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ANTAGONISTIC, STAGE-SPECIFIC SELECTION ON DEFENSIVE CHEMICAL SEQUESTRATION IN A TOXIC BUTTERFLY

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Larvae of the pipevine swallowtail (*Battus philenor*) sequester toxic alkaloids called aristolochic acids from their *Aristolochia* host plants, rendering both larvae and adults chemically defended against most predators. Using a chemically controlled artificial diet, we observed substantial among-family variation in sequestration ability and larval developmental rate in a population occurring in central Texas. Early instar larvae from families that sequester greater amounts of aristolochic acid showed increased survivorship in a field experiment in which cohorts from each family were exposed to natural predators, whereas among-family variation in growth rate did not predict survivorship. Conversely, the aristolochic acid content of adult butterflies was negatively correlated with adult fat content, a fitness correlate. Sequestration ability positively affects the probability of larval survivorship, but at the cost of adult fat content. The costs and benefits of aristolochic acid sequestration vary during the course of the butterfly's development, and these antagonistic selection pressures may explain why variation in sequestration ability persists in wild populations.

KEY WORDS: **Aristolochic acid, *Battus philenor*, gregarious feeding, natural selection, trade-offs.**

Many herbivorous insects sequester toxic chemicals from their host plants rendering them unpalatable to predators. Such chemical defense has been studied extensively, particularly in the Lepidoptera. Indeed many fundamental evolutionary concepts, such as mimicry, have been based on studies of toxic Lepidoptera and their characteristic aposematic coloration (Bates 1862; Müller 1878; Brower 1958a,b). Slater (1877) and Haase (1893) were among the first to propose that host plants were the source of chemicals responsible for unpalatability, however it was not until the seminal works of Brower (1958a,b) and Rothschild et al. (1970) that the efficacy of plant-derived chemical defenses and chemical sequestration was rigorously examined. Since then, a great deal of knowledge has accumulated concerning the variety of chemical toxins sequestered and the diversity of herbivores capable of sequestration (Duffey 1980; Nishida 2002).

The evolution and maintenance of chemical sequestration as a defensive strategy is different from the detoxification and/or excretion of plant defensive chemicals employed by many nonsequestering herbivores because it is affected by selection pressure from both the first and third trophic levels (Price et al. 1980; Rowell-Rahier and Pasteels 1992; Ode 2006) Selection imposed by the first trophic level can affect traits associated with reducing the toxic effect of the chemical, detoxification, tolerance to the toxin (e.g., target site insensitivity), or intraconverting the toxin to less-toxic forms (Harborne 1993; Holzinger and Wink 1996; Berenbaum and Zangerl 1998; Feyereisen 1999; Glendinning 2002). Selection imposed by the third trophic level (i.e., predators and parasites) should favor concentrations of sequestered defensive chemicals that are generally effective against the spectrum of natural enemies the insect might encounter. The sequestration of

plant toxins has been shown to be costly for some insects. These costs include the expenditure of energy associated with handling the toxin, or through the direct effect of the toxin itself on the insect (Malcolm and Zalucki 1996; Camara 1997a; Zalucki et al. 2001; Nishida 2002). Such costs might impose limitations on the upper bounds of variation observed for the amount of host plant chemicals sequestered by insects. The benefits of defensive chemical sequestration are apparent in field and laboratory studies demonstrating predator aversion and avoidance of insects possessing host plant derived chemical defenses (Bowers 1993; Nishida 2002).

