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# Ecological and evolutionary consequences of variation in aristolochic acids, a chemical resource, for sequestering specialist Troidini butterflies

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I am submitting herewith a dissertation written by Romina Daniela Dimarco entitled "Ecological and evolutionary consequences of variation in aristolochic acids, a chemical resource, for sequestering specialist Troidini butterflies." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Ecology and Evolutionary Biology.

James A. Fordyce, Major Professor

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**Ecological and evolutionary consequences of  
variation in aristolochic acids, a chemical  
resource, for sequestering specialist Troidini  
butterflies**

A Dissertation Presented for the  
Doctor of Philosophy  
Degree

The University of Tennessee, Knoxville

Romina Daniela Dimarco

December 2013

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This dissertation is dedicated to my husband Martin for his support, love and his patience, and to my daughter Valentina for giving me balance and perspective.

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## ABSTRACT

Plant-insect interactions provide an opportunity to examine fundamental ecological and evolutionary processes, including mechanisms of species co-occurrence and adaptations to herbivory and predation. An example is the interaction between butterflies in the Troidini tribe (Papilionidae), a group that sequesters chemical defenses from its host plants in the genus *Aristolochia* (Aristolochiaceae).

In this dissertation, I examined the ecological and evolutionary consequences of variation in aristolochic acids, the chemical compounds sequestered by Troidini from its host plants, through a combination of observational, experimental and laboratory studies. I conducted studies at several sites throughout the Americas where different levels of knowledge about this interaction are available. These differences allowed me to ask specific questions in areas where there is ample background information (i.e. North America) and to ask more general, but fundamental questions in areas where little is known (i.e. South America).

In North America, I showed for a Troidini species, *Battus philenor*, that larvae preferentially fed on less tough, younger leaves, and found no evidence that aristolochic acid content influenced larval foraging. For these herbivores, mechanical resistance might be a more important determinant of larval foraging behavior and development compared to plant chemical defenses. In another



study in North America, with data from three consecutive seasons, I found that larger egg clutches of *B. philenor* suffer less predation compared to small clutches. This study suggests that, for eggs protected with toxic chemicals, there is a clear benefit in laying eggs in large clusters in areas with high levels of predator threat.

In Iguazú National Park, Argentina, little is known about Troidini-*Aristolochia* interaction; therefore I studied general and specific aspects of this interaction. Our main finding, in contrast to what has been reported in North America, is that many *Aristolochia* and Troidini are not defended with aristolochic acids, contrary to the currently accepted paradigm that all Troidines are toxic. Studying these species in areas where they are understudied and reach higher diversity gives a more complete picture of this plant-butterfly interaction. My results contribute to furthering our understanding of the role of chemical ecology in shaping evolutionary dynamics and ecological processes.

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**CHAPTER I.**  
**INTRODUCTION**

## Introduction

Plant-insect interactions provide an opportunity to examine fundamental ecological and evolutionary processes, including mechanisms of species co-occurrence and adaptations to herbivory and predation (Ehrlich and Raven 1964, Johnson et al. 2003, Zovi et al. 2008). Plants can impose strong selection pressures on herbivorous insects, and their effects can extend to the third trophic level (Braby 1994, Thompson 1999, Fordyce and Nice 2008). The interaction between butterflies and their host plants has a long history of investigation for understanding the ecological and evolutionary consequences of plant-insect interactions. An example is the interaction between butterflies in the tribe Troidini, a group that sequesters chemical defenses from its host plant (Rausher and Papaj 1983, Sime 2002, Fordyce et al. 2010).

Swallowtail butterflies in the tribe Troidini (Papilionidae) 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). Troidini are found from temperate to tropical areas, but they are predominantly tropical and subtropical, with most species concentrated in the lowland forests of Central and South America and in the Indo-Australian region (Weintraub 1995). There are two Troidini genera in the new world, *Battus* and *Parides*, and members of these

genera are known to possess plant-derived aristolochic acids (Brower 1958, Klitzke and Brown 2000, Fordyce et al. 2005). The butterflies obtain these chemicals as larvae, rendering both larvae and adults chemically defended against most predators, including ladybird beetle larvae, spiders, and birds (Rothschild et al. 1970, Brower 1984, Fordyce 2000) (Fig. I.1.).

The most studied species in the Troidini tribe is the pipevine swallowtail, *Battus philenor*, which is distributed mostly in North America (Rausher 1981, Allard and Papaj 1996, Fordyce 2000, Sime et al. 2000). Examples of studies on this butterfly species include those by Pilson and Rausher (1988) who investigated whether females modify clutch size in response to variation in host plant quality, and by Rausher and Papaj (1983), who studied the oviposition selection of females on conspecific host plants and its effect on larval growth rates, predispersal mortality, and larval size at dispersal. The existence of numerous studies of this species allow us to ask very specific questions about the ecology and evolution of the interaction between *B. philenor* and *Aristolochia* spp., such as the ones explored in chapters II and III of this dissertation (see below).

Although the interaction between the Troidini species *Battus philenor* and *Aristolochia* spp. is documented in North America, there is a paucity of studies in Central and South America, where the greatest diversity of the Troidini tribe is found. Chapter IV of this dissertation explores different aspects of the chemical

ecology of a Troidini community and its *Aristolochia* host plants in an understudied area in South America (see below).

In my dissertation research, I used observational and experimental approaches to quantify and examine different aspects that contribute to the understanding of the ecological and evolutionary consequences of the Troidini tribe and their *Aristolochia* host plant interaction. This research was conducted in three study areas including Texas and Tennessee in the USA, and Iguazú National Park in Argentina.

In Chapter II, I report on the study conducted with Drs. Fordyce and Nice on the effect of aristolochic acids and leaf toughness on larval preference and performance in a population of *B. philenor* in Texas, USA. I also examined if heritable variation in larval sequestration or host plant aristolochic acid content is the predominant determinant of larval chemical phenotype.

In Chapter III I report on the study conducted with Dr. Fordyce on the role egg clutch size has on egg mortality in *B. philenor*. I examined the hypothesis, proposing that larger egg clutches frequently observed in toxic butterflies play a defensive role for eggs by reducing the probability of egg mortality due to predation. I tested this idea in Norris Dam State Park in east Tennessee.

Finally, chapter IV is focused on the chemical ecology of a Troidini community and its *Aristolochia* host plants in Iguazú National Park, Argentina. Since very little is known about this interaction in this area (and actually in many places of the Americas outside of the USA), I started asking a series of very



general natural observation questions that guided us into more specific questions about the chemical ecology of the Troidini-*Aristolochia* interaction.

The studies presented in this dissertation are the first of their kind for this group of toxin-sequestering specialists. By studying aspects of this plant-insect relationship not previously explored, as well as examining the general biology of this herbivore in areas where it reaches higher diversity and abundances and in areas previously understudied (i.e., outside North America), I hope to provide fundamental information to further our understanding of the ecology and evolution of this complex plant-insect interaction.

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## Appendix I: Figures

# Troidini life cycle

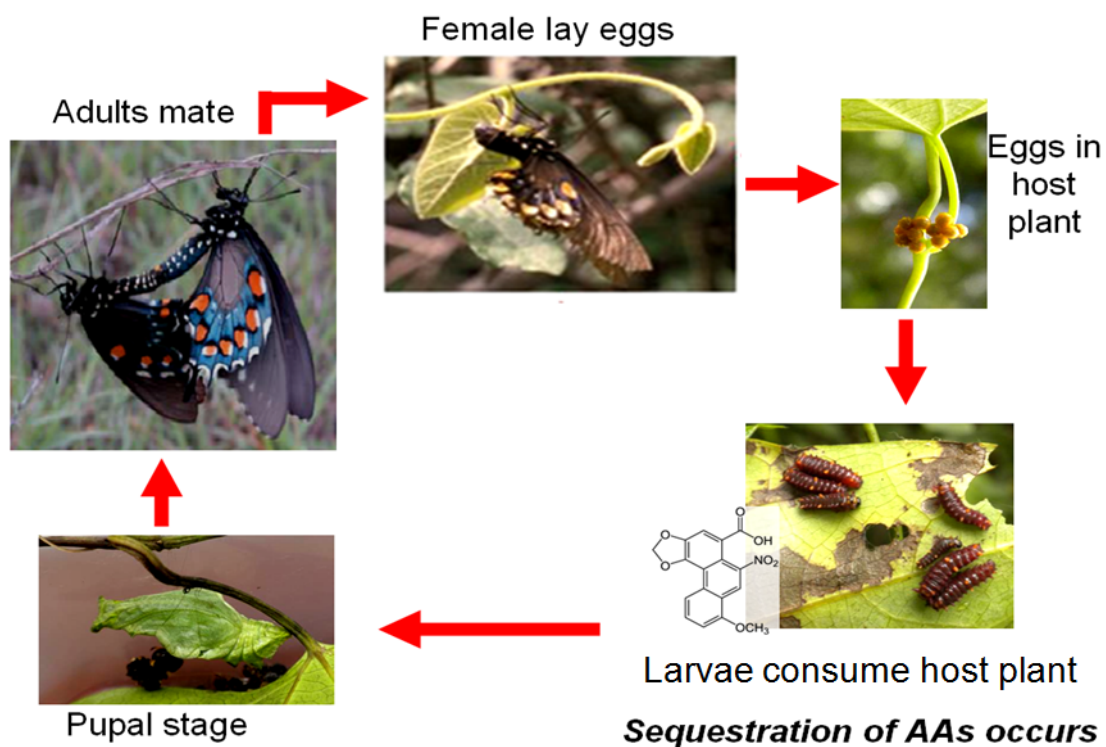


Figure I.1. Typical life cycle of butterflies in the Troidini tribe showing some of the life stages studied in my thesis (adults, eggs and larvae). Troidini butterflies obtain the toxic alkaloids (aristolochic acids, AAs) by feeding on *Aristolochia* plants as larvae, rendering both larvae and adults chemically defended.

