WARNING AND DECEPTION: CHEMICAL, BEHAVIORAL, AND PHYLOGENETIC STUDIES OF APOSEMATIC COLORATION AND MIMICRY

by

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DEDICATION

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TABLE OF CONTENTS

TABLE OF CONTENTS – *Continued*

TABLE OF CONTENTS – *Continued*

TABLE OF CONTENTS – *Continued*

TABLE OF CONTENTS – *Continued*

ABSTRACT

The study of aposematic coloration and mimicry has a long and distinguished history, and has stimulated scientific inquiry in areas as diverse as chemistry, evolution, ecology, and behavior. Yet, many questions regarding signal function and ecological dynamics remain unknown. This dissertation attempts to address some of these questions about how a visual warning signal functions and how the environment changes its efficacy. Using experimental methods, I evaluated the role of luminance contrast in aposematic signaling using milkweed bugs as model prey and Chinese mantids as model predators. The results of this laboratory experiment illustrated that luminance contrast with background can function as an aposematic signal between prey and predator. Predators learned to avoid unpalatable prey sooner and remembered to avoid unpalatable prey for longer when the prey had higher luminance contrast with the background. These results help define what makes a visual signal conspicuous and designate the importance of high luminance contrast in the efficacy of a warning color signal. Another important characteristic of warning coloration is a reason for the advertisement. This is either a physical or chemical defense that confers some protection against predation. In phytophagous insects this is often a chemical defense that is acquired from the insect's host plant. By developing new analytical chemistry methods, I was able to identify and quantify the toxic compounds in both the host plant and the viceroy butterfly, a putative aposematic insect. These results provide a chemical mechanism for previous research that demonstrated that the viceroy was unpalatable to avian predators. Next, I was able to test the role of geographic variation in host plant and viceroy chemical defense and how

that variation compared with the local abundance of a mimicry co-model of the viceroy, the queen butterfly. The results indicated that although host chemical defense did not vary between geographic locations, the viceroy chemical defense and palatability did vary spatially. The viceroy was more chemically defended and more unpalatable in locations where the queen was absent or at low abundance. This result suggests that mimicry and aposematic coloration evolve in a geographic mosaic of co-evolution. Finally, I used molecular phylogenetic approaches to reconstruct and test the evolution of mimicry in the North American admiral butterflies (*Limenitis*: Nymphalidae). One species, *L. arthemis*, evolved the black, pipevine swallowtail mimetic form but later reverted to the white-banded ancestral form. This character reversion is strongly correlated with the geographic absence of the model species and its host plant, not the mimics host plant distribution. These results support the idea that loss of model in a geographic area is not an evolutionary stopping point for a Batesian mimic.

INTRODUCTION

"Oh what a tangled web we weave when we practice to deceive"—Sir Walter Scott

An explanation of the problem and a review of the literature

Signals must be detectable against background noise in order to be effective. Under certain circumstances, natural selection favors visual signals that contrast markedly with their background. Classic examples in the natural world are aposematic coloration and visual defensive mimicry. Aposematic, or warning, coloration is used by noxious organisms to signal their unprofitability to potential predators (Cott 1940; Guilford 1990). Such coloration is typically highly conspicuous. Not only is a conspicuously colored pattern by definition easier for the predator to detect against its background, but it has also been shown to be easier to learn and less likely to be forgotten (Gittleman and Harvey 1980; Roper 1990; Alatalo and Mappes 1996; Roper and Redstone 1987). These multiple benefits to both predator and prey are believed to have facilitated the evolution of aposematic coloration from ancestrally cryptic patterns (Fisher 1930; Sherratt and Beatty 2003).

Visual defensive mimicry is closely related to aposematic coloration, and it is defined as close physical resemblance between unrelated species (Bates 1862; Charlesworth and Charlesworth 1975; Ruxton *et al*. 2004). Mimicry is generally classified as either Batesian or Müllerian. In Batesian mimicry, a rewarding(i.e., palatable) species evolves a physical resemblance to the warning phenotype of a non-

rewarding (i.e., unpalatable) species. This relationship is considered parasitic because the palatable species undermines the effectiveness of the warning signal. In contrast, Müllerian mimicry involves two unpalatable species with both participants sharing a common physical appearance. In a Müllerian system, both species benefit by distributing the mortality costs of predator education, and thus the relationship is generally considered mutualistic (Mallet and Joron 2000). Therefore, correctly classifying the mimicry system has important ramifications regarding the ecology and evolution of the involved participants.

Less well understood are the specific features explaining how warning coloration functions and how the environment changes the efficacy of the aposematic coloration or mimicry (Mappes *et al.* 2005). For example, current theoretical and empirical studies do not explicitly consider what aspects of the signal makes aposematic coloration effective in terms of prey detection, discrimination, and learning. Drawing from our current understanding of animal visual systems and the definition of aposematic coloration itself, aposematic color patterns could differ from cryptic ones in any of a suite of characteristics, including chromatic contrast and luminance contrast. Both are probably important in considering the evolution and maintenance of aposematic signals, but neither has been evaluated separately for conferring the functional benefits of aposematic coloration.

In the study of mimicry, there is a major gap in the theoretical and empirical literature regarding the role that geographic variation plays in the evolution and function of mimicry systems (Thompson 2005). For example, the relative abundances of the

mimetic participants may change geographically especially if the participants have different ecological requirements such as different host plant species. It is assumed that the mimic will go extinct if it occurs in areas without its model, but the mimic could have other responses. It could revert back to its cryptic, ancestral form; it could mimic another model; or it could decrease its own palatability and become aposematically colored. Also it is well documented that noxious prey especially insects vary tremendously in their chemical defenses both spatially and temporally. This variation within a species could have unpredicted effects on the mimetic relationships between species and warrants further investigation.

An explanation of the dissertation format

The research included in this dissertation investigates the functions and patterns of aposematic coloration and mimicry in insects from the level of an individual interaction between prey and predator to the macroevolutionary level. I integrated behavioral, chemical, and phylogenetic approaches to identify the signal components critical in warning coloration and mimicry, how these signals change based on the environmental conditions, and how environmental variation influences evolutionary processes. Four manuscripts are included as appendices.

Appendix A, "Aposematic coloration, luminance contrast, and the benefits of conspicuousness," evaluates the role of high luminance contrast as a visual warning signal between prey and predator. Appendix B, "Isolation, identification, and quantification of potential defensive compounds in the viceroy butterfly and its larval host plant, the Carolina willow," describes a new analytical method and identifies the

chemical defenses of an unpalatable butterfly and its larval host plant. Appendix C, "A mimic without its model: geographic variation in the relative abundance and palatability of the viceroy," assesses the spatial variation of chemical defense, palatability and mimicry roles in a classic butterfly mimicry system. Appendix D, "Once a Batesian mimic, not always a Batesian mimic: mimic reverts back to ancestral phenotype when the model is absent," examines how a Batesian mimic responds in evolutionary time to the absence of its model.

PRESENT STUDY

The methods, results, and conclusions of this study are presented in the manuscripts appended to this dissertation. The following is a summary of the central findings in this document.

Appendix A evaluates if brightness, or luminance, contrast alone can function as an aposematic signal between prey and predator. Many organisms use warning, or aposematic, coloration to signal their unprofitability to potential predators. It is well documented that these conspicuous, unprofitable prey are detected sooner and aversion learned faster by the predator as compared with cryptic, unprofitable prey. Predators also retain memory of the aversion longer when prey is conspicuous. Previous investigations on aposematic signal efficacy have focused mainly on the role of high chromatic contrast between prey and background, whereas little research has investigated the role of high luminance contrast. Our results suggest that the luminance contrast component of aposematic coloration can be an effective warning signal between the prey and predator. Thus, warning coloration can even evolve as an effective signal to color-blind predators.

Appendix B explains a new analytical chemical method for isolating, identifying, and quantifying defensive compounds in an unpalatable butterfly species, the viceroy (*Limenitis archippus floridensis*). We developed liquid chromatography–mass spectrometry–mass spectrometry methods to identify a set of phenolic glycosides shared between the adult viceroy butterfly and the Carolina willow, and solid phase microextraction and gas chromatography–mass spectrometry methods to identify volatile phenolic compounds released from stressed viceroy butterflies. In both approaches, all

structures were characterized based on their mass spectral fragmentation patterns and confirmed with authentic standards. Because these compounds have a generalized defensive function at the concentrations we described, our results are consistent with the Müllerian re-classification put forth by other researchers based on predator bioassay results. It seems that the viceroy butterfly possesses chemical defenses different from its monarch and queen butterfly counterparts (phenolic glycosides vs. cardiac glycosides, respectively), an unusual phenomenon in mimicry.

Appendix C compares geographic differences in palatability and chemical defense within a classic mimic, the viceroy butterfly (Nymphalidae: *Limenitis archippus*), in relation to the presence of its model, the queen butterfly (Nymphalidae: *Danaus gilippus*). The viceroy butterfly varies spatially in its frequency and co-occurrence with its model, the queen butterfly. In southern Florida, the viceroy co-occurs with its co-model, the queen, but in northern Florida, the viceroy occurs without its co-model. In populations where the viceroy occurs without the queen, the viceroy greater concentrations of defensive compounds than viceroy populations without a model even though the host plant chemistry is the same between locations. Also viceroy populations without a model are avoided sooner and remembered longer by an invertebrate predator indicating they are more unpalatable to predators. Thus, the mimicry functions differently depending on the relative abundance of the co-model resulting in a geographic mosaic of seleciton.

Appendix D explores the evolutionary response of a Batesian mimic once the mimicry relationship breaks down. The mimic species could go extinct due to increased

predation; it could evolve a new color pattern to mimic another model species; or it could re-evolve its ancestral, non-mimetic phenotype. We used molecular phylogenetic approaches to reconstruct and test the evolution of mimicry in the North American admiral butterflies (*Limenitis*: Nymphalidae). We confirmed that the non-mimetic, white-banded form is the ancestral phenotype within all admiral butterflies. However, one species, *L. arthemis*, evolved the black, pipevine swallowtail mimetic form, but later reverted to the white-banded ancestral form. This character reversion is strongly correlated with the geographic absence of the model species and its host plant, not the mimic's host plant distribution. Our results support the idea that loss of model in a geographic area is not an evolutionary ending point for a Batesian mimic.