The quantity and quality of sequestered chemical defense has been shown to vary temporally, and within and among populations (Nelson et al. 1981; Urzua and Priestap 1985; Malcolm et al. 1989; Alonso-Mejía and Brower 1994; Ritland 1994; Moranz and Brower 1998; Fordyce et al. 2005, 2006). The consequences of this variation have been examined from the perspective of the costs associated with sequestered chemical defense and the defensive benefits. Explicit tests of the fitness consequences associated with defensive chemical variation, in particular the efficacy with which it deters natural enemies, have primarily focused on how variation in the amount of defensive chemical resources in the diet affects the defense of the herbivore (Dyer and Bowers 1996; Hatle and Spring 1998; Theodoratus and Bowers 1999; Fordyce 2001; Rayor et al. 2007). For example, larvae of the buckeye butterfly (*Junonia coenia*) are less vulnerable to predation when reared on plants with high levels of iridoid glycosides compared to larvae reared on low iridoid glycoside-containing plants (Dyer and Bowers 1996; Camara 1997b; Theodoratus and Bowers 1999). Another source of variation in sequestered chemical defense can result from genetic variation in the ability to sequester plant toxins. Engler-Chaouat and Gilbert (2007) examined variation among *Heliconius* butterfly species in their ability to sequester monoglycoside cyclopentnyl cyanogens from various species of *Passiflora* and autogenously manufacture cyanogenic glycosides de novo. They found that butterflies feeding on plant species with high monoglycoside cyclopentnyl cyanogen content consistently had higher levels of cyanogenic glycoside defenses. They also found that sequestration and de novo synthesis are negatively correlated traits, indicating that variation in sequestration ability is an important determinate affecting host plant breadth. Müller et al. (2003) found heritable variation for glucosinolate sequestration by a sawfly, but concluded that host plant variation was a more important determinate for variation in sawfly defense and, thus, genetic variation for sequestration ability was "invisible to natural selection." To our knowledge, no study has heretofore examined explicitly the relationship between natural variation in sequestration ability and survivorship in wild populations of a sequestering herbivore.

In this study, we examine the relationship between variation in sequestration ability of the pipevine swallowtail, *Battus philenor* (Papilionidae), and survivorship under natural field conditions. Specifically we ask the following: (1) Does variation exist among families for ability to sequester plant toxins? (2) Does variation in sequestration ability affect larval survivorship in the field? (3) Is there evidence of a trade-off in sequestration ability, both from the perspective of larval growth rate and adult fat content? Understanding both the costs and benefits of chemical sequestration under natural conditions will provide insight into the factors that maintain variation for this ecologically important trait.

Materials and Methods

STUDY SYSTEM

The pipevine swallowtail, *B. philenor*, is a specialist on plants in the genus *Aristolochia* (Aristolochiaceae) (Racheli and Pariset 1992), commonly called pipevines. *Aristolochia* contain toxic alkaloids, nitrophenanthrene carboxylic acids, commonly called aristolochic acids, which are unique to the family (Chen and Zhu 1987). Caterpillars of *B. philenor* sequester these toxins rendering both larvae and adults chemically defended against most predators, although predation can be high for the earliest instars (Brower 1958a; Brower and Brower 1962; Platt et al. 1971; Jeffords et al. 1979; Codella and Lederhouse 1989; Fordyce and Agrawal 2001).

This study was conducted in south-central Texas at Freeman Ranch (Hays Co.), a field station operated by Texas State University. The *B. philenor* host plant at this location is *A. erecta*, a semi-erect herbaceous perennial. The butterfly is extremely abundant in the spring, and females spend most of their activity searching for host plants among the dense grass and forbs associated with the oak-scrub habitat characteristic of the Texas Hill Country. Females avoid ovipositing on plants with previously laid eggs (Rausher 1979; Fordyce 2003). Mean clutch size observed for this population is 5.0 ± 0.3 (mean $\pm$ SE) (Fordyce and Nice 2004).

LABORATORY AND FIELD EXPERIMENTS

Wild females were caught in early June 2007 and returned to the laboratory. Each female was placed in a small plastic cup containing a sprig of host plant and permitted to lay eggs. In total, we acquired an adequate number of eggs for experimental replication (>35) from 28 females. The eggs from each female were removed from the plants prior to hatching and each family was placed in a separate plastic container. Eggs were incubated in an environmental chamber at 30°C until hatching.

