

Not all toxic butterflies are toxic: high intra- and interspecific variation in sequestration in subtropical swallowtails

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Abstract. Many herbivorous insects make use of plant secondary metabolites by consuming and storing these toxic compounds in their body tissue or integument, thereby obtaining chemical defense against their natural enemies. Swallowtail butterflies in the tribe Troidini (Papilionidae) sequester toxic alkaloids (aristolochic acids, AAs) from their host plants in the genus *Aristolochia*. Troidine butterflies have been a model group for development of theory on host plant chemical sequestration, but most studies on this group have been limited to a single species in North America. These studies have led, in part, to the paradigm that troidine butterflies are toxic, thereby explaining the numerous mimicry relationships that exist throughout most of their range. Herein, we present one of the first comparative studies in a single location of a community of troidine butterflies. We examined the AA content of five co-occurring troidine butterfly species and their two *Aristolochia* host plants. We found that one *Aristolochia* species, *A. triangularis*, was preferred in choice assays and did not possess quantifiable levels of AA. We also found that most troidine butterflies did not possess quantifiable levels of AAs, but larvae have the ability to sequester AAs when it is present in their diet. A larval preference experiment showed that host plant AA concentration did not influence larval feeding choice. A performance experiment showed that higher levels of AAs in the diet increased larval mortality, which might indicate a cost associated with sequestration of the chemical defense and also might shed some light on why so many troidine butterflies in this community have little or no AAs. We propose that automimicry might be operating in this system and many putative models of this paradigmatic system might not possess plant-derived defensive chemistry.

Key words: aristolochic acid; chemical sequestration; Iguazú; mechanical defenses; mimicry; troidine swallowtails.

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INTRODUCTION

Several groups of insects, including some Lepidoptera, have evolved the ability to sequester plant secondary compounds by ingesting and storing them in their integument or body tissues. Sequestration of defensive compounds provides some herbivores an effective chemical defense against most natural enemies (Duffey 1980, Nishida and Fukami 1989, Dyer 1995, Dyer and Bowers 1996, Fordyce 2001, Sime 2002). Butterflies

that sequester toxic compounds from their host plant often exhibit aposematic or warning coloration to visually advertise their unpalatability to natural enemies. This aposematic coloration has been invoked to explain mimicry complexes where one species is toxic and the other is not (in the case of Batesian mimicry), or both species are toxic (in the case of Mullerian mimicry), physically resemble each other, thereby gaining protection against predators (Brower 1958, Mallet and Joron 1999, see also Aubier et al. 2017).

One group of butterflies that sequester chemical defenses from their host plants are swallowtail butterflies in the Troidini (Papilionidae) tribe (Nishida et al. 1993, Fordyce 2000, Klitzke and Brown 2000, Sime 2002, Papaj et al. 2007). Troidine swallowtails are predominantly tropical and subtropical, occurring in lowland forests of Central and South America, with two genera recognized in the New World, *Battus* and *Parides* (Weintraub 1995). These butterflies specialize on plants of the genus *Aristolochia* (Aristolochiaceae), commonly called pipevines. *Aristolochia* spp. contain toxic alkaloids (nitrophenanthrene carboxylic acids) called aristolochic acids (AAs) that serve as a defense against most insect herbivores (Chen and Zhu 1987, Racheli and Oliverio 1993). These alkaloids are sequestered by troidine larvae from their host plant, rendering both larvae and adults chemically defended against many predators (Brower and Brower 1962, Nishida and Fukami 1989, Sime 2002, Fordyce 2001).

Troidines have been a model group for development of theory on host plant chemical sequestration, mimicry, and tri-trophic interactions (Brower 1958, Brower and Brower 1962, Rausher 1981, Sime et al. 2000, Fordyce and Nice 2008). The most intensively studied troidine species is the pipevine swallowtail, *Battus philenor*, which is largely restricted to North America. Most of the studies on *B. philenor*-*Aristolochia* spp. have been done in areas in the United States where it is not sympatric with other troidine species (Rausher and Feeny 1980, Rausher 1981, Allard and Papaj 1996, Fordyce 2000, Sime et al. 2000, Fordyce et al. 2010, Dimarco et al. 2012). This single case example is largely the basis for the paradigm that all troidines are toxic and usually considered models, thereby explaining the numerous apparent mimicry relationships that exist between troidines and other butterflies. Although the interaction between *B. philenor* and *Aristolochia* spp. is well documented in North America, there is a relative paucity of studies in tropical and subtropical areas (but see, Brown et al. 1980, Klitzke and Brown 2000, Mebs and Schneider 2002, Pinto et al. 2009), where there is a greater richness of troidines and where many species of this group occur in sympatry.