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APPENDIX A

APOSEMATIC COLORATION, LUMINANCE CONTRAST, AND THE BENEFITS OF CONSPICUOUSNESS

Aposematic coloration, luminance contrast, and the benefits of conspicuousness

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Abstract

Many organisms use warning, or aposematic, coloration to signal their unprofitability to potential predators. Aposematically colored prey are highly visually conspicuous. There is considerable empirical support that conspicuousness promotes the effectiveness of the aposematic signal. From these experiments, it is well documented that conspicuous, unprofitable prey are detected sooner and aversion learned faster by the predator as compared to cryptic, unprofitable prey. Predators also retain memory of the aversion longer when prey is conspicuous. The present study focused on the elements of conspicuousness that confer these benefits of aposematic coloration. Drawing on current understanding of animal vision, we distinguish two features of warning coloration: high chromatic contrast and high brightness, or luminance, contrast. Previous investigations on aposematic signal efficacy have focused mainly on the role high chromatic contrast between prey and background, while little research has investigated the role of high luminance contrast. Using the Chinese mantid as a model predator and gray-painted milkweed bugs as model prey, we found that increased prey luminance contrast increased detection of prey, facilitated predator aversion learning, and increased predator memory retention of the aversive response. Our results suggest that the luminance contrast component of aposematic coloration can be an effective warning signal between prey and predator. Thus, warning coloration can even evolve as an effective signal to color blind predators.

Key words: insect vision, mantid, predation, *Tenodera aridifolia sinensis*, warning display

Introduction

Signals must be detectable against background noise in order to be effective. Under certain circumstances, natural selection favors visual signals that contrast markedly with their background. A classic example is aposematic coloration. Aposematic, or warning, coloration is used by noxious organisms to signal their unprofitability to potential predators (Cott, 1940; Guilford, 1990). Such coloration is typically highly conspicuous. Not only is a conspicuously colored pattern by definition easier for the predator to detect against its background, but it has also been shown to be easier to learn and less likely to be forgotten (Gittleman and Harvey, 1980; Roper, 1990; Alatalo and Mappes, 1996; Roper and Redstone, 1987). These multiple benefits to both predator and prey are believed to have facilitated the evolution of aposematic coloration from ancestrally cryptic patterns (Fisher, 1930; Sherratt and Beatty, 2003).

Less well understood are the specific features of the aposematic color signal that promote predator detection, learning and memory retention. Aposematic color patterns could differ from cryptic ones in any of a suite of characteristics, including chromatic contrast and luminance contrast. Chromatic contrast, sometimes called color contrast, refers to differences in hue or chroma between an object and its background (Wyszecki and Stiles, 1982; Adelson, 2000). Luminance contrast refers to differences in amount of light reflected from an object and the background on which it occurs (Wyszecki and Stiles, 1982; Adelson, 2000). The perceptual equivalent of luminance contrast is brightness contrast (Adelson, 2000). Both types of contrast have been shown to be

important in visual communication such as mating displays and seed dispersal by frugivores (e.g. Uy and Endler, 2004; Schmidt *et al.*, 2004, respectively).

Both contrast types also seem important for aposematic coloration signals. For instance, the definition of aposematic coloration often includes references to both chromatic and luminance contrast (e.g. colorful and bright) as key characteristics (Ruxton *et al.*, 2004). Aposematic patterns typically show strong color contrast with background, as compared to cryptic patterns, and the predominant hues in aposematic patterns are red, yellow, or orange, which are distinct from the green or brown hues common in terrestrial environments (Joron, 2003). Aposematic patterns often appear to show significant brightness, or luminance contrast with their background, as compared to cryptic patterns such as the golden poison dart frog or the pipevine swallowtail (Ruxton *et al.*, 2004). Additionally, aposematic color patterns frequently show strong luminance contrast among elements of the pattern such as black and yellow striping of bees and wasps or the black and white patterning of skunks. Such patterning is thought to increase aversion learning of an aposematic signal relative to solid bright colors alone (Bowdish and Bultman, 1993). Thus, luminance contrast is conceivably a viable signal between aposematic prey and predator and may confer the functional benefits of aposematic coloration.

Color contrast is considered more critical to the effectiveness of aposematic coloration signals based on experiments with avian predators. However, these results may not extend generally to all predator species detecting aposematic signals. Predators vary widely in terms of their color discrimination capabilities, ranging from dichromatic

(most mammals and insects) (Goldsmith, 1990; Briscoe and Chittka, 2001, respectively) to tetrachromatic (butterflies and birds) (Briscoe and Chittka, 2001; Goldsmith, 1990, respectively) and even to dodecachromatic (mantis shrimp) (Marshall and Oberwinkle, 1999). Color blind predators can learn to avoid aposematic prey (Berenbaum and Miliczky, 1984; Bowdish and Bultman, 1993), and for these predators, luminance contrast may be very important in assessing warning coloration displays. Thus, prey luminance contrast might readily evolve as an aposematic signal between unpalatable prey and color blind predator.

In this study, we examined if luminance contrast alone can function as an aposematic signal from the standpoint of praying mantids, cosmopolitan predatory insects that co-occur with a variety of aposematically colored prey. Praying mantids are generalist ambush predators relying heavily on visual cues to locate and capture prey (Hurd, 1999; Prete, 1999), and they can learn to avoid aposematic prey (Berenbaum and Miliczky, 1984). However, existing physiological and molecular data suggest that praying mantids have very limited or no color vision (Rossel, 1979; Sontag, 1971; Towner and Gärtner, 1994). These observations raise the possibility that aposematic signals, and the associated benefits of rapid learning and stronger retention, might not be mediated by color contrast per se but by luminance contrast. Using the Chinese mantid as a model predator and the milkweed bug as model prey in controlled laboratory experiments, we sought to determine if luminance contrast alone could function as an aposematic signal and, if so, whether it conferred the predicted benefits of aposematism in terms of rapid learning and improved memory retention of the learned aversion.

Methods and Materials

Experimental arena

All experiments were conducted in a laboratory arena consisting of three components: a rectangular ramp, a square floor, and a cylindrical wall (Figure 1). The ramp began outside of the cylindrical wall, continued through a port in the wall, and continued upward inside the wall at a 30° angle. Both ramp and floor were constructed of wood and covered in poster board paper, while the cylindrical wall was constructed of just the paper. Ramp, floor, and wall were painted a dark uniform gray (see paint reflectance spectra in Figure 2A). The arena was illuminated by three full-spectrum halogen lamps (Solux-Eiko, 50W, 4700^{o}K, 36^{o} field of illumination) (see irradiance spectra in Figure 2B). Each lamp was positioned 23 cm above the highest point of the ramp and 20 cm from the other lamps.

Predators and Prey

Laboratory-reared adult Chinese praying mantids (Mantidae: *Tenodera aridifolia sinensis*) served as the experimental predator. Egg cases were purchased from Carolina Biological Supply Company and reared to adults on two separate occasions: February 2003-May 2003 and February 2004-May 2004. On both occasions, mantids were reared in individual cages on a successive diet of fruit flies, houseflies, and crickets. Mantids did not have access to milkweed bug prey before the experimental feeding trials, and therefore were not experienced with bug shaped prey or distasteful prey. Each mantid was fed two adult crickets every night throughout the period of experimentation.

Laboratory-reared milkweed bugs (Lygaeidae: *Oncopeltus fasciatus*) served as experimental prey. Milkweed bugs are known to sequester cardiac glycosides from milkweed seeds (Isman *et al*., 1977) and to be unpalatable to mantids (Berenbaum and Miliczky, 1984). Milkweed bugs reared on sunflower seeds do not sequester cardiac glycosides (Duffey and Scudder, 1972) and are palatable to mantids (Berenbaum and Miliczky, 1984). Thus, prey palatability was manipulated *in vivo* by rearing bugs for two generations on either sunflower seeds (*Helianthus annuus*: palatable) or milkweed seeds (*Asclepias curassavica*: unpalatable).

Manipulation of prey contrast

Prey contrast against background was manipulated by painting milkweed bugs in either of two shades of gray, and presenting bugs to mantids against a gray-painted background. Prior to being painted, each milkweed bug was chilled in the refrigerator until it was temporarily immobilized. All milkweed bug surfaces except the eyes were then painted with an artist's brush with three coats of the desired paint mixture. The paint was a mixture of black and white non-toxic tempra paint (Prang®). Painting did not affect the ability of the bug to move up the ramp, nor did it affect the prey palatability (Prudic *et al*., unpublished data).

In preparing shades of gray for low-contrast prey, high-contrast prey, and prey background, we used a USB2000 spectrometer with tungsten-halogen light source (Ocean Optics Inc.) to obtain reflectance spectra of sample paint chips. Readings were made with an optical fiber probe directed at the surface at an angle of 45°, and percent

reflectance measured relative to a white Spectralon standard (Labsphere Inc.). We endeavored to use paint mixtures that were achromatic, i.e., grays showing relatively flat reflectance, from 350 to 750 nm (Figure 2A). This range encompasses, at its low end, the minimum wavelength of illumination by experimental lamps (Figure 2B) and, at its high end, the maximum wavelength that insect visual systems are known to detect. In principle, if mantids cannot resolve color, as existing data suggest, use of achromatic paints with flat spectra is unnecessary. For a monochromatic animal, contrast can be assessed simply as the area under the reflectance curve over the range of wavelengths to which the animal is sensitive. However, in the event that mantids do perceive color, use of achromatic paints with flat spectra ensures that we were manipulating prey luminance contrast, but not color contrast. The grays used were reasonably flat in the range from 380-750 nm (Figure 2A). There was a modest degree color contrast in the portion of the spectrum from 350-380 nm. However, the light sources in our experiments provided relatively little illumination in that range (Figure 2B), so it is unlikely the mantids were able to perceive the chromatic differences between the paints even if they are able to discriminate colors.

We quantified luminance contrast between background and prey using a relative luminance contrast index (Endler and Théry, 1996; Uy and Endler, 2004):

$$(I*R_{prey} - I*R_{background}) / (I*R_{prey} + I*R_{background})$$ (integrated over 350-750 nm)

where $I*R_{prey}$ = irradiance x percent reflectance of the prey, and $I*R_{background}$ = irradiance x percent reflectance of the background. This index varies from 0 to 1 where higher values represent a brighter signal. Low-contrast painted milkweed bugs had a 0.19 contrast index while high-contrast painted bugs had a 0.57 contrast index.

Initiation of trials

In all experiments, a trial began by placing a single mantid at the top of the ramp inside the arena wall, such that the mantid's longitudinal axis was perpendicular to the long axis of the ramp. Each mantid was allowed to acclimate for five minutes before trials began. All mantids remained at the top of the ramp for all experiments and trials. A single, painted milkweed bug was placed at the ramp base outside the arena wall, out of view of the mantid. Milkweed bugs consistently walked directly up the ramp at a steady pace through the port in the arena wall and into the mantid's field of view. The milkweed bug invariably continued up the ramp whereupon the mantid's response to the bug was recorded.