To assess among-family variation in sequestration ability, while controlling for the effect of among-plant variation in aristolochic acid content, a fraction (5–8 individuals) of larvae from each female were reared on an artificial diet with a controlled aristolochic acid content (Appendix). Each larva on artificial diet was placed in an individual Petri dish with a piece of artificial diet (approx. $1 \times 1 \times 2$ cm) and placed in an environmental chamber at 30°C and development was monitored daily. These larvae were

permitted to feed for the same duration as the field experiment (4 days), after which they were individually placed in microcentrifuge tubes and frozen until chemical analysis. Variation among families was examined using ANOVA.

To assess among-family variation in survivorship under natural field conditions, we conducted an experiment in which sibling groups of five neonate larvae were placed on wild *A. erecta* haphazardly chosen in the field. Groups of five were chosen because it is the average clutch size observed for this population (Fordyce and Nice 2004). Groups were monitored daily for four days, a time when most individuals had molted through the first and second instar and a majority of the plant was consumed. Missing caterpillars were scored as a death, and some mortality caused by spiders, ants, ladybird beetles, and lacewing larvae was directly observed over the course of the experiment. Although later instar caterpillars are forced to forage for additional host plants, early instars remain on their host plant until it is mostly consumed. In addition to daily mortality, we recorded the instar of the caterpillars on each day. At the end of the experiment caterpillars were collected for chemical analysis. Replication among families varied between three and 10 groups (median = 7). In total, 25 families were included in the field experiment, three of the 28 families being excluded because of insufficient number of caterpillars to obtain at least three replicates.

We used the mean aristolochic acid content and weight of caterpillars reared on artificial diet as a measure of sequestration ability and growth rate of each family. Survivorship in the field was similarly measured as an average across replicates for each family. Correlations between average aristolochic acid content and growth of artificial diet-reared caterpillars and survivorship of field-experiment caterpillars were examined to assess the relationship between sequestration ability and growth rate and survivorship in the field. All correlations herein were explored using Pearson's product moment correlation.

To examine the potential costs of sequestered aristolochic acids, we reared 21 *B. philenor* to adults on artificial diet and examined the relationship between adult aristolochic acid content and fat. Fat content is frequently used as a fitness correlate for butterflies because, like most butterfly species, *B. philenor* is a nectar feeder as an adult and is dependent on fat resources obtained as larvae for reproductive success (Karlsson 1995; Boggs 1997).

ARISTOLOCHIC ACID AND FAT ANALYSIS

Caterpillars and adults were dried under reduced pressure prior to extraction. Adult butterflies were weighed to the nearest milligram and caterpillars were weighed to the nearest 0.1 microgram. Adult butterflies were placed in individual 15-mL centrifuge tubes, homogenized in 5 mL of hexane and sonicated for 20 min at 50°C to remove fat. The fat-containing hexane was decanted into a preweighed test tube. The hexane was evaporated under reduced pressure and the test tube was reweighed, providing a measure of total fat for each sample. Fat content was examined as a percent of total butterfly dry weight. Percent fat content was arcsine-square-root transformed prior to statistical analyses. No aristolochic acid is lost to the hexane (Fordyce et al. 2005). The defatted tissue was extracted two times with 5 mL of 100% ethanol and sonicated for 20 min at 50°C. The ethanol extract was dried under reduced pressure in a test tube leaving a yellow residue. This residue was resuspended in 1 mL of 100% methanol and passed through a 0.45-μm filter in an autosampler vial for HPLC analysis. Caterpillar aristolochic acid was extracted two times in 0.4 mL of 100% ethanol and sonicated for 20 min at 50°C. This extract was similarly dried under reduced pressure. Caterpillar samples were resuspended in 0.04 mL of 100% methanol and placed into total recovery autosampler vials for HPLC analysis.

HPLC analyses were performed using a Waters Alliance HPLC system with a 2996 diode array detector monitoring at 251 nm. Data were acquired and processed using Empower Pro software (Waters Corporation, Milford, MA). Each injection was 10 μl eluted isocratically with a mixture of acetonitrile, water, and 1% HCl (46:39:15) at a flow rate of 1 mL/min on a Symmetry C-18 reverse phase column (3.5 μm, 4.6 × 75 mm) (Waters Corporation). Aristolochic acid content was determined using retention time and absorption spectra of known standards as a reference.