**CHAPTER II.**

**FAMILY MATTERS: EFFECT OF HOST PLANT VARIATION IN  
CHEMICAL AND MECHANICAL DEFENSES ON A  
SEQUESTERING SPECIALIST HERBIVORE**

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The following section is a slightly modified version of a manuscript published in the journal *Oecologia*:

Dimarco, R. D., C. C. Nice, and J. A. Fordyce. 2012. Family matters: Effect of host plant variation in chemical and mechanical defenses on a sequestering specialist herbivore. *Oecologia* **170**:687-693.

The use of “we” in this chapter refers to my co-authors and me. As the lead author of this article, I was responsible for this paper. My primary contributions to this paper included the formulation of ideas, chemical and data analyses, and writing.

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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 inter-plant chemical variation for determining 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 inter-individual 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 inter-plant 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 found no evidence that aristolochic acid content influenced first instar larval foraging. We found that most variation in the amount of aristolochic acids 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.

## 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 a defense against natural enemies (Price et al. 1980; Malcolm and Zalucki 1996; Fordyce 2001). There can be substantial intra-plant variation in the allocation of chemical defenses, and it is reasonable to 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 remains limited understanding of the relationship between intra-plant 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, rather 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 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 determinant 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 been scarcely 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 America *Aristolochias* 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; they are nephrotoxic and hepatotoxic, and are known mutagens (Chen and Zhu 1987). Larvae of *B. philenor* sequester these metabolites, rendering both larvae and adults 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 intra-plant 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 aristolochic acid content of larvae?

## **Materials and Methods**

### **Laboratory and Field experiments**

#### **Intra-plant 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 (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<sup>2</sup>) by averaging three measurements with a force gauge penetrometer (Type 516; Chatillon, Largo, Florida, USA) on fresh leaf material. The leaf penetrometer measures the mass (in grams) 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 as 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 hours. Neonate larvae were randomly assigned to plants and leaf position. After placing larvae on the stem next to their designated starting leaf, the larvae were allowed to move and begin feeding. After 24 hours 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 & AA-II/100 ml ethanol), resulting in nearly



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 hours 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 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). 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 3 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 JMP (v. 9.02) software (SAS Institute 2010) to estimate the among-female variance component.

### **Chemical analysis**

Larvae and leaves were dried under reduced pressure prior to acid extraction. We weighed larvae to the nearest 0.1 microgram 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 minutes at 50 °C, and dried the resultant extract 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). Each injection was 10  $\mu$ l 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 x 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

### **Intra-plant variation in aristolochic acid content and leaf toughness**

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

there is a tendency for less tough, younger leaves to have higher concentrations of aristolochic acid compared to tougher, older leaves.

### **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 hours 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.

### **Effect of aristolochic acid content and leaf toughness on larval performance**

When we examined the effect of leaf chemistry vs. 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. II.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. II.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 ( $F_{1, 29} = 4.885$ ,  $P = 0.035$ ).

## Discussion

We found large intra-plant variation in leaf toughness and aristolochic acid chemistry in *A. erecta* individuals. Regardless of the amount of aristolochic acids 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 with *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 *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 compared to 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 performance 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, compared to 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 intra-plant distribution of aristolochic acids in *A. erecta* was consistent with the predictions of

optimal defense theory, namely that younger leaves with potentially higher fitness value 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 that 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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## Appendix II: Figures

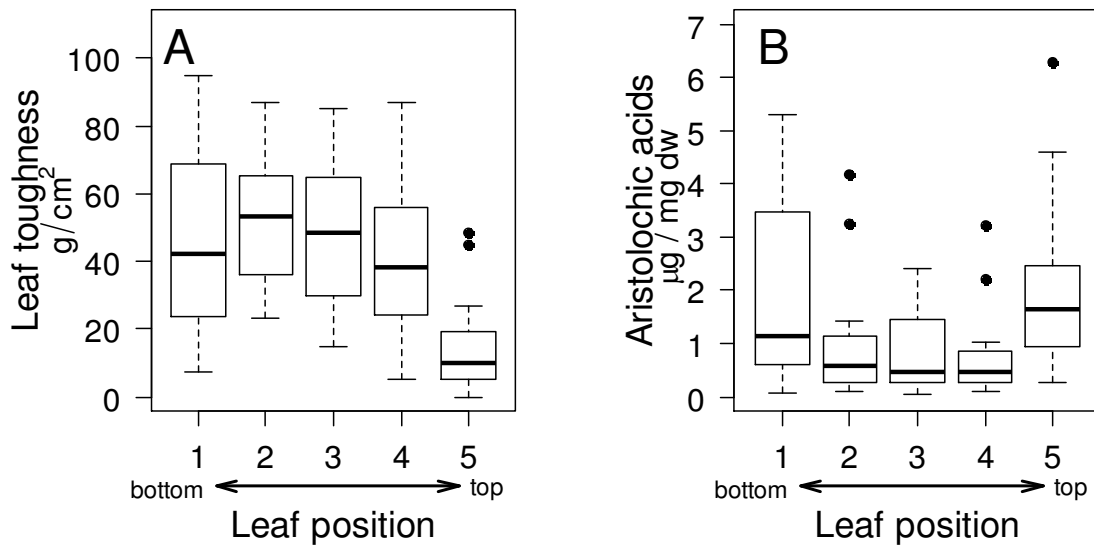


Figure II.1. Boxplots of leaf toughness (A) and total leaf aristolochic acid content (B) in *A. erecta* individuals. Leaves were numbered from 1 to 5 with leaf 1 the oldest and leaf 5 the youngest (tip leaf).

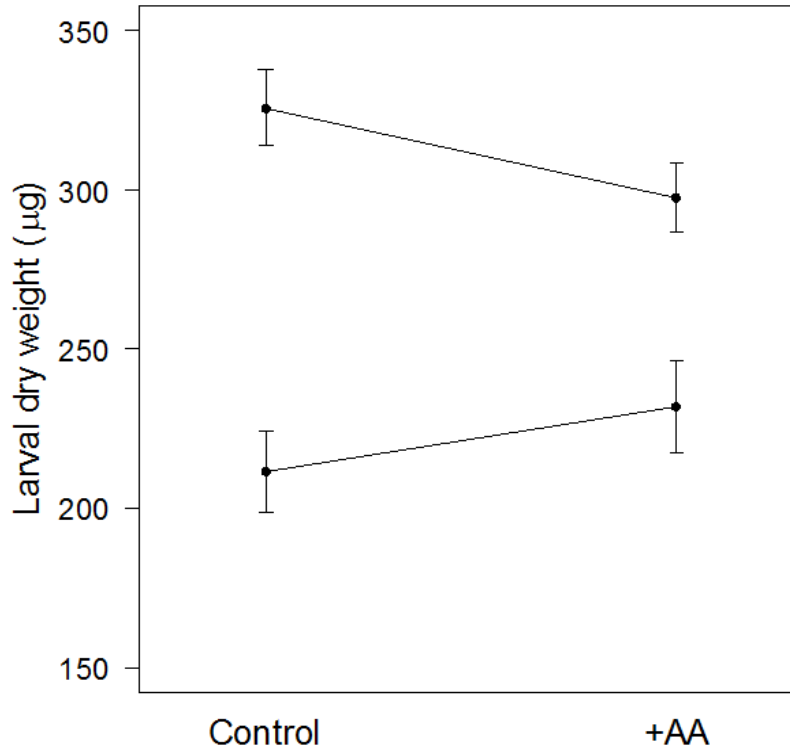


Figure II.2. Larval dry weight ( $\pm$ SE) on less tough and more tough leaves of *A. erecta* without supplemented aristolochic acid (control treatment) or with supplemented aristolochic acids (+AA treatment) ( $F_{(3,76)} = 18.162$   $P < 0.001$ ).