In numerous respects, we tried to make our experiment as 'natural' as possible based on field observations of mantids and their prey. We used live prey and the prey was presented to the mantid in a manner similar to how prey and mantid encounter each other in the field. The mantid was perched above the prey and the prey moved toward the mantid from below the perch without intervention from a human observer (Prete, 1999). Our lighting regime was within the range of natural conditions. The lamps (Solus-Eiko 50W each, 150W total) emitted a continuous spectrum in the visible range which

resembled daylight (Figure 2B). This resulted in an overall irradiance that was moderate relative to the range of field conditions in which we have observed mantids hunting.

Conspicuousness assay

To determine if low-contrast milkweed bugs (0.19 contrast index) were less conspicuous to mantids than high-contrast bugs (0.57 contrast index), we measured latency to orientation and latency to attack for mantids in relation to prey contrast treatment. We compared both first trial treatment differences between mantids and multiple trial treatment differences within mantids. The bugs were reared on sunflower seeds and palatable. Mantids did not display any behavioral responses, such as refusal of prey or regurgitation, suggesting that either paints or sunflower-reared bugs were unpalatable. Each mantid (n=8) was tested with four bugs from each contrast treatment (total=8 milkweed bugs per mantid). The bugs were presented in a random order, and mantids had no prior experience with milkweed bugs. Two trials per mantid per day were conducted, with the successive trials separated by four hours. Mantids are voracious predators (Prete, 1999), and our satiation experiment indicated that the average mantid attacked and consumed 10 palatable, painted milkweed bugs in 30 minutes before refusing prey (Prudic *et al.*, unpublished data). Thus, we assumed mantids had similar hunger levels between the morning and afternoon trials.

Prey detection was inferred when the mantid turned its head from a position perpendicular to the ramp to a position parallel to it, orienting towards the bug and tracking its movement up the ramp. Such orientation movements are striking and

unambiguous in mantids. Latency to orientation was recorded as the time between the bug's appearance in the arena and the mantid's adoption of the orientation posture. Latency to attack was recorded as the time between orientation and the mantid's strike with its raptorial forelegs. We also measured the rate at which the prey moved up the ramp and the time spent feeding by the mantid. Data were log transformed for normalization and then checked for normalcy and homoscedasticity. Transformed data were analyzed using one-way or repeated measures ANOVA (JMP-IN software; SAS Institute, 2002). Means and standard error are reported for significant results ($p < 0.05$).

Learning assay

This experiment compared predator aversion learning rates for low-contrast (0.19 contrast index) versus high-contrast prey (0.57 contrast index). Milkweed bugs were reared on milkweed seeds and unpalatable. Mantids behaved in two ways suggesting milkweed bugs reared on milkweed seeds were noxious. First, all mantids regurgitated at least once after eating part of the milkweed-reared bugs; regurgitation was never observed in mantids eating bugs reared on sunflower seeds. Second, all mantids engaged in high rates of grooming behavior after eating milkweed-reared bugs as compared to sunflower-reared bugs (Prudic *et al*., unpublished data).

Naïve mantids (n=14, 7 per treatment) were assigned randomly either to low-contrast or high-contrast treatments. The experimental protocol was the same as described above except that a single mantid experienced only one prey contrast type. A trial ended either 5 minutes after a mantid attacked and ate the bug, or, if the mantid did

not attack the bug, 5 minutes after the bug entered the arena. After the trial ended, the bug or its remains were removed. If the mantid attacked the bug, it was returned to its holding cage after 2.5 minutes. If the mantid did not attack the bug, it was moved to a second arena after 2.5 minutes, which bore white surfaces instead of gray, and the mantid was allowed to acclimate for another 2.5 minutes. The mantid was then presented with a live, tethered cricket in order to evaluate its hunger status; if the mantid attacked the cricket, it was evaluated as hungry. The mantid was not allowed to feed on the cricket, the cricket being yanked away during the attack sequence. This protocol prevented the mantid from associating its response to the milkweed bug with a cricket reward. A mantid was considered to show an aversion to the bug when it oriented to a bug, failed to attack, but subsequently attacked a tethered cricket, in three consecutive trials. To evaluate if an increase in prey contrast was associated with an increase in aversion learning rate, the number of trials until mantids reached aversion criteria was compared between prey contrast treatments. Data were log transformed for normalization and then checked for normalcy and homoscedasticity. Transformed data were analyzed using one-way ANOVA (JMP-IN software; SAS Institute, 2002). Means and their standard error are reported for significant results ($p < 0.05$).

Retention assay

This experiment evaluated the number of days until the mantid re-attacked a milkweed bug after reaching aversion criteria. We used palatable, sunflower-reared bugs in this experiment for two reasons. First, we wanted to determine if the aversive response

required that bugs had fed on milkweed. We predicted that if this was true, the mantids would re-attack the palatable milkweed bugs on the first trial. Such a response would suggest that cues other than the luminance contrast cue were involved in mediating the aversion, for example an odor of the prey derived from feeding on milkweed. Second, and also important, sunflower-reared bugs were much more plentiful in culture than milkweed-reared bugs. Thus, we were guaranteed to have enough bugs of a singular toxicity to last through numerous retention trials. We have no reason to believe that any observed difference in memory retention between the two contrast treatments would depend on the change in the milkweed bug diet and corresponding palatability.

The same mantids (n=14) used in the learning experiment were tested two days after the day they met aversion criteria (see above) and retested every second day thereafter. One mantid in the low-contrast treatment died over the course of this experiment and was excluded from the analysis. A trial ended either when the mantid attacked and consumed a bug, or when the mantid oriented to a bug, failed to attack, but subsequently attacked a tethered cricket. A mantid was considered to have lost its aversive response when it attacked and consumed a bug. Data were log transformed for normalization and then checked for normalcy and homoscedasticity. Transformed data were analyzed using one-way ANOVA (JMP-IN software; SAS Institute, 2002). Means and their standard error are reported for significant results ($p < 0.05$).

Results

Conspicuousness assay

Mantids attacked all milkweed bugs regardless of prey contrast treatment. In their first encounter with milkweed bugs, mantids oriented sooner to palatable bugs of high-contrast (0.57 luminance contrast index) than to palatable bugs of low-contrast (0.19 luminance contrast index) (one-way ANOVA, 7.05+3.84 sec vs. 22.68+3.77 sec, F1,7 = 8.03, p = 0.019, Figure 3A). Contrast also affected latency to attack, mantids took less time to attack high-contrast bugs during their first encounter (one-way ANOVA, 3.75+1.88 sec vs. 16.77+6.50 sec, F1,7 = 7.46, p = 0.034, Figure 3A).

Data analyzed over multiple trials yielded similar patterns. Over multiple trials, mantids oriented sooner to palatable bugs of high-contrast relative to palatable bugs of low-contrast (repeated-measures ANOVA, 5.04$\pm$1.19 sec vs. 18.33$\pm$1.58 sec, $F_{1,7} = 9.18$, $p = 0.018$). Contrast also affected latency to attack, with mantids taking less time to attack high-contrast bugs during over multiple trials (repeated-measures ANOVA, 7.33$\pm$4.15 sec vs. 18.03$\pm$5.72 sec, $F_{1,7} = 4.13$, $p = 0.034$). For individual mantids, trial number did not affect attack rate (repeated-measures ANOVA, n = 8, $F_{1,7} = 0.80$, p = 0.56). Finally, paint treatment did not affect the movement rate of the milkweed bugs (one-way ANOVA, $F_{1,63} = 0.170$, $p = 0.682$) or the feeding rate by the mantids (repeated-measures ANOVA, n = 8, $F_{1,7} = 1.60$, $p = 0.213$).

Learning assay

Mantids learned to avoid high-contrast unpalatable milkweed bugs more rapidly than low-contrast unpalatable bugs (one-way ANOVA, 4.00+0.71 encounters vs.

6.86+0.71 encounters, $F_{1,13} = 9.14$, p = 0.011, Figure 3B). Two out of seven mantids in the high-contrast treatment demonstrated single trial aversion learning, whereas single trial learning was never recorded in the low-contrast treatment.

In our learning assays, two trials were conducted per day, which resulted in a short inter-prey interval within days, followed by a long inter-prey interval between days. Since inter-prey interval could conceivably affect mantid motivation and learning rate, we conducted a test of time between prey consumption (cricket or bug) on mantid attack rate. Time since last cricket or milkweed bug consumption did not affect mantid attack rate (one-way ANOVA, $F_{1, 13} = 1.93$, $p = 0.110$), so variation in the inter-trial interval did not lead to the observed treatment differences.

Retention assay

All mantids in both treatments (N=7 and 6) initially avoided the sunflower-reared, palatable bugs. This result suggests that mantids had not learned a cue acquired by bugs raised on milkweed; it seems most likely that they learned the luminance-contrast visual cue, although it is possible that other diet-independent cues such as odors were learned as well. All mantids eventually sampled the painted milkweed bugs. Collapsing the data across treatments, aversive responses lasted from 4 to 32 days. Mantids trained on high-contrast bugs retained their aversion almost twice as long as mantids trained on low-contrast bugs (one-way ANOVA, 16.60$\pm$2.03 days vs. 8.57$\pm$1.88 days, $F_{1, 12} = 6.67$, $p = 0.026$, Figure 3C).

Discussion

Aposematic coloration advertises prey unprofitablity to a diversity of predator species. Given the prominent role of hue, it is natural to presume that the benefits of aposematic coloration are due primarily to the distinctive hues typical of warning displays. However, many predator species such as mammals and insects are less sensitive to hue and chromatic contrast (Goldsmith, 1990; Briscoe and Chitka, 2001, respectively). Given the diverse visual capabilities among predators, natural selection may often favor aposematic coloration with generalized signal applicability such as high luminance contrast with background as well as high luminance contrast among components of the coloration pattern.

In order for luminance contrast to be important in the evolution of warning coloration, it should provide the same benefits that have been documented with chromatic contrast. A conspicuous pattern can be costly in the sense that naïve predators can readily detect and attack conspicuous prey. However, benefits of conspicuousness are presumed to offset this disadvantage when prey is unpalatable (Ruxton *et al*., 2004). Two functional benefits of conspicuousness in aposematic coloration have been identified: increased rate of predator aversion learning (Gittleman and Harvey, 1980; Roper and Redstone, 1987; Lindström *et al*., 1999) and improved predator memory retention (Roper and Redstone, 1987; Roper, 1994). In this study, we demonstrated for the first time that both benefits pertain to an invertebrate predator with limited or no color vision. When Chinese mantids were offered high-contrast prey, they detected the prey sooner. The mantids also learned to avoid high-contrast, noxious prey faster and retained

the aversive response longer than mantids trained to avoid low-contrast, noxious prey (Figure 3). Once the aversion was learned, avoidance did not require that bugs had been reared on milkweed, a result consistent with the idea that the mantids learned to avoid bugs on the basis of the luminance contrast cue alone. Our results suggest that warning coloration, specifically the luminance contrast component, could evolve as an effective signal even if a predator lacks sophisticated color vision.