Here, we present one of the first comparative studies in a single location of Troidini-*Aristolochia* relationships in a subtropical rainforest with

multiple troidine butterfly species co-occurring. The present study aimed to further our understanding of the chemical ecology of this model group by addressing the following questions: (1) Do local *Aristolochia* species differ in their mechanical defenses (leaf toughness) and chemical defenses (AA concentration)? (2) Do local co-occurring troidine species differ in their chemical defenses (AA concentration)? (3) Is there variation in larval preference among *Aristolochia* species? and if so, is larval preference affected by AA content? (4) Is larval performance affected by the AA content present in their diet?

MATERIALS AND METHODS

Study site

Iguazú National Park, Argentina, is located in the northeastern Argentina (25.65 S, 54.33 W). Iguazú hosts a diversity of plants and animals, including five species of butterflies in the Troidini group (*Parides agavus*, *Parides anchises nephalion*, *Parides neophilus eurybates*, *Battus polydamas*, and *Battus polystictus*; Canals 2003) and two *Aristolochia* host plant species (*Aristolochia triangularis* and *Aristolochia macroura*). Extensive observations showed that *P. anchises nephalion* and *B. polydamas* are the most abundant species of the five troidine species in the area (R. D. Dimarco, *personal observations*).

Sampling of the local troidine butterflies and *Aristolochia* plants

During April and May of 2008 and from September to March of 2009–2010 and 2010–2011, we sampled individuals of the local troidine community and their *Aristolochia* host plants. During daily trips by two persons, we used nets to collect butterflies and searched for *Aristolochia* plants. We had a total of 191 full days of sampling across all field seasons.

Analysis of leaf toughness and AA concentration in local *Aristolochia* spp.

To examine whether local *Aristolochia* species differ in their mechanical and chemical defenses, we collected leaves from the two *Aristolochia* species, *A. triangularis* and *A. macroura*, to measure leaf toughness and AA concentration. Leaf toughness is an important trait shown to offer mechanical resistant to phytophagous insects (especially

first-instar butterfly larvae) and is correlated with larval performance (Clissold et al. 2009, Pinto et al. 2009, Dimarco et al. 2012). Leaf toughness (g/cm^2) was measured on 30 fresh leaves from each *Aristolochia* species with a force gauge penetrometer (type 516; Chatillon, Largo, Florida, USA) by averaging three measurements per leaf. The leaf penetrometer measures the force needed to puncture a leaf using a 3 mm diameter rod. Mean leaf toughness was compared between *A. triangularis* and *A. macroura* using a *t* test.

To assess variation in AA concentration (μg of AAs/mg of dry weight) of *Aristolochia* plants, we extracted AAs from a total of 114 individual healthy leaf samples collected from the middle part of the stem and that were similar in age, size, and coloration (46 from *A. macroura* and 68 from *A. triangularis*). Leaves from each host plant were brought to the laboratory and were dried under reduced pressure prior to extraction and weighed to the nearest milligram. Aristolochic acids were extracted twice in 5 mL of 100% ethanol in a 10-mL borosilicate test tube and sonicated twice for 20 min at 50°C. The ethanol extracts were dried under reduced pressure and left a yellow residue in the glass tube. 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, Massachusetts, USA). Each injection was 10 μL , eluted isocratically with a mixture of methanol, water, and 1% hydrochloric acid (52:47:1) at a rate of 1 mL/min on a Waters Symmetry C-18 reverse-phase column (3.5 mm, 4.6×75 mm). We identified two AAs (AA I and AA II) based on their retention times and unique absorption spectra. Aristolochic acids I and II were added to obtain the total AA concentration per sample. Aristolochic acid concentration (μg of AAs/mg of dry weight) was quantified by comparing peak retention times and areas to a standard curve generated with pure chemical standards (Sigma Chemical, St. Louis, Missouri, USA) for each AA quantified in this study as described in Fordyce et al. (2005), Fordyce and Nice (2008), Dimarco et al. (2012) among others.