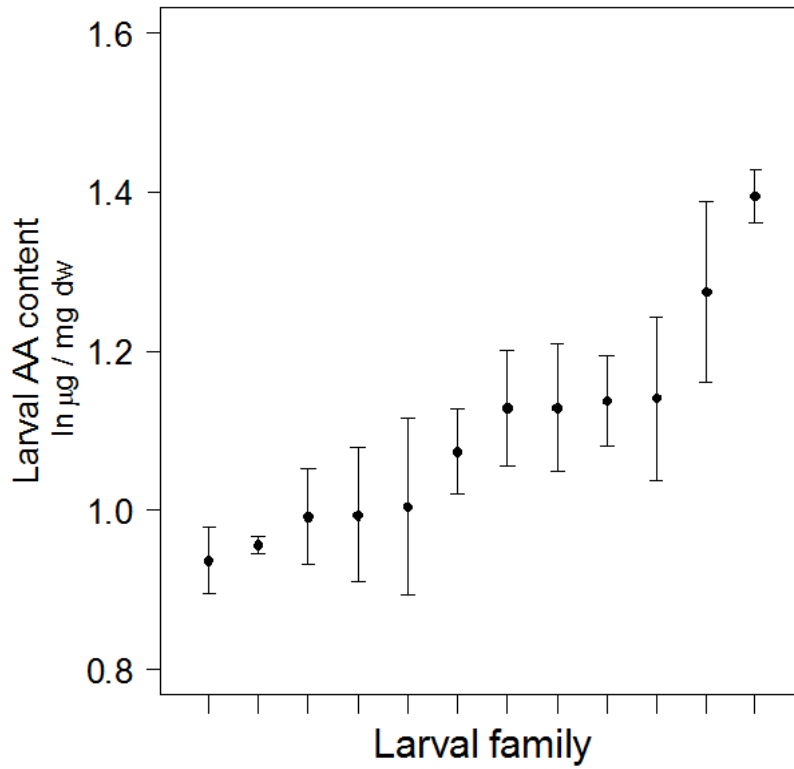


Figure II.3. Among-family variation in sequestered aristolochic acids (ln  $\mu\text{g}$  AA/ mg dry weight  $\pm$ SE) after controlling for among-plant variation in 12 families (full siblings) of *Battus philenor*.

**CHAPTER III.**

**LARGER CLUTCHES OF CHEMICALLY DEFENDED  
BUTTERFLIES REDUCE EGG MORTALITY: EVIDENCE FROM  
*BATTUS PHILENOR***

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The following section is a slightly modified version of a manuscript published in the journal *Ecological Entomology*:

Dimarco, R. D., and J. A. Fordyce. 2013. Larger clutches of chemically defended butterflies reduce egg mortality: evidence from *Battus philenor*. *Ecological Entomology* **38**:535-538.

The use of “we” in this chapter refers to my co-author and me. As the lead author of this article, I was responsible for this paper. My primary contributions to this paper included the formulation of ideas, data collection, chemical and data analyses, and writing.

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## Abstract

Many toxic butterflies lay eggs in clusters and their eggs are aposematically colored to warn predators. The pipevine swallowtail, *Battus philenor* (L.) (Papilionidae), is a specialist herbivore on plants in the genus *Aristolochia*, from which it sequesters toxic alkaloids (aristolochic acids, AAs). Eggs of this group of butterflies are laid singly or in clusters of different sizes, are aposematic and can possess AAs. We conducted a field study during three consecutive summers in Tennessee where we manipulated the exposure of *B. philenor* eggs of different clutch sizes to predators to assess the defensive role of egg clustering. We found that larger egg clutches suffer less predation compared to small clutches, and we failed to detect a relationship between clutch size and AA content in the eggs. Crawling predators seem to play the most important role in egg mortality.

This study suggests that, for toxic eggs, there is a clear benefit in laying eggs in large clusters in areas with high levels of predator threat.

## Introduction

Oviposition strategies of butterflies and other insects vary broadly. Generally, studies examining oviposition behavior focus on the location where clutches are laid by females and on variation in egg clutch sizes (Clark and Faeth 1998, Desouhant et al. 2000, Faraji et al. 2002). After encountering a host plant, a female must choose when, where, and how many eggs to oviposit. Female butterflies oviposit eggs either singly or in clutches with many eggs, and clutch size can vary within and among species (Stamp 1980). Variation in clutch size is thought to be an adaptive mechanism to reduce larval mortality, thereby increasing females' realized fecundity (Courtney 1984, Fordyce and Nice 2004). Aggregative feeding of larvae often occurs as a consequence of egg clustering. A number of hypotheses have been proposed to explain the evolution of egg clustering and the adaptive value of aggregative feeding (Stamp 1980, Fordyce 2005).

Many species of butterflies that lay eggs in clusters are unpalatable and aposematically colored (Stamp 1980, Sillén-Tullberg and Leimar 1988, Hunter 1991). It has been proposed that in chemically defended insects, grouping has the potential to increase the individual per capita survivorship of the prey, even though a group might be more apparent to predators (Clark and Faeth 1998, Hunter 2000). One explanation of this behavior might be enhanced aposematism, because a group of prey that are aposematically colored display a

more conspicuous signal to predators compared to a single aposematic prey, thereby enhancing learning in predators (Sillén-Tullberg and Leimar 1988, Gagliardo and Guilford 1993).

The present study aims to further our understanding on the potential role clutch size might play for egg survival. By conducting field observations and experiments during three consecutive years we ask: 1) Do large egg clutches reduce the risk of predation in this toxic specialist herbivore? 2) Broadly, what are the important invertebrate predators (crawling or flying) of *B. philenor* eggs?

## **Material and methods**

### **Species description**

The pipevine swallowtail, *Battus philenor* L., is a butterfly in the tribe Troidini (Papilionidae) that specializes on plants in the genus *Aristolochia* (Aristolochiaceae) (Racheli and Oliverio 1993). *Aristolochia* contain toxic alkaloids called aristolochic acids (AAs) that serve as defences against most herbivores (Chen and Zhu 1987). *B. philenor* sequester these chemicals as larvae, rendering both larvae and adults chemically defended against many invertebrate and vertebrate predators (Fordyce 2000, Sime et al. 2000). Eggs of *B. philenor* are bright orange, possess AAs on their surface, and are often laid in clusters (Sime et al. 2000). Although eggs are chemically defended, the highest

mortality occurs during the egg and first instar larval stages, due in part to predation (Tatar 1991, Fordyce and Nice 2004). Clutch size can vary within and among populations (Fordyce 2003, Fordyce and Nice 2004). The average clutch size of *B. philenor* in Tennessee is 8.4 eggs per clutch ranging from 1 to 26 eggs per clutch (personal observations), whereas in Texas the average clutch size is 5 eggs/clutch with a range of 1 - 17 eggs/clutch (Fordyce and Nice 2004), and in California is 13 eggs/clutch ranging from 1 - 86 eggs/clutch (Fordyce 2003, 2005). Although *B. philenor* has been widely studied, there is a paucity of empirical studies on the relationship between clutch size of *B. philenor* and egg predation.

### **Study area**

Norris Dam State Park is a 1,634 hectare park, located in east Tennessee (36° 13'N, 84° 5'W). *Aristolochia macrophylla* is the common host plant of *B. philenor* in this area. Oviposition by *B. philenor* in Tennessee begins in May and continues until September.

### **Clutch size and egg predation experiment**

To assess if clutch size explains variation in mortality of *B. philenor* eggs, we conducted a field experiment over three consecutive summers (2009-2011) during which clutch size and accessibility to predators were manipulated. Accessibility to predators was manipulated through the application of Tanglefoot pest barrier (Tanglefoot Company, Grand Rapids, MI, USA) to the stem below

each clutch. The application of Tanglefoot allowed us to elucidate the foraging strategy of important egg predators in this system. Hereafter, we refer to crawling predators as those that are effectively excluded by the application of Tanglefoot, and flying/jumping predators as those that could circumvent the sticky barrier. From extensive observations in the area and in our experiments, we found that velvet mites, *Trombidium holosericeum* (Trombidiidae), are the most common generalist crawling predator of *B. philenor* eggs at our study site.

Egg clutches were obtained by confining individual, wild-caught *B. philenor* females in cages where portions of *A. macrophylla* stems were provided. Clutch sizes range from 1 to 26 eggs, the range observed for naturally laid clutch in the field at this location. Each stem with clutches of variable size was placed back in the field attached to a branch of an *A. macrophylla* plant. In total, we had 54 clutches ranging from 2 to 26 eggs, 20 singleton clutches without Tanglefoot, and 37 clutches and 16 singleton clutches with the addition of Tanglefoot. Clutches were assigned to the Tanglefoot and no Tanglefoot treatment haphazardly. Egg survival was estimated as the proportion of eggs that successfully hatched. The clutches were observed once a day until larvae hatched (on average 7 days after eggs were laid), and predation was recorded by looking at the characteristic predator damage on the eggs (i.e. a small hole in the egg chorion).



## **Chemical analysis of egg's aristolochic acid content**

To determine if AA content of *B. philenor* eggs varies depending on whether eggs were laid in large or small clutches, we analyzed AA content of individual eggs that were laid in the field in clutches of various sizes (n = 25 clutches). An individual egg from each clutch was dried under reduced pressure and weighted to the nearest 0.1 µg. AAs were extracted twice from each egg in 0.75 mL of 100% ethanol and sonicated once for 20 minutes at 50 °C. The resultant extract from each egg was passed through a 0.45 µm filter and dried under reduced pressure. The residue was resuspended in 0.04 ml of 100% methanol and placed into a total recovery autosampler vials for HPLC analysis. HPLC analyses were performed following Dimarco et al. (2012).