The broader implications of our work depend on the nature of mantid vision. If mantids do not discriminate color, our results imply that the functional benefits of conspicuousness in aposematic displays do not require color vision. This inference might seem particularly surprising given the weight that we humans attach to color vision. If mantids do discriminate colors then our results imply that luminance contrast alone is sufficient to promote the faster learning and greater memory retention associated with aposematic coloration. This inference has a broad taxonomic context, because color vision is probably the norm amongst potential predators of aposematically colored prey. The two inferences are subtly different, but both are meaningful. They both suggest aposematic coloration and its benefits do not depend entirely on prey color contrast.

At present, there is no clear consensus on whether mantids have color vision. Color vision requires at least two photoreceptor types with different spectral sensitivities (Kelber *et al*., 2003). In general, this is achieved with the use of two or more opsins that differ in wavelength sensitivity. A molecular study found that mantids possess only a single opsin (Towner and Gärtner, 1994). Similarly, two electrophysiological studies using different techniques found evidence in mantids for a single visual pigment with

maximum sensitivity at human-green wavelengths (Sontag, 1971; Rossel, 1979). These findings suggest that mantids do not discriminate colors; however, a single-opsin pattern is extremely unusual in insects (Briscoe and Chittka, 2001). Even close relatives of mantids, such as cockroaches, have been shown to possess dichromatic vision (Briscoe and Chittka, 2001). Moreover, color vision does not in principle require more than one opsin; it can be achieved in conjunction with a filtering pigment which alters the photic environment of an opsin. The occurrence of such pigments has not been explored in sufficient detail to rule out the possibility of color vision in this group.

Luminance contrast may also enhance communication between prey and predators with color vision. Aposematic displays are generally multimodal (Ruxton *et al.*, 2004), meaning multiple signals are transmitted to the receiver in more than one sensory modality. The deployment of signals in multiple modalities such as olfaction and vision increase the efficacy in aposematic displays (Rowe, 1999). Within a modality, there are usually multiple components. Our results suggest that warning coloration is best regarded and investigated as a visual signal with multiple components. Chinese mantids use luminance contrast information both in learning to avoid unpalatable prey and retaining the aversive response. In a predator with color vision, the simultaneous use of color contrast and luminance contrast might increase the potency of aposematic coloration. Our results indicate that prey luminance contrast with background can confer the benefits of a warning visual signal under carefully controlled laboratory conditions. Future research in aposematic coloration can now address whether prey in nature differ in luminance and chromatic contrast, how the effectiveness of those two components

change with predator guilds and environmental conditions, and the extent of which any differences relate to prey palatability.

Our results, although novel in the realm of aposematic coloration, are consistent with other visual signal studies. Luminance contrast is an important visual signal in sexual signaling such as mating displays and mate preference in various bird species (e.g. Uy and Endler, 2004; Woodcock *et al.*, 2005, respectively). Luminance contrast is also consequential in food selection by foragers. Insect frugivores attend to luminance contrast in foraging decisions especially when fruit is red while avian frugivores attend to chromatic contrast (Schmidt *et al.*, 2004). Primates locate fruit using information from both luminance and chromatic contrast with background (Dominy and Lucas, 2001). Predatory reef fish attack prey with higher luminance contrast more frequently than prey with lower luminance contrast (Losey, 2003). Our results and other examples from the foraging literature attest to a general need to consider how color elements might be tuned to details of a predator-prey interaction, not only in terms of the sensory and cognitive profiles of the predator, but also in the contexts where the encounters occur. Future studies of aposematic coloration are now poised to focus on explicit considerations of a visual ecology perspective and the relative roles of chromatic and luminance in aposematic signal evolution.

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Tables, Figures, and Appendices

Figure text

Figure 1

A cutaway diagram of test arena, shown approximately to scale.

Figure 2

Percent reflectance spectra (A) of experimental grey paints. Background spectrum is indicated by dark-grey line (lowest reflectance), low luminance contrast prey spectrum by medium-grey line (medium reflectance) and high luminance contrast prey spectrum by light-grey line (highest reflectance). Irradiance spectrum (B) of the 50W SoLux-Eiko lamps used in experiments (spectrum supplied by Eiko Ltd.).

Figure 3

Detectability of low luminance contrast and high luminance contrast milkweed bugs (A), as measured by mantids' mean latency to orient in the first trial and over multiple trials. Learning performance by mantids exposed to low-contrast or high-contrast bugs (B), estimated as mean number of trials to reach aversion criteria. Retention of aversive response to low-contrast or high-contrast bugs (C), estimated as mean number of days until a bug is attacked again by mantid.