We estimated the mean AA content of both *Aristolochia* species in a Bayesian framework.

Because the AA concentration of some of the samples was below levels where we could reliably integrate under the UV absorbance curve, we modeled AA content of our samples as being drawn from a zero-truncated normal distribution with $x_{ij} \sim \text{normal}(\mu_j, \sigma_j)$, $x \in (0, \infty)$, where x_{ij} is the AA content of the *i*th sample from the *j*th species. Priors on μ_j and σ_j were $\mu_j \sim \text{normal}(0, 5)$ and $\sigma_j \sim \text{half-Cauchy}(0, 5)$, respectively. Aristolochic acid values below the levels we could reliably integrate were treated as left-censored centered data (Stan Development Team 2015). Parameters were estimated with Hamiltonian Monte Carlo using Stan (Stan Development Team 2015, Carpenter et al. 2017) via the RStan interface in R version 3.2.2 (R Development Core Team 2016) using 10,000 iterations following warm-up on four chains. Convergence toward a stationary distribution was assessed visually using trace plots and the Gelman–Rubin potential scale reduction factor, \hat{R} (Gelman and Rubin 1992, Brooks and Gelman 1998). Values of one are ideal, and $\hat{R} > 1.1$ indicates problems in convergence among the MCMC chains. Highest density intervals of the posterior distributions of parameter estimates were calculated using the HDInterval package 0.1.3 (Meredith and Kruschke 2016) in R. These data were also analyzed in a conventional linear modeling framework, treating AA concentrations below the reliable integration threshold as zeros (Appendix S1).

Analysis of AA concentration in the local troidine

To assess whether different patterns of chemical sequestration exist in co-occurring troidine species, we collected 315 wild adult butterflies that represented four species (136 from *B. polydamas*, 137 from *P. anchises nephalion*, 20 from *P. neophilus euribates*, and 22 from *B. polystictus*). Each adult butterfly was dried under reduced pressure prior to extraction and weighed to the nearest milligram. Fat was extracted from each butterfly by homogenizing butterfly tissue twice in 5 mL of hexane and sonicating for 20 min at 50°C. The fat-containing hexane was placed in a pre-weighed 10-mL borosilicate test tube. We left the hexane to evaporate and quantified fat concentration (g of fat/g of dry weight) by subtracting the weight of the tube with fat from the weight of the pre-weighed tube (Fordyce et al. 2005). The AAs in the defatted tissue were

extracted in 5 mL of 100% ethanol and sonicated for 20 min at 50°C. The extraction was repeated once to ensure AAs removal from each sample. The ethanol extracts were dried under reduced pressure, resuspended in 1 mL of 100% methanol, and passed through a 0.45- μ m filter into an autosampler vial for HPLC analysis. We performed HPLC analyses using the same Waters Alliance HPLC system described above and quantified total AAs as explained in *Analysis of leaf toughness and AA concentration in local Aristolochia spp.* Aristolochic acids were identified based on their retention times and unique absorption spectra. As with the plant material described above, some samples had AA concentrations below levels that we could reliably measure. Therefore, we used a similar Bayesian model as described above, where AA content of our samples was assumed to be drawn from a zero-truncated normal distribution and values below the level that we could reliably measure were treated as censored data. These data were also analyzed in a conventional linear modeling framework, treating AA concentrations below the reliable integration threshold as zeros (Appendix S1).

Larval preference between different *Aristolochia* species and with different levels of AAs

To assess whether the two most common co-occurring troidine species (*B. polydamas* and *P. anchises nephalion*) show variation in preference between the two locally available *Aristolochia* species, and whether AA content influences larval preference, we conducted two larval feeding preference experiments. Assessing larvae preference is relevant because troidine larvae are known to wander long distances between food plants (Rausher 1979). Further, individuals of different *Aristolochia* host species can be observed growing adjacent to one another in some locations at our study site (R. D. Dimarco, *personal observation*). To determine larval preference, we collected 12 *B. polydamas* females and 11 *P. anchises nephalion* females and let them oviposit eggs in the laboratory. After larvae emerged from the eggs, we placed individual larvae (44 *B. polydamas* and 34 *P. anchises nephalion*) in small arenas (10 cm diameter Petri dishes) and provided them with freshly cut leaves of similar size (~25 \times 25 mm) from each *Aristolochia* species (*A. triangularis* and *A. macroura*). Neonate larvae fed for 48 h (leaf

material was replaced after 24 h), and then, we removed remaining leaf material to assess how much of each leaf area was consumed. We quantified leaf area consumption by digitizing the leaves using ImageJ software (Rasband 2003) and then assessing the area missing relative to the total leaf area. A paired *t* test comparing the amount of leaf area consumed of each species was used to characterize larval preference.