## **Statistical analysis**

Data on clutch size and egg survival were analyzed using logistic regression in R 2.14.2 (R development core team 2012). The model included the presence or absence of the Tanglefoot barrier and log clutch size as factors and egg survival as the dependent variable. We also ran a linear regression to see if there was a relation between clutch size and AA content.

## Results

### Clutch size and egg predation experiment

The full model detected an effect of predator exclusion treatment on egg survival ( $z = -4.607$ ,  $P < 0.001$ ); however, the interaction between predator exclusion and clutch size was significant ( $z = 2.473$ ,  $P = 0.013$ ); thus, we ran two separate models to examine how clutch size might affect egg survival. For clutches where crawling predators were excluded, there was no evidence that clutch size explained the probability of egg survival ( $z = -0.513$ ,  $P = 0.61$ ). However, when crawling predators had access to clutches, clutch size affected the probability of egg survival ( $z = 5.792$ ,  $P < 0.001$ ), with larger clutches having a higher probability of survival (Fig. III.1.). The percentage of eggs eaten in the treatment where eggs were protected against crawling predators was 4.6%, while the percentage of eggs eaten in the treatment when crawling predators were not excluded was 55.1%. Based on the parameter estimates of the model, we estimated that egg survival exceeds 50% when a clutch is larger than 6.9 eggs (SE = 0.16).

### Chemical analysis of eggs

We failed to detect a relationship between clutch size and total AA content per egg ( $F_{1,23} = 1.118$ ,  $P = 0.301$ ). We also failed to detect a relationship between

clutch size and AA concentration per egg ( $\mu\text{g}$  of AAs / mg of dry weight) ( $F_{1,23} = 0.206$ ,  $P = 0.654$ ).

## Discussion

We found that, for *B. philenor*, egg predation decreased as a function of clutch size (Fig. III.1.). Further, we found that crawling predators, those that were successfully excluded by the Tanglefoot barrier, are the important predators in this system. Our results are consistent with the hypothesis that larger clutches frequently observed in toxic butterflies plays a defensive role (Stamp 1980). Although enhanced aposematism is frequently championed as the function of aggregation for chemically defended prey (Stamp 1980, Sillén-Tullberg and Leimar 1988, Hunter 1991); the role vision plays for the important predators that were observed in our study is uncertain. For example, it is unclear what role enhanced aposematism might play against the velvet mite (*T. holosericeum*). One possible explanation for the lower incidence of predation on larger clutches might be satiation of the predator. However, this explanation is perhaps not likely given that our experimental clutches remained in the field for a long enough period of time (7 days on average) so there should have been ample opportunity for multiple predator encounters. Moreover, for many large clutches (with more than 20 eggs), only on average 20% of the eggs suffered predation, leaving most

of the siblings untouched. We found no evidence that egg toxicity varies with clutch size. This suggests that larger clutches of toxic eggs might suffer less predation not because of increased toxicity associated with larger clutches, rather because predators reject larger clusters of unpalatable eggs after sampling few members of the clutch (Alatalo and Mappes 1996, Hunter 2000). More research is needed to elucidate the importance of these different factors in explaining the behavior of egg predators.

The existence of variability in egg clutch sizes in different regions and species, and geographic variation in the benefit of being gregarious and toxic at the first instar larval stage among populations of *B. philenor* (Fordyce and Nice 2004) suggest that the benefit of being in aggregation might vary. These differences in oviposition strategies could be affected by ecological factors (e.g. climate, presence of other species of Troidini), and are likely maintained by variation in the types and abundance of local predators.

Our results suggest that predation rate is reduced as a function of increased clutch size, but this fact does not imply that maximizing clutch size is the optimal egg-laying strategy. It is possible that egg clutch size might also affect other factors that can have detrimental effects, such as competition among larvae for food resources. However, evidence that larger clutches benefit toxic eggs can also be found in the fact that non-toxic species of Lepidoptera often lay eggs solitarily and rely on crypsis for defence (Hunter 1991). In species with toxic eggs, it might be expected that under conditions of high levels of predator threat,

larger clutches might be adaptive. The variation observed in clutch size within and between *Troidini* species, might be a response to different levels of predation risk. This study suggests that in toxic organisms, in some circumstances, egg clustering is an effective strategy to reduce predation risk. In areas where egg predation is common, as in our study site, there appears to be a clear defensive benefit in laying eggs in large clusters.

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## Appendix III: Figures

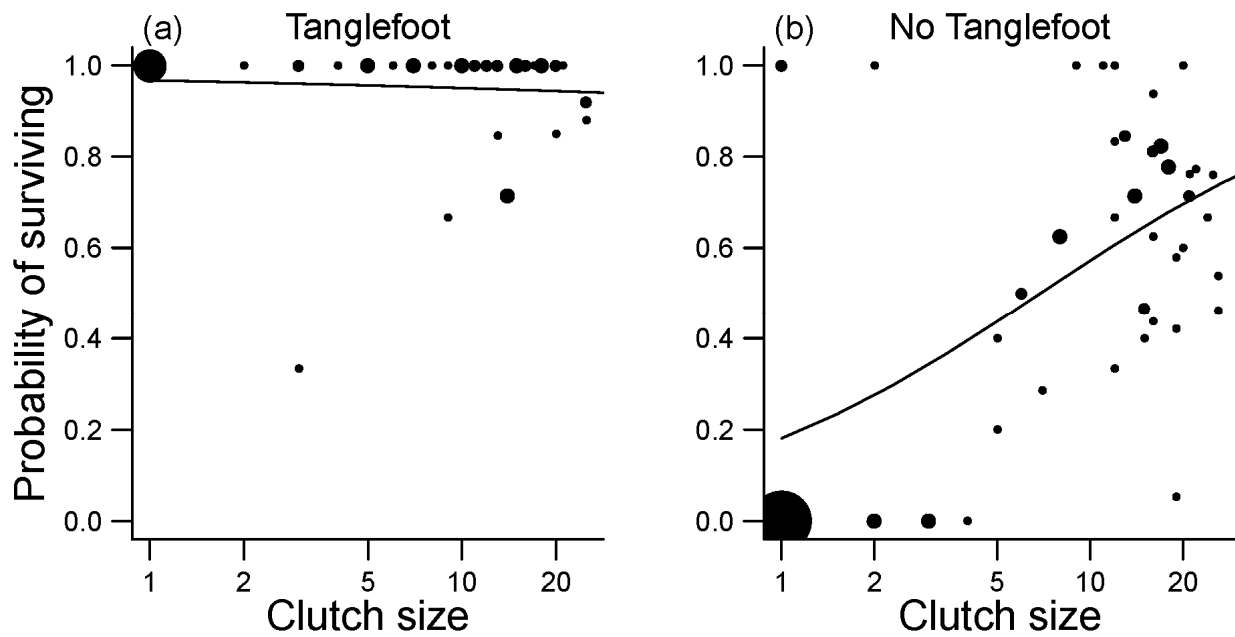


Figure III.1. Probability of survival for individual eggs of *B. philenor* in clutches of different sizes; (a) is clutches protected from crawling predators with the application of Tanglefoot, and (b) are clutches not protected from crawling predators. Dots are scaled to represent duplicate data points. Lines represent the fit of the logistic regression model.

**CHAPTER IV: PATTERNS OF CHEMICAL SEQUESTRATION,  
LARVAL PREFERENCE AND PERFORMANCE IN A SUBTROPICAL  
COMMUNITY OF TROIDINI SWALLOWTAILS AND THEIR  
ASSOCIATED *ARISTOLOCHIA* HOST PLANTS**

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The following section is a version of a manuscript to be submitted for publication.

The use of “we” in this chapter refers to my co-author, James A. Fordyce, and me. As the lead author of this article, I was responsible for this paper. My primary contributions to this paper included the formulation of ideas, data collection, chemical and data analyses, and writing.

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## Abstract

Swallowtail butterflies in the tribe Troidini (Papilionidae), a group that sequesters chemical defenses from its host plant, have been a model group for development of theory on host plant chemical sequestration. Troidini butterflies specialize on plants in the genus *Aristolochia* (Aristolochiaceae), which possess toxic alkaloids called aristolochic acids (AAs), and sequester AAs as larvae, rendering both larvae and adults chemically defended against most natural enemies. Although Troidini butterflies are predominantly tropical, with many co-occurring species concentrated in the lowland forests of Central and South America, most work on this group has focused on a single species in North America at the northern most range of the distribution of this group. The goal of this study is to examine the AA concentration of both swallowtail butterflies and their *Aristolochia* host plants in areas where greater diversity of Troidini butterflies is found. We also examine variation in host plant preference and performance among co-occurring butterfly species reared on different *Aristolochia* host plants. We conducted this study in Iguazú National Park, Argentina where five known Troidini butterfly species and two species of *Aristolochia* co-occur.

