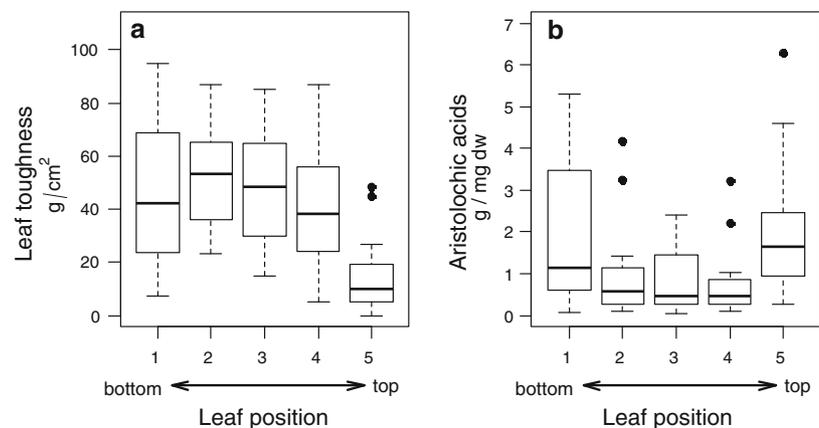
We performed HPLC analyses using a Waters Alliance HPLC system with a 2996 diode array detector and Empower Pro Software (Waters Corporation, Milford, MA, USA). Each injection was 10 μl , eluted isocratically with a mixture of methanol, water, and 1 % acetic acid (52:47:1) at a rate of 1 ml/min on a Waters Symmetry C-18 reverse phase column (3.5 mm, 4.6 \times 75 mm). Aristolochic acids were identified based on their retention times and unique absorption spectra. We quantified aristolochic acid concentrations by comparing peak retention times and areas to a standard curve generated with chemical standards as described in Fordyce and Nice (2008).

Results

Intraplant variations in aristolochic acid content and leaf toughness

Leaf toughness and aristolochic acid content varied with leaf age (Fig. 1). The top-most, youngest leaves were less tough than older leaves ($F_{4, 74} = 18.64$, $P < 0.001$). Aristolochic acid content varied among leaves, with bottom and top leaves having higher concentrations of aristolochic acids than intermediate leaves ($F_{4, 74} = 8.29$, $P < 0.001$). After removing among-plant variation, we found a negative correlation between residual leaf toughness (g/cm^2) and residual leaf aristolochic acid content ($\log \mu\text{g AA}/\text{mg dry weight}$) ($r = -0.41$, $n = 98$, $P < 0.001$). This negative correlation indicates that there is a tendency for less tough, younger leaves to have higher concentrations of aristolochic acid than tougher, older leaves.

Fig. 1 Boxplots of leaf toughness (a) and total leaf aristolochic acid content (b) in *A. erecta* individuals. Leaves were numbered from 1 to 5, with leaf 1 being the oldest and leaf 5 the youngest (tip leaf)



Larval feeding position on *A. erecta*

From analyses of 30 *B. philenor* first-instar larvae, we found that 24 out of 30 larvae (80 %) established a feeding site on the tip leaf after 24 h of feeding in the field ($N = 30$; Wilcoxon signed-rank test $Z = -139.5$, $P = 0.0002$), despite the fact that only 3 of the 30 were initially placed next to a tip leaf.

Effects of aristolochic acid content and leaf toughness on larval performance

When we examined the effect of leaf chemistry vs. that of leaf toughness on larval performance, we found that leaf toughness best predicted larval performance (dry weight) ($F_{1, 67} = 63.24$, $P < 0.01$), whereas we failed to find an effect of leaf chemistry on larval performance ($F_{1, 67} = 0.11$, $P = 0.73$). In this manipulative experiment, we failed to detect a significant interaction between aristolochic acid supplementation and leaf toughness on larval performance ($F_{1, 67} = 2.95$, $P = 0.112$) (Fig. 2).

Heritable variation in larval sequestration versus variation in host-plant chemistry

When we examined the relative importance of larval family variation for sequestration vs. the variation of host-plant chemistry, we found that 44.3 % of the variation in larval aristolochic acid content was explained by family identity. This serves as our broad-sense heritability estimate for sequestration ability (Fig. 3). To explore how much of the remaining variation in larval chemistry was explained by host-plant chemistry, we performed a mixed model ANCOVA of plant chemistry on the residual variation in larval chemistry after removing the effect of family. Here, we found that plant chemistry explained an additional 14 % of the variation in larval aristolochic acid content ($F_{1, 29} = 4.885$, $P = 0.035$).