Because the two locally available *Aristolochia* species differed in AA concentration and leaf toughness (see *Results*), we conducted a second larval preference experiment to examine the role AAs play in larval choice. We used only *A. triangularis*, which has more tender leaves and was typically devoid of or contained only trace amounts of AAs. For this experiment, we manipulated *A. triangularis* leaf chemistry by adding AAs to *A. triangularis* leaves of similar sizes. We sprayed one half of each *A. triangularis* leaf with a mixture of equal amounts of AA I and AA II (saturated solution, 100 μ g/100 mL ethanol) obtained from Sigma Chemical. The supplemented leaves were analyzed in the laboratory, and the addition of the AAs solution to *A. triangularis* leaves made it reach similar AA concentrations to that found naturally in *A. macroura* (the plant species with higher leaf toughness and levels of AAs). The other half of the leaf was sprayed with 100% ethanol as a control. We left the ethanol to evaporate before leaf tissue damage occurred, leaving leaves with an AA supplemented and a control side. We placed 20 neonate larvae from each butterfly species (*B. polydamas* and *P. anchises nephalion*) in individual arenas and provided them with the *A. triangularis* leaf with manipulated AA content (with and without the addition of AAs). As a measure of larval preference, we quantified the leaf area consumed by each larva after 48 h of feeding (replacing leaf material at 24 h) as described in the previous experiment. We tested for differences in the amount of leaf area consumed between *A. triangularis* leaf halves (with and without the addition of AAs), using a paired *t* test.

Larval performance and its relation to presence of AAs in their diet

To evaluate whether larval performance is affected by the AA content in their diet, we reared individual *B. polydamas* and *P. anchises nephalion*

larvae under the following three treatments: (1) *A. macroura* leaves with their natural levels of AAs (i.e., typical levels of AAs found in an *Aristolochia* species); (2) *A. triangularis* leaves with their natural levels of AAs (i.e., no detectable or trace amounts of AAs); and (3) *A. triangularis* leaves with the addition of AAs. To modify *A. triangularis* AA content, we sprayed leaves with a mixture of AA I and II (saturated solution, 100 µg/100 mL ethanol; see full methods in previous sections). To control for effects of ethanol, we sprayed 100% ethanol on unmodified *A. triangularis* and *A. macroura* leaves (treatments 1 and 2). In the laboratory, we obtained eggs from wild-caught *P. anchises nephalion* ($n = 23$) and *B. polydamas* ($n = 8$). Eggs were pooled for each butterfly species. Each individual egg was placed in a Petri dish, and each neonate larva was reared individually. We consider each rearing container as an experimental unit. We obtained information from 70 neonates of *P. anchises nephalion* and 24 neonates of *B. polydamas*. The difference in the number of neonates between the two butterfly species was due to differences in adult female abundances at the time of the experiment. We assessed the variation in survivorship, adult fat content, and adult dry weight across all three feeding treatments (*A. macroura* leaves, unmodified *A. triangularis* leaves, and *A. triangularis* leaves supplemented with AAs). Troidine butterflies, as most other butterfly species, are nectar feeders as adults, so fat accumulated during the larval stages is an important fitness correlate (Boggs 1997). Also for this sequestering group of butterflies, it has been found that adult fat content is negatively affected by AAs sequestration (Fordyce and Nice 2008). To obtain the fat content from the adult butterflies, we followed the procedure explained above (see *Analysis of AA concentration in the local troidine*).

We compared survival to the adult stage for both butterfly species reared on either of the three different diets (*A. triangularis*, *A. macroura*, *A. triangularis* + AAs) using a generalized linear model with binomial errors. Adult fat content and adult dry weight among the three diets for each butterfly species were analyzed using ANOVA. Aristolochic acid content was modeled using the Bayesian framework described above.

All original data, R code, and STAN code to reproduce the results presented here are provided in Appendix S2.