We found that one of the two host plant species used by Troidini possessed the alkaloids that these butterflies sequester as larvae. Individuals of *Aristolochia triangularis*, a very abundant species commonly used by Troidini

butterflies, did not possess detectable levels or had trace amounts of AAs. In the larval preference experiments, larvae showed preference for *A. triangularis* individuals over *Aristolochia macroura*. Further, contrary to the currently accepted paradigm, in our study system most Troidini individuals do not possess AA chemical defense, showing that toxicity itself can be polymorphic within a population. This suggests that not only are these butterflies involved in a Batesian mimicry complex, they might also be involved in automimicry, where butterflies without AAs are benefiting from the presence of toxic con-specifics. We also found that larvae chose to feed on tender leaves when given the choice; however, host plant AAs did not influence larval feeding choice. Experimental evidence showed that higher levels of AAs in the diet increased larval mortality, which might indicate a cost associated with sequestration of the chemical defense for protection against natural enemies, and might explain the observed polymorphism for sequestration.

By studying Troidini species in regions previously understudied (i.e., outside of North America), and in areas where they reach higher diversity, we are obtaining a more complete picture of the chemical ecology of this model group.

## Introduction

Plants employ a number of strategies to deter insect herbivores. These strategies can involve the investment in mechanical defenses (e.g., trichomes, increased leaf toughness and latex production) or the production of chemical defenses that could deter herbivores (Hulley 1988, Pérez-Harguindeguy et al. 2003, Clissold et al. 2009). Herbivores, however, also have evolved strategies for circumventing physical and mechanical plant defenses (Rathcke and Poole 1975, Dussourd and Eisner 1987, Hulley 1988) and overcoming plant chemical defenses either through tolerance to plant toxins (e.g., cytochromes p-450) (Schuler 1996, Li et al. 2000) or by possessing substantially reduced sensitivity towards the toxin (e.g. target-site insensitivity) (Holzinger et al. 1992, Agrawal et al. 2012). Additionally, some herbivores sequester these plant secondary compounds, not only giving them the ability to consume the plant, but also obtaining chemical defense against their natural enemies (Duffey 1980, Malcolm and Zalucki 1996, Fordyce 2000, Karban and Agrawal 2002, Nishida 2002).

Co-evolutionary interactions between plants and herbivores can be complex. Plants and herbivores can engage in an “evolutionary arms race”, where plant defenses (e.g. production of toxic secondary compounds) and herbivore counter defenses to overcome plant defenses continue to escalate (Thompson 1999, Karban and Agrawal 2002, Musser et al. 2002). Plants can also invest in other types of defenses that might augment the effect of the

chemical ones to deter insect herbivores. For example, they might invest in traits that provide mechanical resistance, such as a tougher leaf, to reduce damage by specialized insect herbivores that sequester the plant-synthesized toxic compounds (Dimarco et al. 2012). There are several groups of insects that are able to sequester plant secondary compounds by ingesting and storing them in the integument or body tissue. The sequestration of these toxic compounds provides the insect with a defense against their natural enemies (Duffey 1980, Opitz and Müller 2009). For herbivores, there might be a cost associated with the ability to sequester a plant's toxic compounds, which could produce a tradeoff between an increased ability to defend against predators but decreased overall performance defined as a measure of offspring survival, growth, or reproduction. (Camara 1997, Rieger et al. 2004, Fordyce and Nice 2008).

One of the best-studied groups of butterflies that sequester chemical defenses from their host plants is swallowtail butterflies in the Troidini (Papilionidae) group (Nishida et al. 1993, Fordyce 2000, Klitzke and Brown 2000, Sime 2002, Papaj et al. 2007). Troidine swallowtails are predominantly tropical, with most species concentrated in the lowland forests of Central and South America and in the Indo-Australian region (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). There



are two Troidini genera in the New World, *Battus* and *Parides*, and the other genera in the Indo-Australian region. Members of this group are largely tropical or subtropical and are known to possess plant-derived aristolochic acids. These alkaloids are sequestered by larvae from their host plant, rendering both larvae and adults chemically defended against many predators, such as ladybird beetle larvae, spiders, and birds (Brower 1958, Rothschild et al. 1970, Brower 1984, Klitzke and Brown 2000, Fordyce et al. 2005).

The most intensively studied Troidini 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 USA where it is not sympatric with other Troidini species (e.g. Rausher and Feeny 1980, Rausher 1981, Allard and Papaj 1996, Fordyce 2000, Sime et al. 2000, Fordyce et al. 2010, Dimarco et al. 2012). Although the interaction between *B. philenor* and *Aristolochia* spp. is well documented in North America, there is a paucity of studies in tropical areas where the Troidini group is notably more diverse and where many species of this group co-occur (but see, Brown et al. 1980, Klitzke and Brown 2000, Mebs and Schneider 2002).

Here, we present one of the first comparative studies in a single location of Troidini-*Aristolochia* relationships in subtropical forests with multiple Troidini species. In this study, we examined the interaction between five co-occurring Troidini butterflies species and their associated *Aristolochia* host plants in Iguazú National Park, Argentina. We consider that by studying Troidini butterflies and

their *Aristolochia* host plants in an understudied rainforest in South America, we may be able to contribute to the understanding on this complex plant-insect interaction. Our goal was to address general questions about variation in host plant quality and chemical sequestration, and the ecological processes that might produce these patterns. We address the following: 1) Do local *Aristolochia* species differ in their leaf toughness and aristolochic acid concentration? 2) Do different patterns of chemical sequestration exist in co-occurring Troidini species? 3) Is there variation in larval preference among *Aristolochia* species? and if so, is larval preference affected by aristolochic acid content?: 4) Is larval performance affected by the aristolochic acid 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 Troidini species in the area.

### **Sampling of the local Troidini 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 Troidini community and their *Aristolochia* host plants. During daily trips by two persons, we used nets to collect butterflies and searched for *Aristolochia* plants. When we found *Aristolochia* plants we took leaf tissue samples and recorded their location. This sampling allowed us to obtain preliminary estimates about the relative abundance of butterfly and plant species and provided samples for chemical analyses. We had a total of 191 full days of sampling across all field seasons.

### **Analysis of aristolochic acid concentration and leaf toughness in local *Aristolochia* spp.**

We collected leaves from the two *Aristolochia* species, *Aristolochia triangularis* and *Aristolochia macroura* to measure leaf toughness and aristolochic acid concentration. Leaf toughness ( $\text{g/cm}^2$ ) was measured on 30 fresh leaves from each *Aristolochia* species with a force gauge penetrometer (type 516; Chatillon, Largo, FL, USA) by averaging three measurements per leaf. Measuring leaf toughness is important because it is a trait shown to offer mechanical resistant to phytophagous insects (especially first instar butterfly larvae) and is correlated with larval performance (Clissold et al. 2009, Dimarco et

al. 2012). The leaf penetrometer measures the force needed to puncture a leaf using a 3 mm diameter rod.

To assess variation in aristolochic acid concentration ( $\mu\text{g}$  of AAs / mg of dry weight) of *Aristolochia* plants we extracted AAs from a total of 114 individual leaf samples (46 from *A. macroura* and 68 from *A. triangularis*). Leaves from each host plant were dried under reduced pressure prior to extraction and weighed to the nearest milligram. AAs 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  $\mu\text{m}$  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  $\mu\text{l}$ , 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 x 75 mm). We identified AAs based on their retention times and unique absorption spectra and quantified AA concentration by comparing peak retention times and areas to a standard curve generated with chemical standards as described in Fordyce and Nice (2008). We tested for differences in leaf toughness and leaf AA concentration ( $\mu\text{g}$  of AAs / mg of dry weight) between the two *Aristolochia* species using t-tests (JMP v. 9.02) software (SAS Institute 2010).

## **Analysis of aristolochic acid concentration in the local Troidini**

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*). To extract the AAs, each adult butterfly was dried under reduced pressure prior to extraction and weighed to the nearest milligram. The AAs were extracted from the defatted butterfly tissue (see below defatting methods) 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 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 x 75 mm). Aristolochic acids were identified based on their retention times and unique absorption spectra. 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 chemical standards.

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

To assess if the two most common co-occurring Troidini species (*B. polydamas* and *P. anchises nephalion*) show variation in use of the two locally available *Aristolochia* species, and if aristolochic acid content influence larval preference, we conducted two larval feeding preference experiments. Assessing larvae preference is relevant because Troidini 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 regions (R. 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 from each *Aristolochia* species (*A. triangularis* and *A. macroura*). Neonate larvae fed for 48 hours (leaf material was replaced after 24 hours) 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 Image J software (Rasband 2003) and then assessing the area missing relative to the total leaf area. We tested for differences in preference between the two *Aristolochia* host plant species using a Wilcoxon signed-rank test JMP (v. 9.02, software, SAS Institute 2010). We tested for

differences in percentage of leaf area consumed between the two host plants species using also a Wilcoxon signed-rank test.