Figure 1

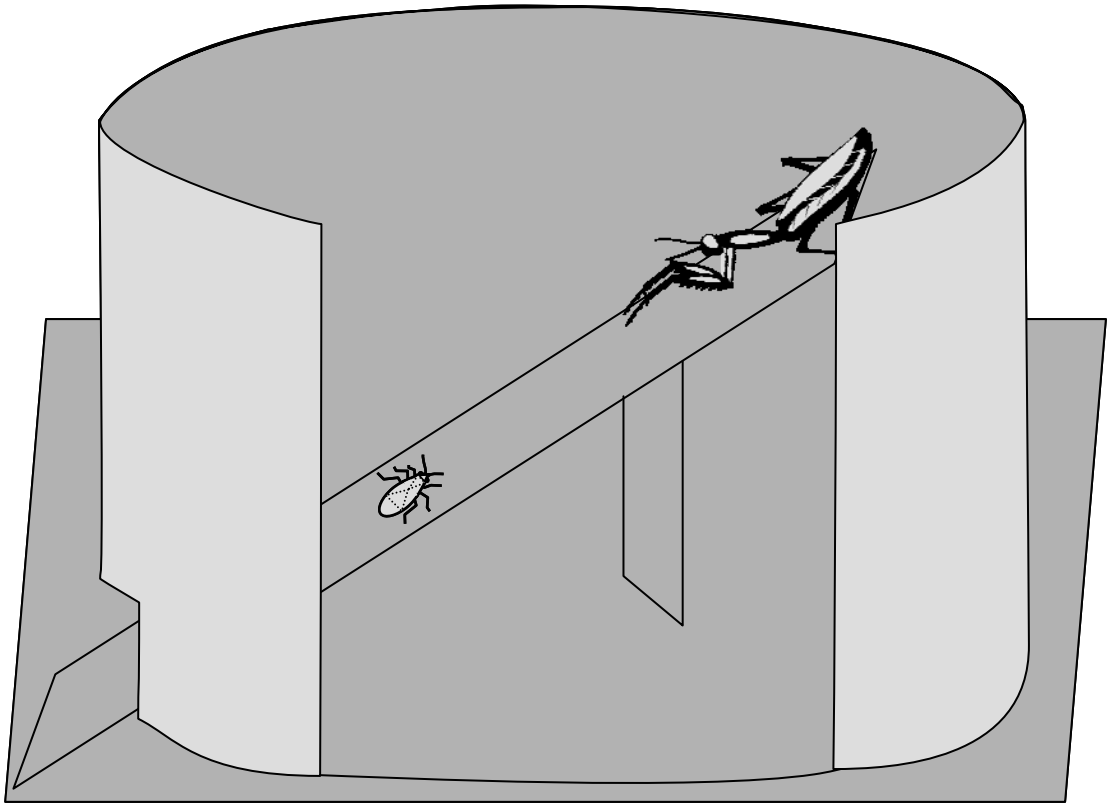


Figure 2

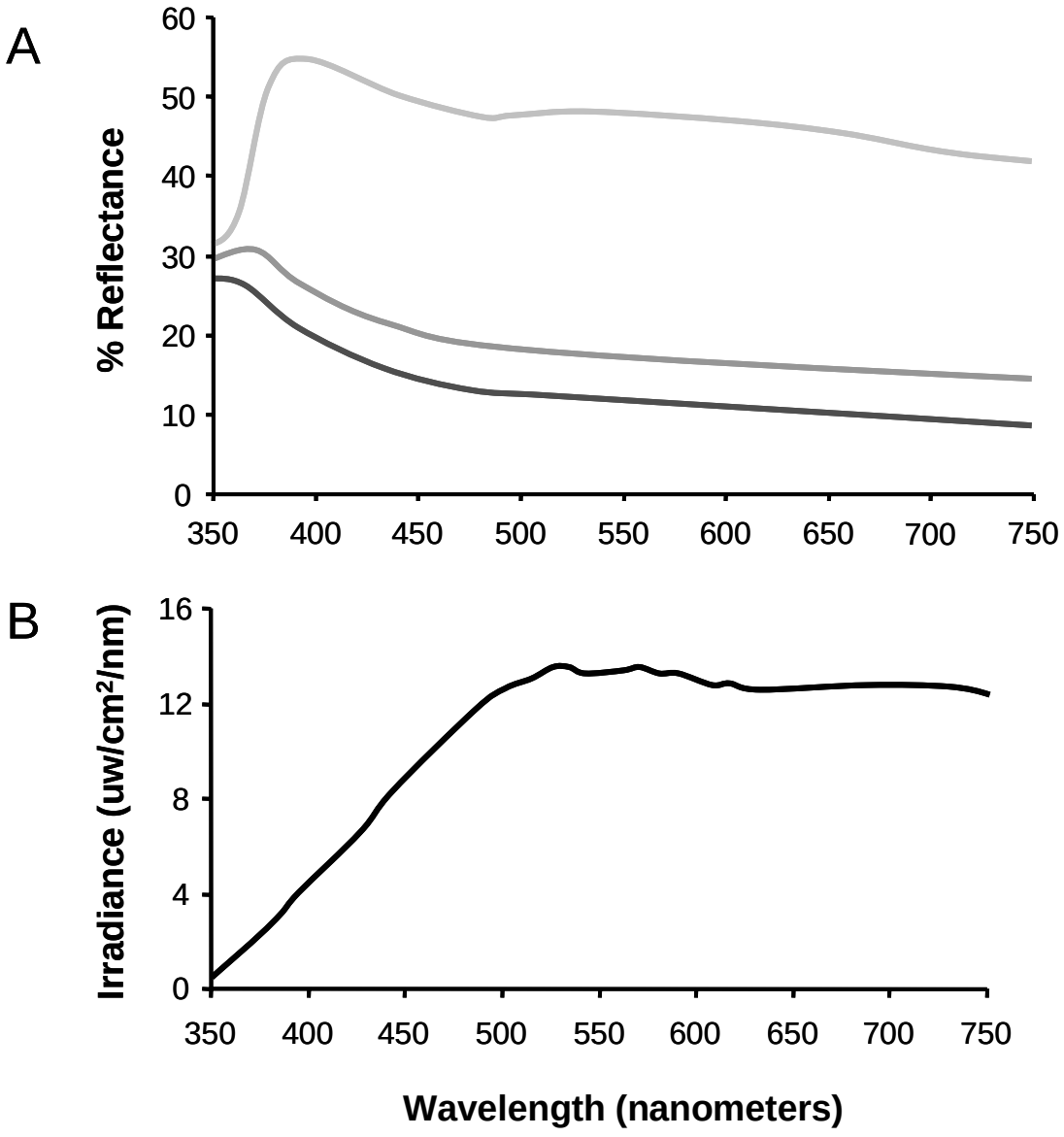
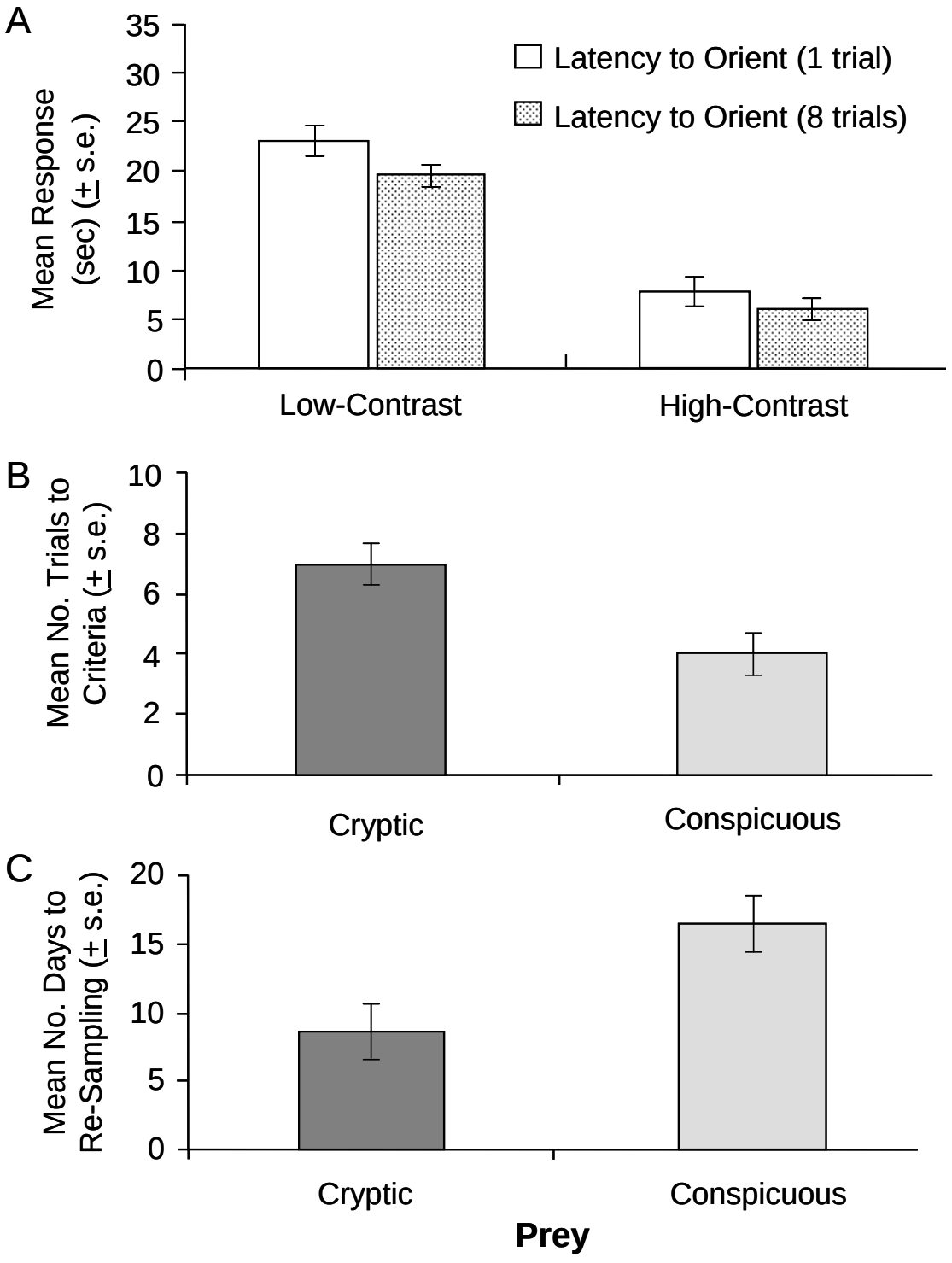

A
% Reflectance
60
50
40
30
20
10
0
350
400
450
500
550
600
650
700
750
B
Irradiance (uw/cm²/nm)
16
12
8
4
0
350
400
450
500
550
600
650
700
750
Wavelength (nanometers)

Figure 3


A
Mean Response (sec) (± s.e.)
35
30
25
20
15
10
5
0
Latency to Orient (1 trial)
Latency to Orient (8 trials)
Low-Contrast
High-Contrast
B
Mean No. Trials to Criteria (± s.e.)
10
8
6
4
2
0
Cryptic
Conspicuous
C
Mean No. Days to Re-Sampling (± s.e.)
20
15
10
5
0
Cryptic
Conspicuous
Prey

APPENDIX B

ISOLATION, IDENTIFICATION, AND QUANTIFICATION OF POTENTIAL DEFENSIVE COMPOUNDS IN THE VICEROY BUTTERFLY AND ITS LARVAL HOST-PLANT, CAROLINA WILLOW

ISOLATION, IDENTIFICATION, AND QUANTIFICATION OF POTENTIAL DEFENSIVE COMPOUNDS IN THE VICEROY BUTTERFLY AND ITS LARVAL HOST-PLANT, CAROLINA WILLOW

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Abstract

The viceroy-monarch and viceroy-queen butterfly associations are classic examples of mimicry presented in nearly all introductory biology textbooks. These relationships were originally classified as Batesian, or parasitic, but were later reclassified as Müllerian, or mutalistic, based on predator bioassays. The Müllerian re-classification implies that the viceroy is unpalatable because it too is chemically defended like the queen and the monarch. However, unlike the queen and the monarch, the viceroy defensive chemistry has remained uncharacterized. We demonstrate that the viceroy butterfly (*Limenitis archippus*, Nymphalidae) not only sequesters non-volatile defensive compounds from its larval host-plant, the Carolina willow (*Salix caroliniana*, Salicaceae), but also secretes volatile defensive compounds when disturbed. We developed LC/MS/MS methods to identify a set of phenolic glycosides shared between the adult viceroy butterfly and the Carolina willow, and SPME and GC/MS methods to identify volatile phenolic compounds released from stressed viceroy butterflies. In both approaches, all chemical structures were characterized based on their mass spectral fragmentation patterns and confirmed with authentic standards. The phenolic compounds we found are known to deter predator attack in other prey systems including other willow-feeding insect species. Since these compounds have a generalized defensive function at the concentrations we described, our results are consistent with the Müllerian re-classification put forth by other researchers based on their bioassay results. It seems the viceroy butterfly possesses different chemical defenses than its monarch and queen

butterfly counterparts (phenolic glycosides vs. cardiac glycosides, respectively), an unusual phenomenon in mimicry warranting future study.

Key Words: chemical defense, *Limenitis archippus*, LC/MS/MS, Nymphalidae, *Salix caroliniana*, Salicaceae, SPME-GC/MS.

Introduction

Visual defensive mimicry, defined as close physical resemblance between unrelated species (Bates, 1862), is an enduring example of adaptation by natural selection (Fisher, 1958; Charlesworth and Charlesworth, 1975; Ruxton *et al.*, 2004; Thompson, 2005). Mimicry is generally classified as either Batesian or Müllerian. In Batesian mimicry, a rewarding (i.e. palatable) species evolves a physical resemblance to the warning phenotype of a non-rewarding (i.e. unpalatable) species. This relationship is considered parasitic because the palatable species undermines the effectiveness of the warning signal. In contrast, Müllerian mimicry involves two unpalatable species with both participants sharing a common physical appearance. In a Müllerian system, both species benefit by distributing the mortality costs of predator education, and thus the relationship is generally considered mutualistic (Mallet, 1999). Therefore, correctly classifying the mimicry system has important ramifications regarding the ecology and evolution of the involved participants.

The monarch (*Danaus plexippus*) and queen (*Danaus gilippus*) butterflies (Nymphalidae: Danainae) arrived at the forefront of biological interest as Batesian models of the viceroy butterfly (*Limenitis archippus*) (Nymphalidae: Limenitinae) based on predator bioassays (Brower, 1958a; 1958b). In these behavioral experiments, monarch and queen butterflies were found to be more unpalatable than viceroy butterflies to avian predators. Furthermore, monarch and queen larvae feed on milkweeds (Asclepiadaceae), and both insect species are able to sequester and retain bitter and toxic cardenolides present in milkweeds into their adult stage (reviewed in Ackery and Vane-

Wright, 1984; Brower *et al.*, 1984). Cardenolides are known to contribute to prey unpalatability, resulting in avoidance learning by both vertebrate (Glendinning, 1992) and invertebrate (Berenbaum and Miliczky, 1984; Prudic *et al.*, 2007) predators. Conversely, viceroys were originally shown to be relatively palatable (Brower, 1958a; 1958b), and were not known to sequester noxious compounds from their larval host-plants, willows and poplars (Nishida, 2002). However, subsequent behavioral bioassays led to a re-evaluation of these relationships. In those assays, viceroy butterflies were found to be equally or more unpalatable to avian predators than their monarch and queen counterparts and this shared unpalatability increased the rate of predator aversion learning (Ritland and Brower, 1991). These results led the authors to conclude that viceroy butterflies were Müllerian, not Batesian, mimics with queens and monarchs.

Ritland and Brower's conclusions imply that viceroy butterflies, like monarch and queen butterflies, are protected by chemical compounds that render them unpalatable to predators; however, these putative defensive compounds were not known. This absence of knowledge about underlying defensive mechanism provoked criticisms of the predator bioassay results and interpretations (Guilford, 1991). In light of these criticisms, a chemical explanation for viceroy unpalatability would go far in deciding whether viceroys are Müllerian or Batesian mimics and understanding their ecological interactions and evolutionary trajectories. If the viceroy is a Batesian mimic, then it should not possess chemical defenses. However, if the viceroy is a Müllerian mimic, then it should have chemical defenses (Rothschild, 1991). We sought to determine if the viceroy was indeed chemically defended and thus consistent with the Müllerian classification.