RESULTS

Analysis of leaf toughness and AA concentration in local Aristolochia spp.

The two studied *Aristolochia* plants differed in their leaf toughness and AA concentrations. *Aristolochia macroura* leaves are 11 times more tough than *Aristolochia triangularis* leaves. *A. triangularis* leaves have a mean toughness of 21.04 g/cm² compared to 212.14 g/cm² for *A. macroura* leaves ($t = -32.27$, $df = 30.74$, $P < 0.001$). *A. triangularis* had low AA concentrations, with 76 of 91 samples below HPLC integration levels, whereas *A. macroura* individuals ($n = 23$) had AA levels comparable to those reported for North American *Aristolochia* species (Dimarco et al. 2012; Fig. 1). *A. macroura* was more likely than *A. triangularis* to have AA concentrations at levels that could be reliably quantified ($\chi^2 = 53.929$, $df = 1$, $P < 0.001$). Analysis of these data in a conventional null hypothesis testing framework resulted in similar inference (Appendix S1).

Analysis of AA concentration in the local troidine

We found substantial variation in AA concentration among the four species of butterflies (Fig. 2). Most individuals of the abundant *Battus polydamas* and the less-common *Battus polystictus* had low concentrations of AA, with 103 of 136

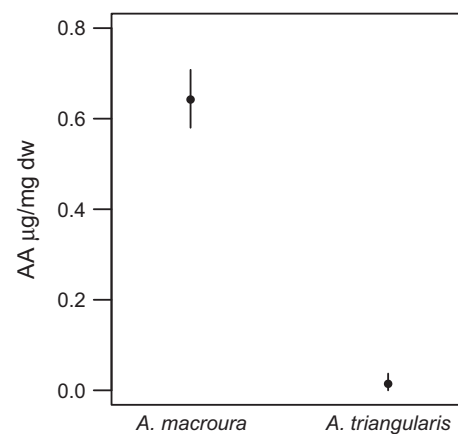


Fig. 1. Mean aristolochic acid (AA) concentration (µg of AAs/mg of dry weight) in leaves of *Aristolochia macroura* and *Aristolochia triangularis* found in Iguazú National Park, Argentina. Dots and bars indicate the median and 95% highest density interval of the posterior for the mean AA concentration, respectively.

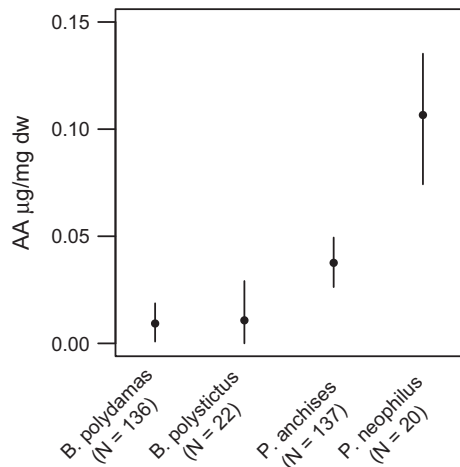


Fig. 2. Mean aristolochic acid (AA) concentration (μg of AAs/mg of dry weight) in adults of four butterfly species found in Iguazú National Park, Argentina. Dots and bars indicate the median and 95% highest density interval of the posterior for the mean AA concentration, respectively.

B. polydamas and 20 of 22 *B. polystictus* having AA concentrations below HPLC integration levels. *Parides anchises* had AA concentrations below integration levels in 68 of 137 individuals, whereas only one of 20 *Parides neophilus* had AA levels below the HPLC integration level. Only *P. neophilus* had estimates of mean AA concentration where 95% highest density interval was comparable to North American *Battus philenor* (Fig. 2; Fordyce et al. 2005). The other three species had substantially less. Analysis of these data in a conventional linear modeling framework and treating AA levels below the reliable quantification threshold as zeros resulted in similar statistical inference (Appendix S1).

Larval preference for different *Aristolochia* species and with different levels of AAs

Both *B. polydamas* and *P. anchises nephalion* larvae preferred to feed on *A. triangularis* over *A. macroura* (*B. polydamas*: $t = 4.11$, $df = 35$, $P < 0.01$; *P. anchises nephalion*: $t = 3.34$, $df = 24$, $P < 0.01$; Fig. 3A, B). We failed to detect differences in leaf area removed between AA supplemented leaves and control *A. triangularis* leaves by *B. polydamas* ($t = 1.70$, $df = 18$, $P = 0.11$) and *P. anchises nephalion* ($t = 1.33$, $df = 20$, $P = 0.20$; Fig. 3C, D).