Results

The total aristolochic acid content and the aristolochic acid concentration (μg aristolochic acid per gram dry weight) was highly correlated in both adults ($r = 0.87$; $P < 0.001$) and caterpillars ($r = 0.936$; $P < 0.001$). All aristolochic acid analyses hereafter are based on concentrations. Aristolochic acid concentration of adults and caterpillars reared on artificial diet was log transformed to reduce deviations from normality. Sequestration ability varied among families ($F_{27,141} = 3.402$; $P < 0.001$). Among-family variation (i.e., broad sense heritability) accounted for 39.5% of the total variation in sequestered aristolochic acids (Fig. 1A). Dry weight of caterpillars reared on artificial diet was used to examine among-family variation in growth rate. Larval weights were log transformed to normalize data. Growth rate varied among families ($F_{27,141} = 3.579$; $P < 0.001$). Among-family variation accounted for 40.6% of the total variation in growth (Fig. 1B). We examined the relationship between sequestration ability and growth rate of families and failed to detect a significant correlation ($r = 0.272$; $P = 0.170$).

We explored various measures of family success in the field to establish if a relationship exists between survivorship, sequestration ability, and growth rate. Variation in sequestration ability was positively correlated with caterpillar survivorship to the second instar (usually occurring by the third day) ($r = 0.4398$;

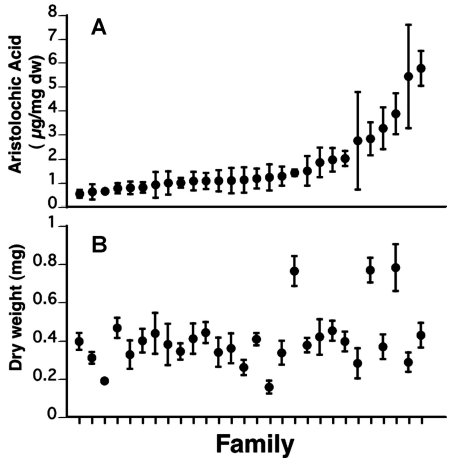

Figure 1. Among-family variation in (A) sequestration of aristolochic acid and (B) growth after four days raised on artificial diet. Families ranked from lowest to highest sequestration ability. Circles indicate family mean (±1 SE).

$P = 0.028$). We found no relationship between growth rate variation and survivorship to the second instar ($r = 0.156$; $P = 0.457$). Our experimental larvae were placed in the field in groups of five, which mimics the average clutch size observed in nature. Because kin selection is often proposed as a mechanism favoring gregariousness in toxic caterpillars (Stamp 1980), we examined the relationship between a family's sequestration ability and the probability of at least one individual surviving to the second instar and found a positive relationship (Fig. 2A; $r = 0.632$; $P < 0.001$). We found no relationship between family growth rate and group persistence to the second instar ($r = 0.008$; $P = 0.971$). The number of replicate groups differed among families, however, the number of replicates for each family was not related to cohort survival ($r = -0.281$; $P = 0.174$), thus the relationship detected between group survival and aristolochic acid sequestration ability was not an artifact of unbalanced replication.

Mean survivorship for groups across families was 29.5% (SE = 3.6). The survivorship observed for individual groups ranged from 0 to 100%. Although it is possible that groups with 100% survivorship were rejected by predators they encountered, it is also possible, and perhaps more likely, that these groups simply never encountered predators over the course of the experiment. To account for this possibility, we examined the relationship between family sequestration ability and survivorship in the field over the course of the experiment, restricting our analysis to replicates where at least one mortality event occurred. On average, there was a positive relationship between a family's sequestration ability and the number of individuals that survived per group over the course of the experiment (Fig. 2B; $r = 0.424$; $P = 0.035$). This result is consistent with the above analyses, but perhaps more informative because it only includes groups where some mortality occurred.