A critical first step in such chemical characterization was determining candidate compounds associated with unpalatability. Many different chemical classes have been found to contribute to the unpalatability of Lepidoptera, including aristolochic acids, cardenolides, cyanogenic glycosides, iridoid glycosides, and pyrrolizidine alkaloids (Nishida, 2002). Although, they have not previously been identified in any butterfly species, we considered phenolic glycosides and their chemical relatives as the most likely candidate compounds for viceroy butterfly unpalatability because (1) these compounds are ubiquitous anti-herbivore compounds of the Salicaceae (willows and poplars), the larval host-plants of the viceroy butterfly (Palo, 1984; Tahvanainen *et al.*, 1985; Lindroth *et al.*, 1988b); (2) these compounds are sequestered by other insects of the willow-feeding guild, such as the beetles *Pharatora vitellinae* and *Chrysomela* spp. (Coleoptera: Chrysomelidae) (Pasteels *et al.*, 1988; Kopf *et al.*, 1998); and (3) these compounds when present in insects deter attack by generalist predators (Pasteels *et al.*, 1986; Rank *et al.*, 1996; Müller *et al.*, 2006). Using high performance liquid chromatography-mass spectrometry (LC/MS), and gas chromatography-mass spectrometry (GC/MS), we investigated whether (1) viceroy butterflies sequester non-volatile defensive phenolic glycosides from their larval host-plants; whether (2) viceroy butterflies emit volatile defensive phenolic compounds when disturbed; and whether (3) both the identity and the concentrations of the compounds we found have been shown to be predator deterrents in other bioassay experiments.

Methods and Materials

Natural history

The viceroy butterfly is a member of the genus *Limenitis* (Nymphalidae, Limenitidinae). Three of the four members of this genus in North America are involved in some type of mimicry relationship with vastly different butterfly species (Prudic *et al.*, 2002 and references therein). According to phylogenetic analysis, the ancestral phenotype for all North American *Limenitis* is a black ground color with a white dorsal band (Mullen, 2006). The viceroy adult phenotype is highly diverged from this ancestral pattern with an orange or brown ground color with black markings. Viceroy butterflies (*Limenitis archippus*) range over much of North America, and they exhibit regional variation in adult color pattern (Scott, 1986) which correlates with the geographic distribution of their models (Ritland and Brower, 2002). In areas where the monarch is prevalent, the viceroy exhibits a tawny orange ground color with black markings (*L. a. archippus*, *L. a. lahontani*), like the monarch. Conversely, viceroy butterflies in areas where the queen butterfly is more frequent exhibit a color pattern similar to the queen, which is a more brownish-orange ground color with black markings (*L. a. floridensis* and *L. a. obsoleta*). Viceroy larvae primarily feed on *Salix* and *Populus* (Salicaceae), unlike monarch and queen larvae which feed on members of the Asclepidaceae (Scott, 1986). In Florida, where the biological collections for this study were done, the viceroy butterfly (*L. a. floridensis*) is believed to mimic the queen, not the monarch butterfly, and the viceroy larvae feed almost exclusively on the Carolina willow, *Salix caroliniana* (Ritland and Brower, 1991).

Butterfly and host-plant collections

Adult viceroy butterflies and their larval host-plants were collected between July 20-21, 2004 from two locations in Florida, USA: Green Meadow Road, Lehigh Acres, Lee County, N 26 31.69, W -81 40.67 and Newnan's Lake, Gainesville, Alachua County, N 29 38.19, W -82 12.00. These collection sites are very close to the viceroy collection sites used in previous predator bioassays (Brower, 1958b; Ritland and Brower, 1991). For the LC/MS analysis, four samples each of butterflies and willow were collected from both locations. A butterfly sample consisted of 10 adults (either all male or all female) (~ 1 g dry weight), while a willow sample consisted of 16 young leaves, two leaves from eight different plants (~ 10 g dry weight). All samples were weighed, then air-dried at room temperature for one week and re-weighed. For the GC/MS analysis, live butterflies were individually sampled using both solid phase micro-extraction and direct sampling of the putative defensive secretion in the laboratory. Butterflies were caught in the field and analyzed the next day before being fed. There were five males and five females per site, and each individual butterfly secretion was analyzed separately.

Authentic standards

Benzoic acid, benzaldehyde, salicylaldehyde, and salicin standards were purchased from Sigma Chemical Co. (USA). A salicortin standard was provided by C. Orians (Tufts University, USA), and a tremulacin standard was purchased from AApin Chemical Ltd. (Abingdon, Oxon, UK).

High performance liquid chromatography – mass spectrometry

Sample extraction and preparation: The Green Meadow Road samples had the following dry weight: willow 1 (11.11 g), willow 2 (10.90 g), male-viceroy 1 (0.89 g), female-viceroy 1 (0.91 g). The Newnan's Lake samples weighed the following: willow 3 (9.24 g), willow 4 (10.88 g), male-viceroy 2 (0.99 g), female-viceroy 2 (1.17 g). Whole butterflies including their wings were used in the extraction. The dried samples were ground to a fine powder before methanol extraction. Methanol (50 ml viceroy, 200 ml willow) was added to each sample, sonicated for 30 minutes, and then allowed to soak overnight. The mixture was partitioned with an equal volume of hexane, three times. The methanol fraction was concentrated to dryness, weighed, and 1 mg samples were re-suspended in 1 ml of HPLC grade methanol, then filtered through a 0.45 µm PTFE filter (Whatman) for analysis.

Identification of non-volatile defensive compounds: Chromatograms were obtained using an Agilent 1100 HPLC system (quaternary gradient pump, diode array detector, thermostated column compartment, and autosampler) with a 4 µm Waters Nova Pack Sentry guard column (3.9 x 20 mm) and Waters Nova Pak reverse phase C18 column (4.6 x 150 mm). Each sample (20 µl) was injected into a methanol-water gradient flowing at 1 ml/min (N = 8) (solvent gradient described in Table 1). UV signals were observed at 200, 230, 277, and 300 nm. For LC/MS, analyses of each sample were obtained using an Agilent 1100 HPLC system tandem with Agilent MSD-Trap-SL ion trap mass spectrometer. The LC parameters were the same as above. The MS acquisition parameters were positive ESI mode, drying gas temperature 350°C, drying gas flow rate

10 l/min, nebulizer pressure 35 psi, HV capillary 3500 V, HV end plate offset -500V, capillary current 24.4 nA, current end plate 1138.6nA, RF amplitude capillary exit 158.5 V, and skimmer 40.0 V. Compounds were identified by comparing their retention times and mass spectra (MS and MS/MS) with those of authentic standards.

Quantification of non-volatile defensive compounds: Quantification was done by external standard method using a six-point standard curve with standards ranging from 0 to 2.0 mg/ml. Calibration curves were constructed for the three phenolic compounds using the LC-MS protocol described above. For each compound, a characteristic product ion was chosen from its MS/MS as its quantification ion (Table 2). Peak integration and quantification were performed automatically using Agilent Chemstation software (version A.10.01). The same samples used in identification analyses were re-run twice for quantification to ensure consistency within a sample. The concentrations were considered consistent if run 1 and run 2 were within 10% of each other. If not, then the sample was re-injected until the two runs reached the consistency criteria. However, only the concentration of the first run was used for reporting and statistical analyses (N = 4 willow samples; N = 4 viceroy samples). All statistical analyses were performed in JMP-IN software (SAS Institute, 2002) using one-way ANOVAs.

Gas chromatography – mass spectrometry

Identification of defensive volatiles: The viceroy butterfly secretes a malodorous fluid from its abdomen when disturbed. To characterize the volatile compounds a predator would encounter when it disturbs a viceroy, stable flex PDMS-DVB coated solid phase

micro-extraction (SPME) fibers (Supelco, USA) were used to sample the volatiles in the headspace above the abdomen of both stressed and normal butterflies (Borg-Karlson and Mozuraitis, 1996). To simulate a predatory event, a butterfly was pinned horizontally by the wings between a glass plate and a glass cylinder with one small opening (400 ml beaker) (Supplemental Material Fig. 1). This arrangement positioned the butterfly legs parallel to the glass plate without any surface for the butterfly to perch on. A SPME fiber holder with an averted fiber was placed in the small opening for one hour sampling the area directly above the struggling butterfly and its secretion. To collect the volatile compounds emitted by an unstressed butterfly, the same individuals were placed in the same container and sampled as described above; however, the butterfly was not pinned down to simulate a predation event. The butterfly was able to wander, or more often perch, on the glass plate. Separate SPME fibers were used for stressed and normal sampling events. The glassware was cleaned between sampling bouts with methanol. Each butterfly was sampled twice for each treatment to ensure consistency of the results (N = 20). These data were collected using a Varian Saturn 2100T GC/MS and a Chrompack capillary column (CP Sil 8 CB; 30 m x 0.25 mm; 0.5 μm film thickness). The initial column oven temperature was set for 80°C increasing to 210°C at 10°C/min.; the injector/transfer line/trap temperatures were 230°C /250°C /200°C, respectively; and the electron voltage was set at 70 eV. Ultra high purity (UHP) helium was used as the carrier gas at a flow rate of 1.0 ml/min. Each volatile sample was injected directly into the chromatograph after desorbing from the SPME fiber for 15 sec in the GC injector

(230°C). Compounds were identified by comparison of retention times and mass spectra to authentic standards and the NIST 02 MS library.

Quantification of defensive volatiles: Volatile compounds were quantified by the external standard method using a six-point standard curve with standards ranging from 0.005-5.0 µl/ml. Calibration curves from triplicate injections of 2.0µl were obtained using the GC-MS protocol above. Peak integration and quantification were performed automatically using Saturn 2100 Workstation software. The same insects used for compound identification were re-analyzed for compound quantification; however, for this analysis, the secretion was sampled directly from the abdomen of the butterfly using a glass capillary (Supplemental Material Fig. 2). Two µls of the secretion was collected from a disturbed butterfly and dissolved in 2.0 µl of ethyl acetate with 1.0 µl of 0.25 M p-chlorotoluene as the internal standard. Then 2.0 µl of this solution was injected directly into the GC column. Each butterfly sample was run on the GC twice for consistency. The concentrations were considered consistent if run 1 and run 2 were within 5% of each other. If not, then the individual butterfly was re-sampled until the two runs reached the consistency criteria. However, only the concentration of the first run was used for reporting and statistical analyses (N = 20). All statistical analyses were performed in JMP-IN software (SAS Institute, 2002) using one-way ANOVAs.