Larval performance and its relation to presence of AAs in their diet

We found that larvae of *B. polydamas* and *P. anchises nephalion* had a lower probability of surviving when reared on *A. macroura* leaves or *A. triangularis* leaves with supplemented AAs (probability of survival 0.29 and 0.26, respectively), compared to larvae reared on *A. triangularis* (probability of survival 0.63; $\chi^2 = 10.96$, $df = 2$, $P < 0.01$). We failed to detect a difference in larval survivorship between *B. polydamas* and *P. anchises nephalion* ($\chi^2 = 0.10$, $df = 1$, $P = 0.75$). For surviving individuals, we failed to detect an effect on adult fat content with the addition of AAs in larvae diet, in both butterfly species (*P. anchises nephalion* $F_{2,25} = 0.05$, $P = 0.95$; *B. polydamas* $F_{2,6} = 0.02$, $P = 0.99$). We also failed to detect an effect on adult dry weight with the addition of AAs (*P. anchises nephalion* $F_{2,25} = 0.31$, $P = 0.74$; *B. polydamas* $F_{2,6} = 1.53$, $P = 0.29$).

Based on the AA concentration of adult butterflies obtained, we found that *P. anchises* and *B. polydamas* larvae have the ability to sequester AAs when it is present in their diet. *P. anchises* sequestered substantially more AAs compared to *B. polydamas* when reared on either *A. macroura* or *A. triangularis* with supplemented AAs. Both *P. anchises* and *B. polydamas* had nearly negligible amounts of AA when reared on control *A. triangularis* (Fig. 4). Analysis of these data in a conventional linear modeling framework and treating AA levels below the reliable quantification threshold as zeros resulted in similar statistical inference (Appendix S1).

DISCUSSION

We found that one abundant *Aristolochia* species and most individuals of the two most common swallowtail butterfly species do not possess AAs or contained only trace amounts. Larval preference does not appear to be affected by leaf AA content; rather, leaf toughness appears to be the more important determinant for *Battus polydamas* and *Parides anchises* larval preference. This is consistent with Dimarco et al. (2012) who found that, for *Battus philenor*, leaf toughness was more important in determining the preference of early-instar larvae compared to AA content. Our analyses showed that larvae fed indiscriminately in the treatments with or without addition of