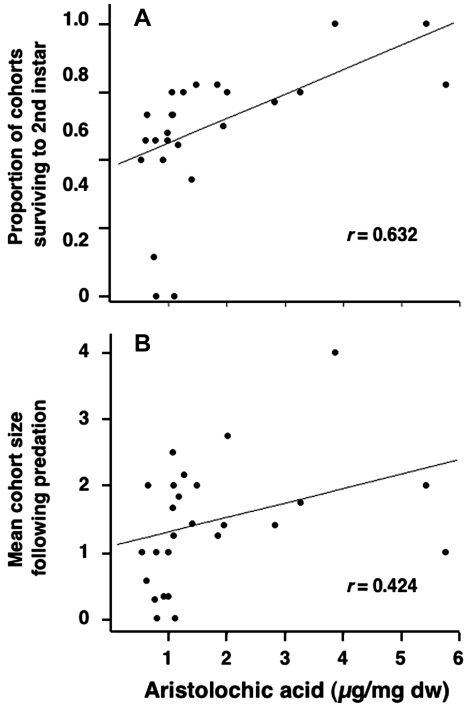

Figure 2. Relationship between aristolochic acid sequestration ability and larval survivorship in the field. Each dot represents a family average. Aristolochic acid sequestration ability is based on the mean concentration of aristolochic acids of individuals reared on a control diet. (A) The proportion of cohorts with at least one individual surviving to the second instar. (B) The average number of individuals surviving per cohort (initial cohort size = 5) for groups that experienced at least one predation event. Correlations (Pearson product-moment) analyses based on log-transformed aristolochic acid concentrations. Line fitted using linear regression.

Aristolochic acid concentration of adult butterflies reared on artificial diet differed between males ($N = 9$; 62.96 μg AA/g dw ± 8.81 (mean ± SE)) and females ($N = 12$; 99.79 μg AA/g dw ± 8.74 (mean ± SE)) (t-test; $t = 3.297$; df = 19; $P = 0.004$). The observed difference between male and female aristolochic acid content is consistent with previous examinations of *B. philenor* adults (Sime et al. 2000; Fordyce et al. 2005). Overall fat content was 14.6% ± 0.9 (mean ± SE) of the butterfly dry weight and did not differ between the sexes (t-test; $t = 0.107$; df = 19; $P = 0.916$). An analysis of covariance (ANCOVA) on aristolochic acid content using sex as a factor and fat content as a covariate failed to detect a significant interaction term between sex and fat ($F_{1,17} = 0.699$, $P = 0.415$), but did find an effect of sex ($F_{1,17} = 13.835$, $P = 0.002$) and fat ($F_{1,17} = 6.303$, $P = 0.023$). The effect of sex was removed from aristolochic acid content and percent fat content by examining the residuals of a one-way analysis of variance (ANOVA) with sex as the effect. Examination of the residuals indicated a negative relationship between adult toxicity and adult fat content ($r = -0.492$; $P = 0.024$), implying a cost associated with the sequestration of aristolochic acids (Fig. 3).

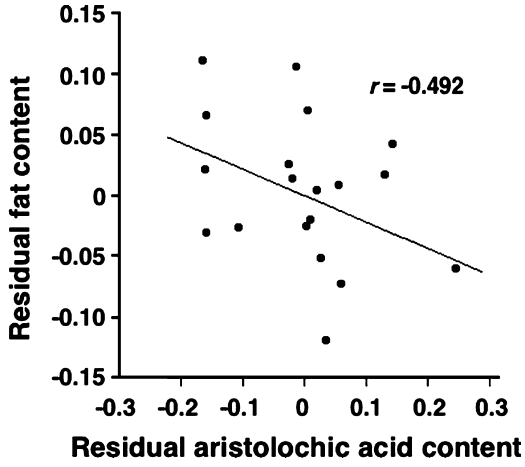

Figure 3. Adult fat content and sequestered aristolochic acid. Correlation (Pearson product-moment) on residuals after removing the effect of sex. Line fitted using linear regression.