Because the two locally available *Aristolochia* species differed in AA concentration and leaf toughness (see result section), 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 aristolochic acids 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 µg / 100 ml Ethanol) obtained from Fisher Scientific Company. The addition of the AAs solution to *A. triangularis* leaves made it reach similar natural levels of AAs found 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 (see Fordyce 2001). 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). After 48 hours of feeding (replacing leaf material after 24 hours), we removed the uneaten leaf material. We recorded on which half of the leave each larvae fed and we quantified the leaf area consumed by each larvae as described in the previous experiment. We

tested for differences in preference between the two *A. triangularis* leaf halves (with and without the addition of AAs) using a Wilcoxon signed-rank test JMP (v. 9.02, software, SAS Institute 2010). We tested for differences in percentage of leaf area consumed between *A. triangularis* leaves halves, also using a Wilcoxon signed-rank test.

### **Larval performance and its relation to aristolochic acids presence in their diet**

To evaluate the effect of aristolochic acid content on larval performance we reared individual *B. polydamas* and *P. anchises nephalion* larvae under the following three treatments: 1) *A. triangularis* leaves with the addition of AAs; 2) *A. triangularis* leaves with their natural levels of AAs (i.e., no detectable or trace amounts of AAs); and 3) *A. macroura* leaves with their natural levels of AAs (i.e., typical levels of AAs found in an *Aristolochia* species). This was not a full factorial experiment since it was not possible to reduce the amount of AAs in *A. macroura*. To modify *A. triangularis* AA content, we sprayed leaves with a mixture of aristolochic acid 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 2 and 3). We assessed the variation in survivorship, adult fat content, and adult dry weight across all three feeding treatments. We collected 22 *P. anchises nephalion* and 8 *B. polydamas* females and let them oviposit eggs in



the laboratory. Eggs were separated from their clutch and pooled per butterfly species. Each individual egg was placed in a Petri dish. 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. To obtain the fat content from the adult butterflies, each individual was dried under reduced pressure prior to extraction and weighed to the nearest milligram. The fat was extracted from each individual butterfly twice by homogenizing in 5 ml of hexane, and sonicated 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.

We analyzed survivorship with a Chi-square test to assess differences among the different treatments (*A. triangularis* + AAs, *A. triangularis*, and *A. macroura*) and butterflies species (*P. anchises nephalion* and *B. polydamas*). Our response variable was the number of neonate larvae that reached the adult stage. Adult fat content, adult dry weight and AA content were analyzed with ANOVAs to assess the differences among the three different feeding treatments of the two species.

## Results

### **Analysis of aristolochic acid concentration and leaf toughness in local *Aristolochia* spp.**

The two studied *Aristolochia* plants differed in their AA concentrations ( $t = 17.47$ ,  $DF = 112$ ,  $P < 0.0001$ , Fig. IV.1.), with most individuals of *A. triangularis* having no detectable or trace amounts of AA, and *A. macroura* having AA levels similar to the ones reported for North American *Aristolochia* species.

*A. triangularis* leaves are notably more tender than *A. macroura* leaves. *A. triangularis* leaves have a mean toughness of  $1.51 \text{ gr/cm}^2$  versus  $39.78 \text{ gr/cm}^2$  of *A. macroura* leaves ( $t = 18.89$ ,  $DF = 82$ ,  $P < 0.0001$ ).

### **Analysis of aristolochic acid concentration in the local Troidini**

We found significant differences in AA concentration among the different butterfly species ( $F_{3, 311} = 14.4$ ,  $P < 0.0001$ ). We found large intra- and interspecific variation in AA concentration across butterflies. Most individuals of the abundant *Battus polydamas* ( $N = 136$ ) and the rare *B. polystictus* ( $N = 22$ ) had undetectable or very low AA levels. The abundant species *P. anchises nephalion* ( $N = 137$ ) had high average levels of AAs, but also had large intraspecific variation with AA concentration of individuals. The less abundant species *P. neophilus euribates* ( $N = 20$ ) had the highest mean AA levels and less intraspecific variation than other species (Fig. IV.2.). Sample size of each species

was variable (ranging from  $N = 20$  to  $N = 137$ ), which reflects the variable abundances of the different butterflies species. Results from a post hoc Tukey HSD test showed that *P. neophilus eurybates* have the highest levels of AAs and *B. polystictus* and *B. polydamas* having the lowest levels of AAs (see Fig. IV.2.).

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

We found a significant larval preference for the two locally available *Aristolochia* host plant species. Both *B. polydamas* and *P. anchises nephalion* larvae preferred to feed on *A. triangularis* ( $S = -604.5$ ,  $DF = 60$ ,  $P < 0.001$ ) and consumed a greater amount of *A. triangularis* leaves (*B. polydamas*:  $S = -207$ ,  $DF = 34$ ,  $P < 0.001$ ; *P. anchises nephalion*:  $S = -133.5$ ,  $DF = 26$ ,  $P < 0.001$ ) (Fig. IV.3.). This result is consistent with our field observations, where larvae of both species were more commonly seen feeding on *A. triangularis* than on *A. macroura*.

We found that *B. polydamas* and *P. anchises nephalion* larvae show no preference between *A. triangularis* leaf halves with and without the addition of AAs ( $S = -70$ ,  $DF = 38$ ,  $P < 0.2678$ ). Area removed by *B. polydamas* showed no significant differences between the two treatments ( $S = -32$ ,  $DF = 18$ ,  $P = 0.2101$ ). Area removed by *P. anchises nephalion* showed also no significant differences ( $S = -43$ ,  $DF = 20$ ,  $P = 0.1337$ ) (Fig. IV.4.).

## Larval performance and its relation to aristolochic acids presence in their diet

We found that larval survivorship of *B. polydamas* and *P. anchises nephalion* decreased by more than 50% when reared on *A. macroura* leaves or on *A. triangularis* leaves with the addition of AAs than when reared on *A. triangularis* leaves without the addition of AAs ( $\chi^2 = 10.95$ ,  $DF = 2$ ,  $P = 0.0042$ ). Survivorship was 2.2 times higher in individuals fed on *A. triangularis* leaves without the addition of AAs than in the other treatments. In these treatments we failed to detect a difference in larval survivorship when comparing *B. polydamas* and *P. anchises nephalion* ( $\chi^2 = 0.072$ ,  $DF = 1$ ,  $P = 0.788$ ). When comparing the two treatments with high levels of AAs (*A. macroura* vs. *A. triangularis* with the addition of AAs), we failed to detect a difference in larval survivorship ( $\chi^2 = 0.081$ ,  $DF = 1$ ,  $P = 0.776$ ). These results indicate that a cost associated with feeding on leaves with aristolochic acid might exist.

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*  $F_{2, 23} = 0.409$ ,  $P = 0.668$ ; *B. polydamas*  $F_{2, 7} = 0.417$ ,  $P = 0.67$ ). We also failed to detect an effect on adult dry weight with the addition of AAs (*P. anchises nephalion*  $F_{2, 23} = 0.09$ ,  $P = 0.913$ ; *B. polydamas*  $F_{2, 7} = 1.66$ ,  $P = 0.256$ ). When we looked at AA content, we found that *P. anchises nephalion* and *B. polydamas* larvae have the ability to sequester AAs when it is present in their diet. We found a treatment effect, and a post-hoc Tukey's test showed that when

larvae fed on *A. macroura* or on *A. triangularis* with the addition of AAs; the adult butterflies have similar and significantly higher AAs levels than in the treatment with *A. triangularis* with no addition of AAs (*P. anchises nephalion*  $F_{2, 22} = 25.8$ ,  $P < 0.0001$ ; *B. polydamas*  $F_{2, 6} = 107.9$ ,  $P < 0.0001$ ). In the treatment with *A. triangularis* with no addition of AAs, we found that larvae had no detectable or very low levels of AAs.

## Discussion

We found that one abundant plant and most individuals of the two most common swallowtail butterfly species do not possess detectable levels of aristolochic acids (AAs) or contain trace amounts. Larval preference does not appear to be affected by leaf AA content; instead, leaf toughness seems to be more important in deterring first instar *B. polydamas* and *P. anchises nephalion* larvae from feeding on their host plant. This agrees with Dimarco et al. (2012) who found that, for *B. philenor* leaf toughness was more important in determining the preference of early instar larvae compared to aristolochic acid content. Our analyses showed that larvae fed indiscriminately in the treatments with or without addition of AAs on *A. triangularis* leaves. This host plant species possesses tender leaves and largely lacks aristolochic acids. 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 aristolochic acid 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 may shed some light on why so many Troidine swallowtails in Iguazú did not possess AAs. The lack of AAs in wild caught adults might be also explained by females choosing to lay egg on *A. triangularis*. This could also be the case for other Troidine swallowtails from other regions that feed on *Aristolochia* species (e.g. *A. galeata*, *A. momandul*, *A. elegans*) (Klitze and Brown 2000, Mebs and Schneider 1996, Urzúa & Priestap 1985) that have trace amounts or non detectable amounts of AAs, so AAs are not available to be sequestered by the developing larvae.