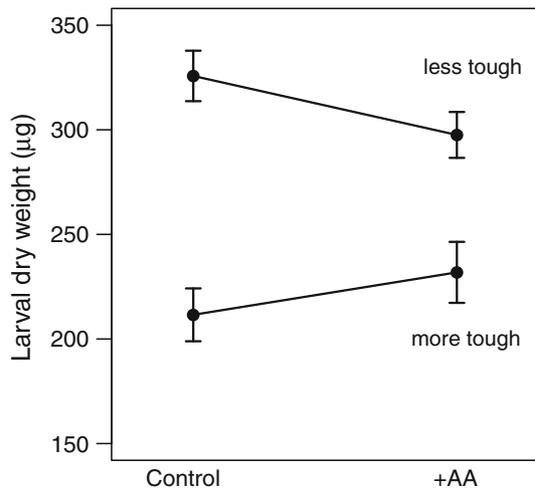


Fig. 2 Larval dry weight (\pm SE) on less tough and more tough leaves of *A. erecta* without supplemented aristolochic acid (control treatment) or with supplemented aristolochic acids (+AA treatment) ($F_{3,76} = 18.162$, $P < 0.001$)

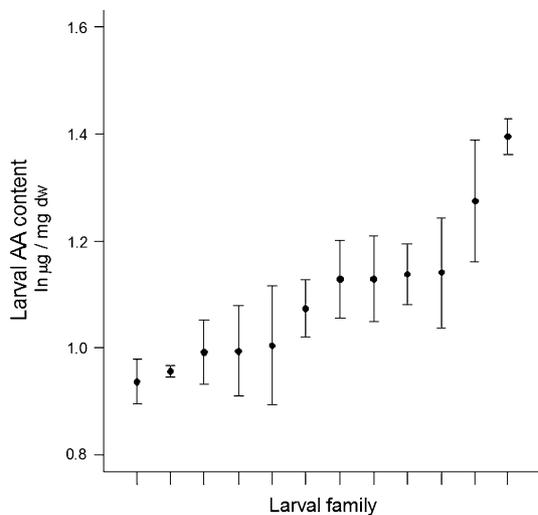


Fig. 3 Among-family variation in sequestered aristolochic acids (ln μ g AA/mg dry weight \pm SE) after controlling for among-plant variation in 12 families (full siblings) of *Battus philenor*

Discussion

We found high intraplant variations in leaf toughness and aristolochic acid chemistry in *A. erecta* individuals. Regardless of the amount of aristolochic acid present in the leaves, first-instar larvae of *B. philenor* preferred to feed on younger, tender leaves. This indicates that leaf toughness is likely an important characteristic of plant resistance to herbivory by *B. philenor* larvae, as has been observed frequently in other insect groups (Matsuki and Maclean 1994). In *A. erecta*, leaf toughness showed a consistent pattern, with younger leaves being less tough than older leaves. Using the same procedures as employed for *A. erecta*, we

also found a similar pattern in two other *Aristolochia* species commonly used by other populations of *B. philenor* (unpublished data). It is important to note that many factors change with leaf position, due to ontogeny. Therefore, leaf toughness may not be the sole factor responsible for the patterns observed in this study because other factors might co-vary with leaf ontogeny. However, from previous studies, we know that leaf toughness plays a fundamental role in deterring early-instar larvae of other butterfly species (Zalucki et al. 2002). In previous examinations of *B. philenor* larval behavior, Fordyce and Agrawal (2001) found that a structural defense (trichomes) on *A. californica* notably reduced the rate of herbivory of early-instar larvae. This result also suggested that defenses other than chemical ones can be important agents for deterring herbivory by a specialist herbivore.

Although the *A. erecta* leaves analyzed in this study showed different concentrations of aristolochic acids, with younger leaves containing higher concentrations of aristolochic acids, leaf aristolochic acid content appears to play a less important role in predicting first-instar larval performance than leaf toughness. This is an interesting result given that aristolochic acids are an important defensive resource for *B. philenor*, providing them with a chemical defense against natural enemies (Fordyce 2001; Sime 2002; Fordyce and Nice 2008). In our system, we found that aristolochic acid content had no measurable effect on *B. philenor* first-instar larval performance, contrary to at least one other well-studied system involving chemical sequestration, the interaction between monarch butterflies (*Danaus plexippus*) and their milkweed host plant (*Asclepias* spp.) (Zalucki et al. 1990; Zalucki and Brower 1992). It would be interesting to examine if later instars of *B. philenor* show the same performance and consumption patterns, since they may be less influenced by leaf toughness given their larger size.

Surprisingly, we found that a substantial amount of variation in larval aristolochic acid chemistry was explained by larval family, despite considerable variation in aristolochic acid content among individual plants. This suggests that heritable variation for sequestration ability is an important determinant of larval aristolochic acid concentration and adult chemotypes. This is at odds with the hypothesis that plant chemical variation will overshadow insect genetic variation for sequestration as proposed by Müller et al. (2003), and suggests that the ability to sequester can likely respond to natural selection, despite the variation in aristolochic acid content observed among individual plants.

The role that plant chemical defenses play in the foraging decisions and performances of herbivores has often been emphasized (Zangerl and Bazzaz 1992; VanDam et al. 1996; Zangerl and Rutledge 1996; Asplund et al. 2010).

In this study, we found that leaf toughness was a better predictor of first-instar larvae preference and performance than plant chemical defense (aristolochic acids), and although there was substantial variation in plant aristolochic acid content, larval family was a better predictor of larval aristolochic acid content. In both field observations and experiments, larvae were more likely to feed on the young, actively growing leaves near the tips of the stems, regardless of the amount of aristolochic acids present. Although the intraplant distribution of aristolochic acids in *A. erecta* was consistent with the predictions of optimal defense theory (namely that younger leaves with potentially higher fitness values contained higher levels of the putative anti-herbivore defense), there was no evidence that the distribution of these chemicals affected larval preference and performance. This study shows that for highly specialized herbivores which sequester chemical defenses from their host plants, mechanical resistance, such as leaf toughness, might be a more important determinant of herbivore foraging patterns than chemical defenses.

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