Results

In the LC/MS assays, we characterized the tissue chemical profiles of the Florida viceroy (*Limenitis archippus floridensis*) and its larval host-plant Carolina willow (*Salix caroliniana*). Of the five peaks shared between the viceroy and willow extracts, three peaks corresponded to phenolic glycosides known to be unpalatable to either herbivores or their predators: salicin, salicortin, and tremulacin (Fig. 1; Table 2). These compounds were found in both the willows and butterflies across sites and between sexes. However, geographic location and butterfly sex did not significantly affect the concentration levels of any of the three phenolic glycosides (one-way ANOVAs, $P > 0.05$) (Table 3).

In the GC/MS assays, we characterized and quantified the volatile chemical profile of a viceroy during a predation simulation and compared it to the volatile profile of a viceroy at rest. During these collection bouts, unstressed viceroy never emitted a secretion, but the stressed butterflies always did. Using SPME in the stressed butterfly, we identified four compounds not found in the unstressed butterfly: benzaldehyde, benzoic acid, methyl salicylate and salicylaldehyde (Table 3). These compounds were identified both in the headspace surrounding the provoked butterfly and in its secretion. Again, all four compounds were found in butterflies across geographic location and between butterfly sexes. There were also significant differences in geographic concentrations in benzaldehyde ($F_{1,19} = 10.24$, $P = 0.001$), benzoic acid ($F_{1,19} = 12.35$, $P < 0.001$), and salicylaldehyde ($F_{1,19} = 11.91$, $P < 0.001$) (Table 3). Butterflies from the northern Florida site, Newnan's Lake, had higher concentrations than butterflies from the southern Florida, Green Meadow Road.

Discussion

Originally, the viceroy butterfly was widely regarded as a palatable, Batesian mimic without any form of chemical defense (Brower, 1958a; 1958b). However, subsequent predator bioassays suggest that the viceroy butterfly is unpalatable and thus chemically defended against predation. These results led to a re-classification of the system as Müllerian (Ritland and Brower, 1991). In this study, our results are consistent with the Müllerian, not the Batesian, classification. We have shown adult viceroy butterflies possess compounds that function as defensive compounds in other willow-feeding insects. We found three phenolic glycosides (salicin, salicortin, and temulacin) in the larval host-plant, the Carolina willow, and the adult viceroy (Fig. 1; Table 2). It is well known that salicin can be a sample preparation artifact; however, our sample preparation protocol limited this possible bias (Julkunen-Tiitto and Sorsa, 2001). All three phenolic glycosides were found in the butterflies and the willows from both populations and in both sexes of butterflies, but there were no statistical differences in compound concentration between either geographic location or butterfly sex (Table 3). We also found the viceroy emitted four compounds (benzaldehyde, benzoic acid, methyl salicylate and salicylaldehyde) when disturbed in a simulated predation event. All these compounds were found in butterflies from both locations and in both sexes; although, concentrations of benzaldehyde, benzoic acid, and salicylaldehyde were statistically higher in butterflies from northern Florida compared to butterflies from southern Florida (Table 3). However, future sampling focusing on more populations is needed to verify

these preliminary patterns of geography and sex on viceroy chemical defense profiles and concentrations.

This is the first time that Carolina willow (*Salix caroliniana*) has been chemically characterized for phenolic glycosides, and the three phenolic glycosides we documented resemble the chemical profiles of other willow species (Nyman and Julkunen-Tiitto, 2005; Palo, 1984) and other members of the Salicaceae in general (Lindroth *et al.*, 1988a; Lindroth and Hemming, 1990). Typically, when tremulacin is found in willows, it is accompanied by salicortin and salicin (Julkunen-Tiitto, 1989). The Carolina willow had average levels of salicin, salicortin, and tremulacin compared to other *Salix* spp. (Soetens *et al.*, 1998), and these quantities have been shown to deter generalist herbivores (Palo, 1984; Tahvanainen *et al.*, 1985).

This is also the first example of adult butterflies containing phenolic glycosides (compare with Nishida, 2002) which are, in this case, probably derived via sequestration from the larval host-plant. Although our study did not address if these compounds deter predation on viceroys, the results from many other bioassay experiments strongly suggest this is a likely function. Willow-derived phenolic glycosides, including the compounds we found, are known for their deterrent and toxic effects on a variety of insect predators (reviewed in Bairlein, 1997). Salicin, when present in leaf beetles at concentrations similar to those found in the viceroy butterflies, deters predators (Pasteels *et al.*, 1986). Salicortin and tremulacin have been evaluated together, not individually, for their defensive properties against predators. When these two compounds are present in gypsy moth caterpillars (Lepidoptera: Liparidae) at concentrations similar to the viceroy, they

are deterrent and toxic, and they negatively affect diet choice in insectivorous songbirds (Müller *et al.*, 2006).

The volatile compounds, benzaldehyde, benzoic acid, and salicylaldehyde, are well-documented chemical defenses of willow-feeding leaf beetles against a variety of generalist predators (Smiley *et al.*, 1985; Pasteels *et al.*, 1988; Denno *et al.*, 1990). The concentrations of these three compounds we documented were lower than in some other studies; however, they are within the range of concentrations that are effective in conferring resistance to predation (Pasteels *et al.*, 1988; Denno *et al.*, 1990). Methyl salicylate (wintergreen oil) has not been evaluated as a deterrent to avian or invertebrate predators. However, it is described as the most poisonous of the salicylates; doses less than 1 g have killed small children weighing 10 kg (Michael and Sztajnkrycer, 2004). Concentrations as low as 2 mg/ml also induce nausea and vomiting when ingested by mice (Davidson *et al.*, 1961). It is not presently known how viceroys acquire these volatile compounds. Some of these compounds are known to occur in the foliar scent of willows (Dötterl *et al.*, 2005), and thus they may be sequestered from the larval host plant or acquired through adult nectar feeding. Regardless of the exact mechanism, our data demonstrate that viceroy butterflies contain known toxic and deterrent phenolic compounds, although further predator behavioral bioassays are required to evaluate the relative roles of each compound and their combined effect in viceroy chemical defense.

Our chemical results are consistent with other researchers' re-classification of the viceroy-queen and viceroy-monarch butterfly mimicry as Müllerian. Viceroys likely share the costs of predator education with their co-mimics. Unlike other well-studied

Müllerian mimicry systems such as the one involving *Heliconius* butterflies, the shared appearance and unpalatability between the viceroy and the monarch and queen did not arise through either common ancestry or shared host-plant use. Thus, comparing these two types of Müllerian systems will provide a powerful framework for understanding the relative roles of host-plant use, sequestration, and genetic architecture of wing coloration in Müllerian mimicry and aposematic coloration. This system is also one of the few Müllerian examples where the participants unmistakably vary in defensive chemistry: the adult viceroy butterfly sequesters defensive phenolic glycosides from its larval host-plant, while the queen and monarch butterflies sequester defensive cardiac glycosides from their larval host-plant (Brower *et al.*, 1984). Although these compound classes differ structurally and functionally, recent experimental evidence indicates that such chemical differences could actually facilitate Müllerian mimicry evolution by increasing rates of predator aversion learning and remembering (Skelhorn and Rowe, 2005). Although much more research remains, our findings provide a likely chemical mechanism explaining previously reported viceroy unpalatability, and these results are consistent with the Müllerian re-classification of this classic mimicry system.

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Tables, Figures, and Appendices

TABLE 1 Liquid chromatography solvent gradient with flow rate of 1.00 ml/min

Time (min)	% Water + 0.05% Acetic Acid	% Methanol + 0.05% Acetic Acid
0.00	100%	0%
3.50	80%	20%
7.00	55%	45%
15.00	50%	50%
19.00	5%	95%
20.00	0%	100%
26.00	0%	100%
29.00	85%	15%

TABLE 2 Compound specific LC/MS and LC/MS/MS identification and quantification results of viceroy butterfly and its larval host-plant. For salicin, the extracted ion was 309 *m/z* [M + Na] from 5.0-7.0 min, for salicortin the extracted ion was 442 *m/z* [M + H_2O] from 8.0-10 min, and for tremulacin 546 *m/z* [M + H_2O] from 18.0-20.0 min.

Compound	Retention Time (min)	Diagnostic Ions (*m/z*) Parent:Daughters	Willow Conc. ± Std Dev (mg/g dry wt.) (N = 4)	Viceroy Conc. ± Std Dev (mg/g dry wt.) (N = 4)
Salicin	6.8	309:185[a], 277	3.55 ± 0.91	3.04 ± 2.02
Salicortin	8.9	442:263[a], 268, 245	25.36 ± 2.93	6.56 ± 3.74
Tremulacin	18.6	546:267[a], 373	34.90 ± 3.54	26.69 ± 15.91

[a] quantification ion

TABLE 3 Concentration averages of non-volatile phenolic glycosides in willow and viceroy butterfly (total, geographic location and sex) (LC/MS) and concentration averages of volatile phenolics released from stressed butterflies (total, geographic location and sex) (GC/MS)

Compound	**Willow Conc. ± Std Dev**		**Viceroy Conc. ± Std Dev**		
	Total Conc.	**Location**	**Total Conc.**	**Location**	**Sex**
Benzaldehyde[a]	NS	NS	4.31 ± 2.56	2.17 ± 0.82 (GMR) vs. 6.45 ± 1.73 (NL) [c]	3.42 ± 2.10 (M) vs. 5.21 ± 2.74 (F)
Benzoic Acid[a]	NS	NS	0.14 ± 0.13	0.03 ± 0.01 (GMR) vs. 0.25 ± 0.09 (NL) [c]	0.15 ± 0.13 (M) vs. 0.13 ± 0.11 (F)
Methyl Salicylate[a]	NS	NS	28.01 ± 10.60	27.64 ± 2.24 (GMR) vs. 28.33 ± 10.11 (NL)	26.48 ± 10.39 (M) vs. 29.54 ± 11.03 (F)
Salicin[b]	3.55 ± 0.91	3.19 ± 0.88 (GMR) vs. 3.90 ± 1.10 (NL)	3.04 ± 2.02	2.12 ± 1.29 (GMR) vs. 4.97 ± 1.12 (NL)	2.55 ± 2.30 (M) vs. 3.55 ± 3.14 (F)
Salicortin[b]	25.36 ± 2.93	24.11 ± 2.38 (GMR) vs. 26.61 ± 3.72 (NL)	6.56 ± 3.74	6.52 ± 3.83 (GMR) vs. 9.75 ± 1.31 (NL)	5.67 ± 4.52 (M) vs. 7.45 ± 4.57 (F)
Salicylaldehyde[a]	NS	NS	6.12 ± 4.89	0.82 ± 0.01 (GMR) vs. 9.99 ± 4.04 (NL)[c]	6.86 ± 6.18 (M) vs. 5.38 ± 3.26 (F)
Tremulacin[b]	34.90 ± 3.54	36.5 ± 3.37 (GMR) vs. 33.35 ± 4.00 (NL)	26.69 ± 15.91	12.94 ± 2.58 (GMR) vs. 40.18 ± 3.37 (NL) [c]	24.46 ± 18.83 (M) vs. 28.67 ± 19.60 (F)

[a] measured in µg/µl (Total, N=20; Geography, N=10; Sex, N=10)
[b] measured in mg/g dry wt. (Total, N=4; Geography, N=2; Sex, N=2)
[c] significant difference (one-way ANOVA, $P < 0.05$)
F=Female, GMR=Green Meadow Road, M=Male, NL=Newnan's Lake, NS=Not Sampled

Figure Text

FIGURE 1

Extracted ion chromatograms of shared phenolic glycosides of Carolina willow and viceroy butterfly compared to authentic standard mixture. (A) Standard solution containing three phenolic glycosides: salicin (peak 1), salicortin (peak 2), and tremulacin (peak 3), (B) Carolina willow sample 1 from Green Meadow Road site, (C) Adult viceroy butterfly sample 1 (10 males) from Green Meadow Road site. Specific site information is provided in the methods and materials section.