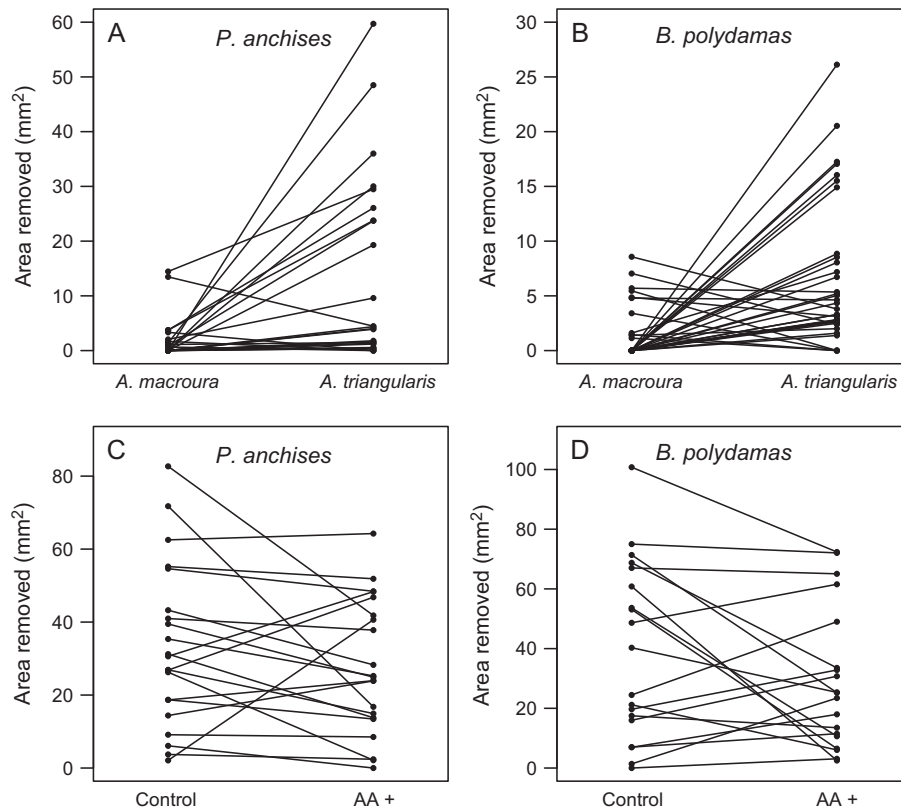


Fig. 3. Results from the two larval preference experiments by the two studied butterfly species, *Parides anchises nephalion* and *Battus polydamas*. Segments connect points that represent the amount consumed by a caterpillar in each replicate. The upper plots (A, B) show the area removed (in mm²) of fresh leaves of *Aristolochia macroura* and *Aristolochia triangularis* by larvae of each studied butterfly species. Both butterfly species removed significantly more area of *A. triangularis* leaves than *A. macroura* leaves. The bottom plots (C, D) show the area removed (in mm²) of fresh *A. triangularis* leaves that had aristolochic acids (AAs) added to half of the leaf (+AAs) and no AAs addition to the other half (control). We failed to detect differences between the two treatments for both butterfly species.

AAs on *Aristolochia triangularis* leaves. This host plant species possesses tender leaves and largely lacks AAs. This result suggests that, if the opportunity to feed on a more tender host plant is given, larvae might choose to feed on this host plant irrespective of its AA content.

Although AAs did not influence larval feeding choice, larval performance was affected by the amount of AA present in their diet. In the treatment with leaves with supplemented AAs, survivorship was lower compared to treatments with trace amounts or no detectable levels of AAs (i.e., larvae reared only on *A. triangularis*). These results might indicate that a cost associated with feeding on leaves with AA might exist (but see Pinto et al. 2009) and it may also shed

some light on why so many adult troidine swallowtails in Iguazú did not possess AAs. Adult troidine swallowtails might possess other chemical defenses that could be produced without sequestering it from plants as larvae. However, we did not search for other chemical defensive compounds, since there is not previous evidence of their existence, but further research may be needed on this topic.

Some troidine swallowtails from other regions that feed on *Aristolochia* species (e.g., *A. galeata*, *A. momandul*, *A. elegans*) also have none or trace amounts of AAs, so AAs are not available to be sequestered by the developing larvae (Urzúa and Priestap 1985, Mebs and Schneider 2002, Klitzke and Brown 2000). The general lack of AAs in

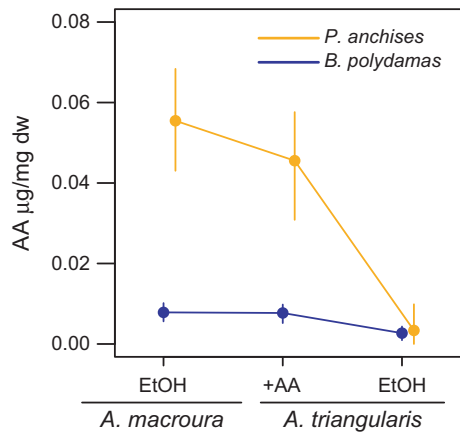


Fig. 4. Estimates of mean aristolochic acid (AA) content for *Parides anchises* and *Battus polydamas* reared on three different diets. Dots indicate median and bars indicate 95% highest density interval of the posterior for the mean AA concentration. Bars indicate 95% highest density interval of the posterior for the mean AA concentration.

wild-caught adults in Iguazú might be a consequence of females preferentially ovipositing on *A. triangularis*, or due to differences in abundances of the two host plant species. Further studies are needed on *Aristolochia* species abundances and on the oviposition pattern of these troidine female butterflies to fully understand this. The absence of an *Aristolochia* host plant species free of AAs in the studied temperate areas might explain why there are less variation and high levels of AAs in the associated butterflies (Fordyce 2000, Sime et al. 2000), whereas the high variation of AA content in *Aristolochia* species from the studied tropical and subtropical areas can allow for high variation in AA content in the co-occurring sequestering troidine swallowtails.