Discussion

There exists substantial among-family variation in sequestration ability and growth rate for *B. philenor*. Given the observed difference between adult male and female sequestered aristolochic acids, it is possible that the observed variation among families in sequestration ability reflects variation in sex ratios among families. However, this is unlikely as significant deviations from a 1:1 sex ratio have not been observed in captive reared families (Fisher's combined probability test: $\chi^2 = 29.05$, df $= 40$, $P = 0.9$). Variation in sequestration ability had observable fitness consequences, as illustrated by the positive relationship between sequestration ability and survivorship in the field. Although previous studies have demonstrated that variation in sequestered defenses affects survivorship in the field, these studies have focused on the consequences of chemical resources available among different host plant species. This is the first demonstration that natural variation within populations feeding on a common host plant species can directly affect larval survivorship in the field. This is contrary to the suggestion of Müller et al. (2003) that host plant variation might swamp genetic variation for sequestration ability, and thus sequestration as a trait might be "invisible to natural selection." This contradiction might be reconciled by the fact that we examined the consequences of sequestration ability for the earliest instars, where larval mortality is high and dispersal to other host plants is unlikely, whereas Müller et al. (2003) examined sequestration ability for the final stages of development in a species with a high turn-over rate of sequestrates (Müller and Wittstock 2005). Thus, host plant variation and other environmental factors might become increasingly important determinates of sequestered toxins as development progresses, however, among-family variation for sequestration ability was an important factor affecting early instar larval survivorship for *B. philenor*.

Variation in growth rate observed among families was not related to larval survivorship. This is surprising, as it seems to contradict the predictions of the slower-growth higher-mortality hypothesis, which posits that early developmental stages of insects pass through a "window of vulnerability" where they are particularly vulnerable to natural enemies (Feeny 1976; Clancy and Price 1987) and other environmental factors (Fordyce and Shapiro 2003). Thus, slower growth should result in higher predation rates, which is commonly observed in mobile, leaf-surface-feeding herbivores like caterpillars (Williams 1999). Stamp (1986) described in particular the vulnerability of first instar *B. philenor* caterpillars compared to later stages, yet we detected no effect of growth rate on survivorship. At least two factors might explain this observation. First, as suggested by Müller et al. (2003) in regards to sequestration ability, inter- and intraplant variation in nutritional quality might swamp out the among-family variation in growth rate observed on a controlled diet. Second, although the first instar has been regarded as the most vulnerable stage for *B. philenor* (Stamp 1986; Fordyce 2003; Fordyce and Nice 2004), the actual window of vulnerability might extend into the second or third instar.

We examined survivorship in the field using various metrics and each showed a similar positive relationship between sequestration ability and survivorship in the field. Analysis of groups in which at least one predation event was recorded is perhaps the most important result, as it directly relates to the concept of kin selection and the evolution of gregarious feeding in the Lepidoptera. Gregarious feeding has been proposed to evolve subsequent to the evolution of chemical defense and aposematism, and might function as a strategy to enhance aposematism (Sillén-Tullberg 1988; Tullberg and Hunter 1996), although other mechanisms might favor gregariousness (Stamp 1980; Fordyce 2005). That enhanced aposematism is the mechanism favoring gregariousness deserves the following considerations. First, many gregarious lepidoptera, including *B. philenor*, are most densely aggregated in the first instar, where they do not exhibit aposematic coloration. Second, experimental tests of enhanced aposematism have focused on avian predators with keen color vision (e.g., Gamberale and Tullberg 1998), and have largely ignored the kinds of invertebrate predators frequently observed feeding on *B. philenor* caterpillars. Our field experiment suggests an alternative, although not mutually exclusive, hypothesis regarding a benefit of feeding in groups of siblings for chemically defended caterpillars. Specifically, that some individuals of a family are sacrificed and that the chemical defense present in the sacrificed individuals affect the foraging of predators and influence the vulnerability of remaining larvae. Most invertebrate predators of early instar *B. philenor* caterpillars kill, or mortally wound, the individual before rejecting it as a prey item (J.A. Fordyce, pers. obs.; but see Sime 2002). Fordyce (2001) showed that lacewing larvae presented with *B. philenor*

caterpillars reared on a high aristolochic acid diet exhibited increased handling time, and ultimately increased mortality, compared to those presented with caterpillars reared on a low aristolochic acid diet. Thus, gregariousness might be favored for toxic lepidoptera if it leads to predators foraging elsewhere after they experience one individual, regardless of the degree to which an aposematic display is enhanced. This hypothesis is consistent with our observation that groups from families with greater sequestration ability had more individuals survive when at least one individual was killed.