This potential tradeoff between chemical defenses and survivorship may be one reason why automimicry might be happening in this system. Automimicry complexes develop when chemical defenses are costly (as is the case with AAs). Since there is no individual survival benefit in being toxic, some individuals of a population (the cheaters) produce little or nothing of the costly defense. These individuals can still get protection because other members of the same population that are similar in appearance (the non-cheating individuals) are defended and deter predators (Brower et al. 1970, Brower et al. 1975, Tuskes

and Brower 1978, Daly et al. 2012). In tropical and subtropical areas, where species richness is typically higher than in temperate areas, cheating might be more common given the high richness of phenotypically similar-butterflies but relatively low abundance of each species. High richness and low abundance could make distinguishing between truly toxic individuals and their mimics harder to learn for predators (which are also highly diverse).

Sequestration of aristolochic acids has been proposed to be costly through a reduction in adult fat content in Troidine. A previous study reported a negative relationship between *B. philenor* adult AA content and fat content, suggesting a cost associated with the sequestration of the aristolochic acids by *B. philenor* larvae (Fordyce and Nice 2008). However, we failed to detect an effect of the amount of AA in adult fat content and adult dry weight, suggesting that this cost of adult fat content may not exist in our study system.

The main limitation of this study is that it has been conducted in at a single site (Iguazú National Park). However, preliminary results from Costa Rica (Dimarco and Fordyce, unpublished data) suggest that lack of sequestered AAs in this group might be the rule, not the exception. In concordance with results from Iguazú, we found that some Costa Rican *Aristolochia* and Troidini 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 aristolochic acids in adult butterflies. Another study by Mebs and Schneider (1996) in South-East Asia, also found also high variability in

aristolochic acid 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 (1996) 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 this phenomenon.

In North America, *B. philenor*, the most abundant and best studied Troidini species, possess AAs as does their locally available *Aristolochia* host plant (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 temperate areas can differ from results obtained in warmer and more diverse environments. 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 and warmer climates, 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 Troidini 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.



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## Appendix IV: Figures



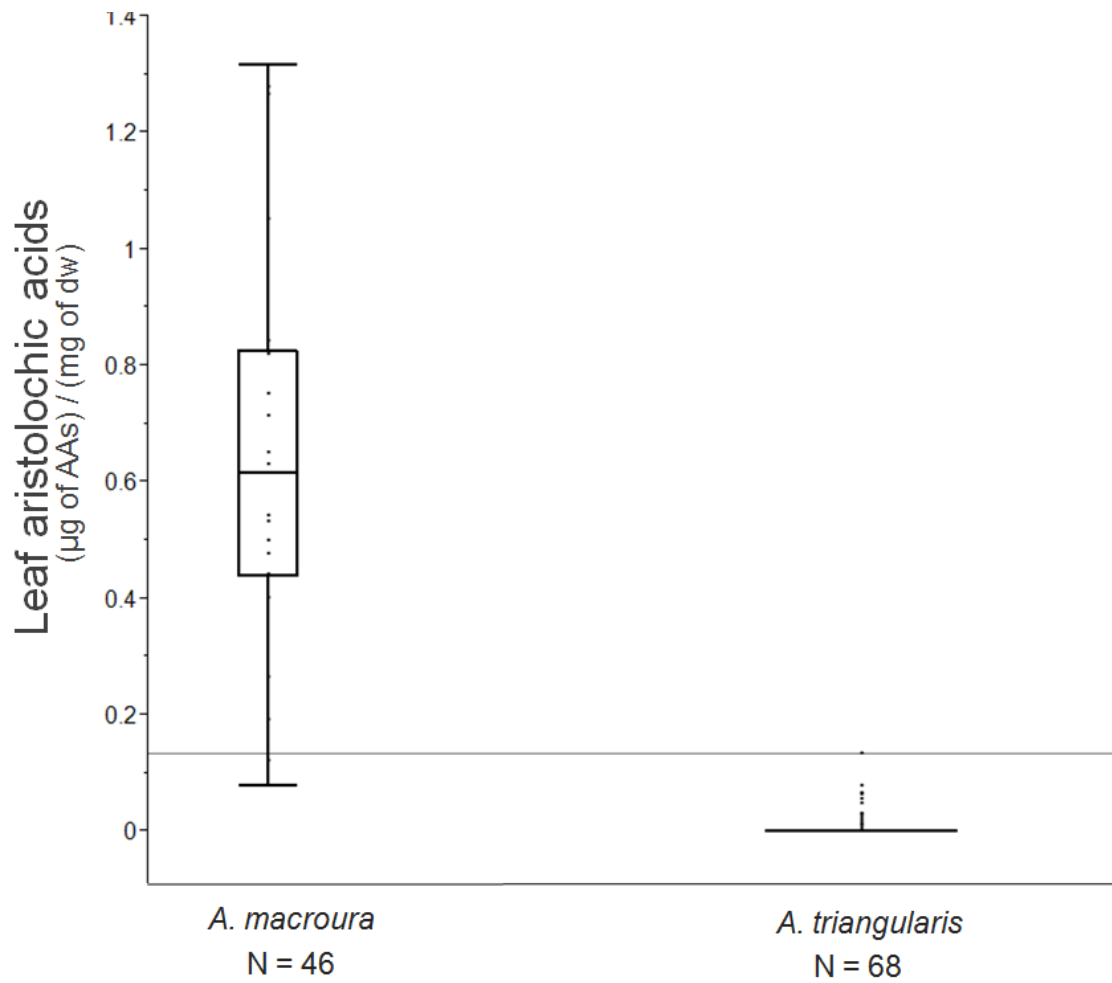


Figure IV.1. Aristolochic acid concentration ( $\mu\text{g}$  of AAs / mg of dry weight) in leaves of *A. macroura* and *A. triangularis* found in Iguazú National Park. We found significant differences in aristolochic acid content between the two plant species.

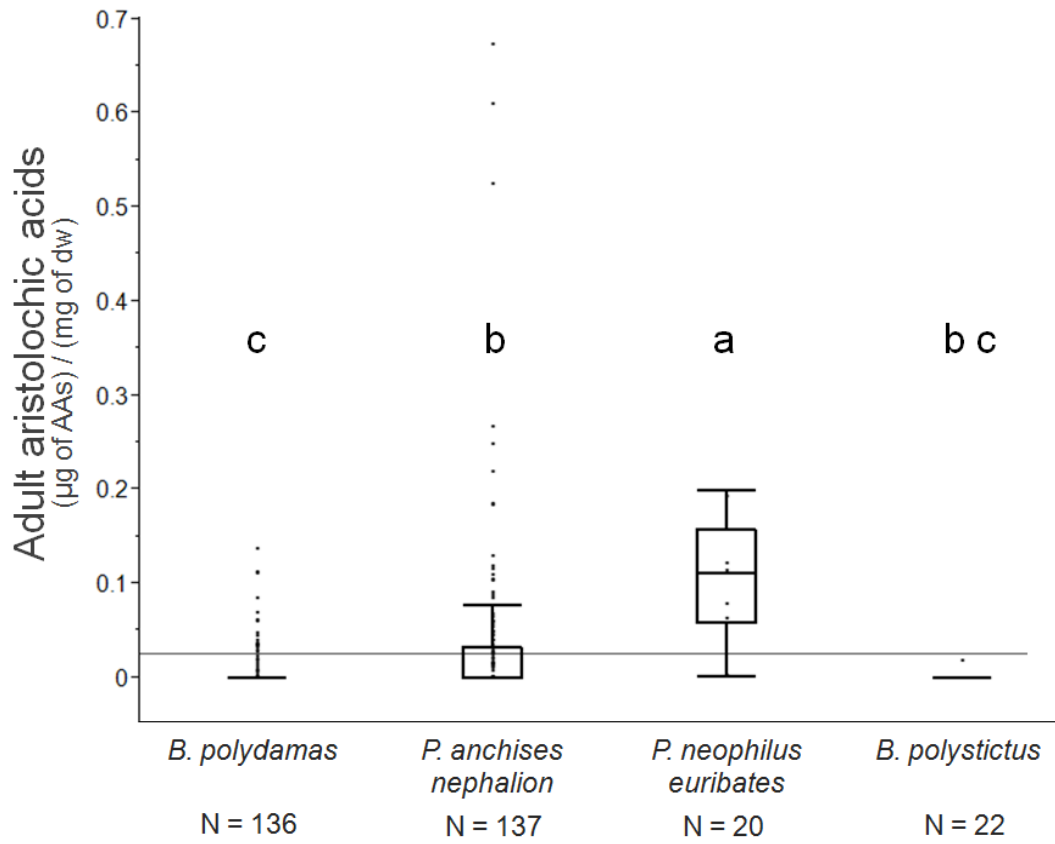


Figure IV.2. Aristolochic acid concentration ( $\mu\text{g}$  of AAs / mg of dry weight) in adults of four butterfly species found in Iguazú National Park, Argentina. Different letters show significant difference following a post-hoc Tukey's test.