Automimicry complexes (Batesian mimicry within a species) could develop when chemical defenses are costly (as is the case with AAs; Brower et al. 1970, Fordyce and Nice 2008, Daly et al. 2012). Since there is no individual survival benefit in being toxic, some individuals of a population produce little or nothing of the costly defense (the automimics). These individuals can still get protection because other members of the same population that are similar in appearance are defended and deter predators (Brower et al. 1970, 1975, Tuskes and Brower 1978, Daly et al. 2012, Aubier et al. 2017). The potential tradeoff

found in our studied system in Iguazú between sequestering chemical defenses and larval survivorship might be explained by automimicry.

In North America, *B. philenor* is the troidine toxic model species for the Batesian mimicry complex, where five putative Batesian mimics occur (*Papilio troilus*, *Papilio polyxenes*, the melanic female form of *Papilio glaucus*, *Limenitis arthemis astyanax*, and female *Speyeria diana*; Brower 1958, Platt et al. 1971, Prudic and Oliver 2008). However, due to the highly unpredictable mimetic environment, the existence of such phenomenon in the butterfly species studied herein is unlikely, and Müllerian mimicry seems more plausible (Aubier et al. 2017). Since there are multiple possible models exhibiting a range of AA concentrations, unlike *B. philenor* in North America, distasteful troidine individuals that share similar warning signals and the mutual benefit of being avoided by common predators might produce a Müllerian mimicry complex rather than a Batesian one.

Handling toxic AAs have been proposed to be costly. A previous study on *B. philenor* suggested a cost associated with the sequestration of AAs by a reduction in adult fat content (Fordyce and Nice 2008). In the troidine butterflies study herein, we failed to detect an effect on adult fat content and adult dry weight in relation to the amount of AA sequestered. However, larval survivorship was reduced when reared on plants with higher AA content, whether it be plants that naturally possess higher levels of AA, as is the case for *Aristolochia macroura*, or experimentally augmented AA, as in our experiment with *A. triangularis*. This suggests that there might be a cost for these butterfly species associated with the presence of AAs. However, it is not known whether this cost is balanced by a defensive benefit when natural enemies are present.

Although this study was conducted at a single site (Iguazú National Park), the lack of sequestered AAs has also been observed in other communities of troidine swallowtails. Preliminary results from Costa Rica (R. D. Dimarco and J. A. Fordyce, unpublished data) suggest that lack of sequestered AAs in this group might be the rule, not the exception. Concordant with results from Iguazú, we found in Costa Rica that most individuals of *Aristolochia constricta* (60 out of 64) and *Aristolochia pilosa* (51 out of 64) and some individuals of the troidine species studied (*Parides*

iphidamas, *Parides sesostris*, *Parides childrinae*, and *B. polydamas*) do not possess AAs. In a study of neotropical troidine collected in different regions of Central, South America, and the Caribbean, Klitzke and Brown (2000) found variable concentrations of AAs in adult butterflies. Another study by Mebs and Schneider (2002) in South-East Asia also found high variability in AA concentration among troidine adults, with some individuals containing no detectable or trace amounts. Although these studies found similar results to the one presented here, they were limited by small sample sizes. Klitzke and Brown (2000) used an average of eight adults per species collected from different sites throughout Central and South America, and Mebs and Schneider (2002) had an average of three adults per species collected from different sites in South-East Asia. More detailed studies in other areas of the Americas and Asia are needed to fully understand variation in sequestered AA content.

Some regions of the world are notably more studied than others, producing a substantial bias in ecological knowledge that can be problematic (Martin et al. 2012). Working in understudied areas that have higher biological diversity, such as Iguazú National Park, has its difficulties, but more studies in these areas may be needed to get a more complete picture of plant–insect interactions. This seems to be the case for understanding interactions, like that of the troidine butterflies and their *Aristolochia* host plants, which occur from tropical to temperate areas and from sites with a rich ecological knowledge to sites that have been rarely studied.

In North America, *B. philenor*, the most abundant and best-studied troidine species, possess AAs, as do all their locally available *Aristolochia* host plants examined thus far (Sime 2002, Fordyce and Nice 2008, Fordyce et al. 2010). Our study sheds light on the fact that, for the same butterfly group, results from well-studied areas can differ from the results obtained in less studied areas, and the paradigm of chemically defended troidines through the sequestration of AA from their host plants might not accurately reflect nature.

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