There was an inverse relationship between adult percent fat content and aristolochic acid content, indicating that sequestration of aristolochic acids might be costly for *B. philenor*. Our use of artificial diet in a controlled laboratory environment was motivated by the desire to control for other factors that undoubtedly affect adult fat content. For example, pupal diapause has been shown to be costly in the currency of adult fat content for California *B. philenor* (Fordyce et al. 2006). In Texas, larvae are required to actively search for additional host plants and contend with nearly lethal high temperatures (Nice and Fordyce 2006), which likely impose a high metabolic cost that might be reflected in adult fat content. It is unknown if nutritional quality and aristolochic acid content of the plants covary and if this might affect the trade-off between adult aristolochic acids and fat content. The negative correlation between aristolochic acid and fat content for adults reared on our standardized diet indicates that one factor affecting adult fat content is a cost associated with sequestration. The mechanism underlying this cost is unknown. Evidence from the congener *B. polydamas* (Urzúa and Priestap 1985) and the more distantly related *Troides amphrysus* (Nishida et al. 1993) suggests that sequestering larvae modify and/or metabolize some aristolochic acid, which might expend energy that carries over to the adult stage in the form of reduced energy stores. Previous examinations of the effect of aristolochic acids on larval growth of *B. philenor* failed to detect an effect, even on diets containing six-times the concentration naturally occurring in the host plant (Fordyce 2001). Future study is warranted on the underlying physiology of aristolochic acid sequestration by *B. philenor* to elucidate the mechanism(s) underlying costs exhibited by adults.

Contrasting selection pressure for aristolochic acid sequestration ability between larvae and adults might explain the variation we observed among families for this trait. The probability of caterpillar survivorship increases with aristolochic acid sequestration ability. Thus, natural selection should favor an increase in sequestration ability. In contrast, selection on adults might favor reduced sequestration ability in favor of increased fat content. One factor that might favor increased aristolochic acids in adults is that eggs have aristolochic acids contributed by the female (Fordyce et al. 2006). However, the defensive efficacy of aristolochic acids in eggs has yet to be examined, and it is unknown if sequestered aristolochic acids are a limiting resource for ovipositing females, although females do lose aristolochic acids over the course of their adult life, whereas males do not (Fordyce et al. 2006).

Variation in the ability to sequester aristolochic acids has observable consequences for *B. philenor* caterpillars and measurable costs for adults. The ability to sequester plant toxins is widespread in phytophagous insects, yet little is known about the evolutionary dynamics of this trait in wild populations. This study demonstrates that the ability to sequester plant defensive chemicals is a dynamic trait that might experience contrasting selection pressures imposed by susceptibility to predators and the costs imposed by plant defensive chemistry.

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APPENDIX

Artificial diet for rearing *Battus philenor*.

Broth:	Dry ingredients:
14 g agar	5 mL Flax seed oil
17 g sucrose	5 mL Wheat germ oil
0.7 g sorbic acid	10 g powdered white beans
1.4 g ascorbic acid	18 g powdered *Aristolochia* leaves
2.0 g cholesterol	160 g cellulose
1.0 g choline chloride	40 g brewers yeast
0.9 g KCl	10 g casein
0.9 g MgCl	5 mL Aristolochic acid solution (EtOH)
0.25 g NaCl	
0.1 g CaCl	

Mix Broth in 700 mL of water until ingredients dissolve. Add hot broth to dry ingredients. Mix until homogeneous. Addition of aristolochic acid is optional. When cooled to 45°C add 0.17g Streptomycin dissolved in 1 mL water. Spread out diet on sheets of waxed paper. Flatten until diet is 1-cm thick. Refrigerate.