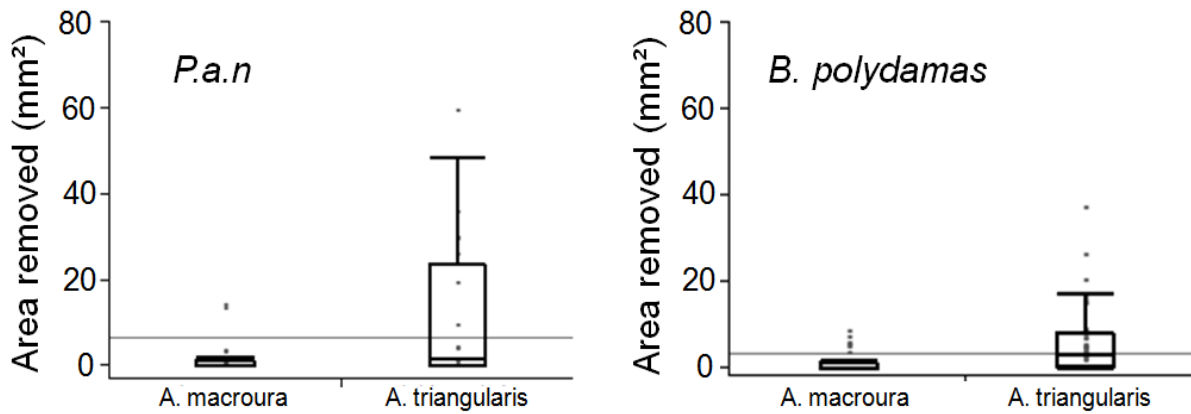


Figure IV.3. Results from a preference experiment between fresh leaves of *A. macroura* and *A. triangularis* by the two butterfly species, *Parides anchises nephalion* (*P.a.n*) and *Battus polydamas*. The box plots presented here show the area removed (in mm<sup>2</sup>) by larvae. Both butterfly species removed significantly more area of *A. triangularis* leaves than *A. macroura* leaves.

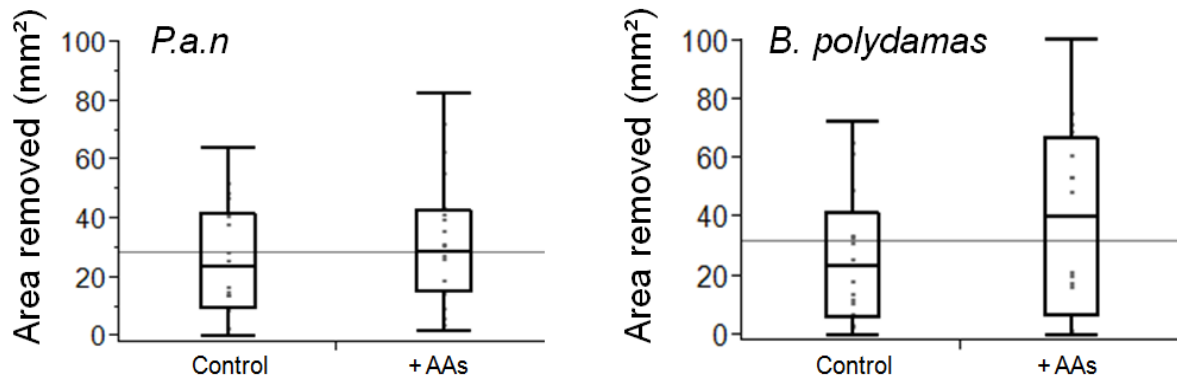


Figure IV.4. Results from a preference experiment by the two studied butterfly species, *Parides anchises nephalion* (*P.a.n*) and *Battus polydamas* between fresh leaves of *A. triangularis* that had aristolochic acids added to half of the leaf (+AAs) and no AAs addition to the other half (control). The box plots presented here show the area removed (in mm<sup>2</sup>) by larvae of each species. We failed to detect differences between the two treatments in both butterfly species.

**CHAPTER V.**  
**CONCLUSION**

## Conclusion

In this dissertation, I examine the ecological and evolutionary consequences of the interactions between swallowtail butterflies in the Troidini tribe (Papilionidae) and their host plants in the genus *Aristolochia* (Aristolochiaceae). Butterflies in the tribe Troidini specialize on plants of the genus *Aristolochia*. Plants in the family Aristolochiaceae are known to possess toxic alkaloids (nitrophenanthrene carboxylic acids) called aristolochic acids that serve as a defense against most insect herbivores (Chen and Zhu 1987, Racheli and Oliverio 1993). Troidini butterflies sequester these toxic alkaloids as larvae rendering both larvae and adult chemically defended against most predators (Rothschild et al. 1970, Sime et al. 2000, Fordyce 2001). Through a combination of observational, experimental, and laboratory studies with different Troidini-*Aristolochia* species combinations, I am able to further our understanding of how plant chemical and mechanical defenses affect evolutionary dynamics and ecological processes for butterflies including mechanisms of species co-occurrence and adaptations to herbivory and predation. I conducted my research in two biogeographical regions. My research in North America (Texas and Tennessee, USA) focused on the Troidini butterfly *B. philenor* and its *Aristolochia* host plants, for which there is ample background information. I also conducted research in South America (Iguazú, Argentina), which is a region with high

diversity and several co-occurring butterfly species of the Troidini tribe but a paucity of research.

In Chapter II, reports a study of the role of two *Aristolochia erecta* traits, leaf toughness and leaf chemistry, on the larval foraging decision and performance of the Troidini butterfly, *Battus philenor*. I also examined the importance of plant chemical variation in determining the chemical phenotype of larvae in a Troidini-*Aristolochia* system from Texas, USA. I found that *A. erecta* had substantial intra- and interplant variation in leaf toughness and in aristolochic acid content. The top (youngest) leaves were less tough and had higher concentrations of aristolochic acid than older leaves. Despite this substantial variation in plant aristolochic acid content among individual plants, *B. philenor* heritable variation for sequestration was a better predictor of larval aristolochic acid content indicating that an individual's genetic ability to sequester the toxic alkaloids has a greater influence than variation in plant chemistry. I also found that *B. philenor* first-instar larvae preferentially fed on less tough, younger leaves and avoided tougher, older leaves, but I found no evidence that aristolochic acid content influenced first instar larval foraging. Furthermore, larval performance (measured as larval dry weight) was better predicted by leaf toughness than aristolochic acids content.

My study of the defensive role that *B. philenor* clutch size plays on egg survival in Tennessee, USA is reported in Chapter III. It has been noted before that toxic species tend to lay eggs in clutches, and not in singletons as other

species and their eggs are aposematically colored to warn predators (Stamp 1980, Sillén-Tullberg and Leimar 1988). I showed that larger egg clutches suffer less predation than small clutches; however, I failed to detect a relationship between clutch size and aristolochic acid content in the eggs. Furthermore, crawling predators seem to play the most important role in *B. philenor* egg mortality. My research suggests that there is a clear benefit in laying eggs in large clusters in areas with high levels of predator threat.

In Chapter IV, I report my findings on Troidini-*Aristolochia* interactions in a subtropical forest in Misiones, Argentina, an area with no previous information on this system. I found that one of the most abundant *Aristolochia* host plant species, *A. triangularis*, did not possess detectable levels of aristolochic acids or only contained trace amount of the toxic alkaloids. Further, contrary to the currently accepted paradigm that all Troidini butterflies are toxic, and have numerous mimicry relationships; in our study system, most Troidini butterflies did not possess an aristolochic acid chemical defense, showing that toxicity can be polymorphic within a population. This suggests that not only are these butterflies being involved in a Batesian mimicry complex, they might also be involved in automimicry, such that butterflies without aristolochic acids are benefiting from the presence of toxic co-specifics (Brower et al. 1967, Brower et al. 1970). These were unexpected results since the *Aristolochia*-Troidini system is a textbook example of an interaction between a toxic plant and its sequestering herbivores. I also found that when given the opportunity to feed on a more tender host plant,



these butterflies choose to feed on tender leaves regardless of their aristolochic acid content. Experimental evidence showed that higher levels of aristolochic acid in the diet increased larval mortality, which could be a mechanism behind the apparent automimicry in these species. The observed increase in larval mortality might also indicate a tradeoff between fitness and sequestration of the chemical defense for protection against predators.

By studying the Troidini-*Aristolochia* interaction in three different ecosystems I have discovered some generalities. In the studies reported in Chapters II and IV, I found that, for highly specialized herbivores that sequester chemical defenses from their host plants, mechanical resistance, such as leaf toughness, seems to be a more important determinant of herbivore foraging patterns than chemical defenses. These results are from Texas (USA) and Iguazú (Argentina), which are very different localities in several aspects (e.g., different biogeographical regions with different climates and the presence or not of other co-occurring species of Troidini). My findings, that feeding preferences is determined more by plant mechanical resistance, might be the rule rather than the exception for this highly specialized group of butterflies.

My work in an area where the Troidini tribe has higher diversity helps form a more complete picture of the chemical ecology of the Troidini-*Aristolochia* complex. It seems evident that future work should explore different aspects of the interaction between Troidini and *Aristolochia* in understudied geographical areas as this may allow us to make new discoveries and develop a better

understanding of plant-insect interactions (Lindenmayer et al. 2010, Martin et al. 2012).

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## VITA

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