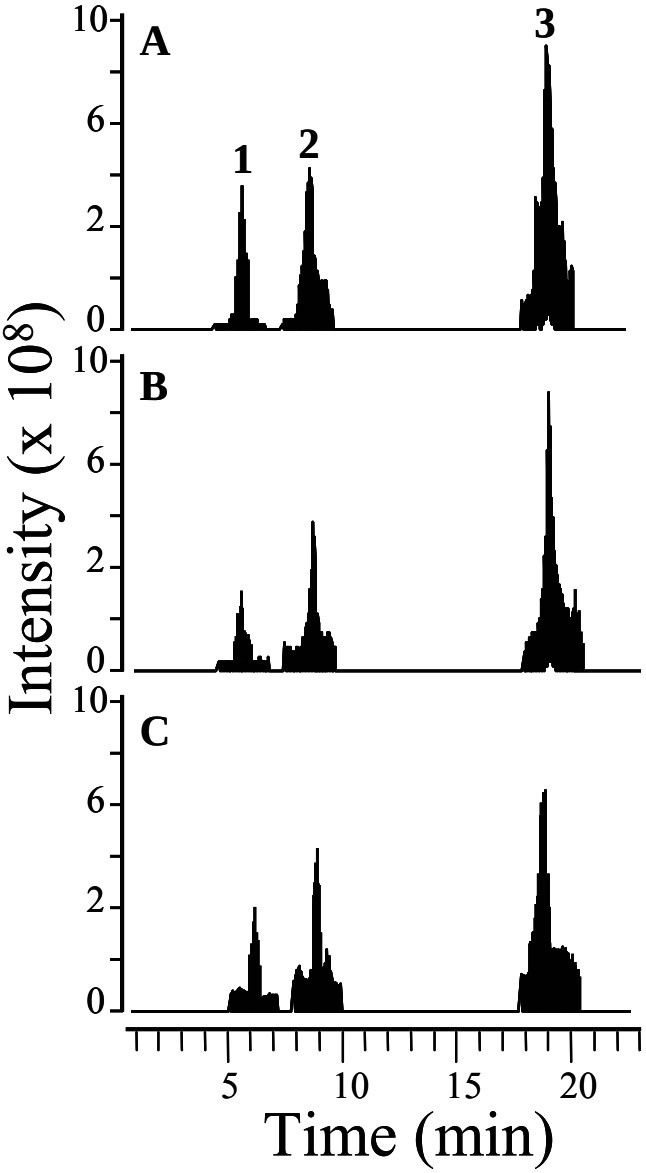
SUPPLEMENTARY MATERIAL FIGURE 1

Digital image of solid phase micro-extraction (SPME) setup. The position of the butterfly pinned between the glass plate and the beaker simulated a predation event. In a non-predatory event, the butterfly was allowed to perch freely on the glass plate during the SPME sampling bout.

SUPPLEMENTARY MATERIAL FIGURE 2

Digital image of secretion when butterfly experiences a predation event. One μl of the secretion was collected using a glass capillary for the GC-MS quantification analyses.

FIGURE 1



SUPPLEMENTARY MATERIAL FIGURE 1

SUPPLEMENTARY MATERIAL FIGURE 2

APPENDIX C

A MIMIC WITHOUT ITS MODEL: GEOGRAPHIC VARIATION IN THE RELATIVE ABUNDANCE, CHEMICAL DEFENSE AND PALATABILITY OF THE VICEROY

A mimic without its model: geographic variation in the relative abundance, chemical defense and palatability of the viceroy

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Abstract

The study of mimicry often takes either a prey perspective or a predator perspective. Both approaches have been useful to identify mechanisms and patterns in mimicry, but they are seldom used in combination. Recent critiques have drawn attention to untested assumptions in both approaches that may have major impact on our understanding of mimicry and visual warning signals. Specifically, there needs to be a better understanding of how geographic variation affects the origin and function of mimicry. I compared geographic differences in palatability and chemical defense within a classic Müllerian mimic, the viceroy butterfly (Nymphalidae: *Limenitis archippus*) in relation to the presence and abundance of its co-model, the queen butterfly (Nymphalidae: *Danaus gilippus*) across Florida. I evaluated the effect of geographic location on mimicry function by (1) surveying eight viceroy populations, measuring the relative abundance of viceroys and queens in each population; (2) documenting geographic variation in chemical defense of viceroys; and (3) comparing these data to geographic variation in viceroy palatability using invertebrate predators. The results suggest that viceroys vary spatially in both frequency and co-occurrence with queens. As latitude increased, viceroys increased their relative abundance between locations while queens decreased their relative abundance. Viceroys had their greatest relative abundance in northern Florida while queens had their greatest relative abundance in southern Florida. All viceroys across sites had similar defensive chemical profiles. However, the concentrations of these defensive compounds were higher in viceroys from populations with fewer queens even though the host plant chemical concentrations did

not differ among locations. Viceroys from populations with fewer queens were also more unpalatable. Predators learned to avoid these viceroys faster and remembered to avoid them longer. Thus, viceroys vary spatially in their chemical defenses and unpalatability depending on the relative abundance of its co-model, the queen, in a given location. These findings illustrate that there is a geographic mosaic of selection in mimicry relationships and the presence or absence of a co-model plays a very important role in the functioning of mimicry.

Key words: chemical defense, Lepidoptera, *Limenitis archippus*, phenolic glycosides, viceroy

Introduction

Prey species employ an extraordinary array of defenses against predators, including chemical toxicity and protective coloration. Investigating these defenses has led to major advances in the fields of behavior, chemistry, ecology, and evolution. A classic example of prey defense is the phenomenon of defensive mimicry, in which one prey species gains protection from predation through the evolution of superficial phenotypic similarities to another prey species with some anti-predator defense (Bates 1862). Alternatively, both prey species could both benefit by resembling each other and sharing the cost of predator education (Müller 1879) Regardless of the precise relationship, mimicry has been regarded as a classic co-evolutionary relationship between the two prey species that is mitigated by selection from the predator (Ruxton *et al.* 2004).

Considerable effort has been paid to exploring scenarios that could generate mimicry relationships in nature. Multiple theoretical models and empirical studies have advanced our understanding of the origin and evolution of mimicry (reviewed in: Ruxton *et al.* 2004), but these usually take either a prey perspective exclusively or a predator perspective exclusively. A prey perspective focuses on the signal-sender such as the model, co-model, or mimic species. Common areas of investigation are frequency–dependent selection, evolution and development, population genetics, and relative abundance or either model or mimic (e.g. Joron *et al.* 2006). These investigations usually are based on field collections and observations of the prey only. Conversely, a predator approach focuses on the signal-receiver, the predator, who is learning or has learned to avoid noxious prey based on their visual appearance. Common areas of investigation are

predator learning, predator memory retention and how memory retention is influenced by prey palatability (e.g. Rowe *et al.* 2004). These investigations tend to be laboratory-based experiments that use model predator species (usually birds) and artificial prey. Both these approaches to the study of mimicry have been fruitful; however, they are seldom integrated. Moreover, attention has been recently drawn to untested assumptions in both these approaches that may have major impact on our understanding of mimicry and visual warning signals (Turner 1984; Speed 1993; Joron and Mallet 1998; Mappes *et al.* 2005).

Neither viewpoint alone can reveal how geographic variation in relative abundance of the participants or geographic variation in palatability or chemical defense within a species could affect how mimicry originates and functions. Yet, in other species interactions, describing and understanding geographic variation has advanced our knowledge of how these interactions evolve (reviewed in: Thompson 2005). Mimicry by definition involves multiple concurrent interactions between prey and their predators so geographic variation could be particularly important in generating different ecological and evolutionary pathways. Three lines of empirical evidence suggest that geographic variation is important to consider in mimicry. First, there is limited documentation of geographic variation in chemical content within mimetic species (e.g., Malcolm and Brower 1989; Moranz and Brower 1998). Second, a mimetic species that was traditionally presumed palatable can be unpalatable to predators depending on where it is from (e.g., Ritland and Brower 1991; Ritland 1994; Ritland 1995). Finally, field surveys have shown that a mimic species can occur in geographic locations without its model

(e.g. Ritland 1995; Prudic *et al.* 2002; Pfennig *et al.* 2007). Examining this geographic variation in the field could provide new information regarding how mimicry functions and evolves.

A classic example of mimicry between the queen (*Danaus gilippus*: Nymphalidae) and the viceroy (*Limenitis archippus*: Nymphalidae) butterflies provides a unique opportunity to investigate the role of geographic variation in relative abundance and palatability and their potential effects on mimicry. The queen has been described as both a Batesian model (Brower 1958) and a Müllerian co-model (Ritland 1994) based on palatability experiments with predators. The queen is also known to vary spatially in its defensive chemical content and relative abundance (Brower 1984; Moranz and Brower 1998). In addition, the viceroy is purported to be the Batesian mimic of the queen in some locations (Brower 1958), while in other populations, the viceroy is less palatable than the queen to avian predators (Ritland and Brower 1991). The viceroy has also been shown to contain noxious phenolic compounds similar to the defensive compounds in other willow-feeding insects, and the concentrations of these compounds vary geographically (Prudic *et al.* 2007). Because of these features, this mimicry relationship provides a unique opportunity to investigate geographic variation in relative abundance, chemical defense, and palatability within a species and how it may affect the mimicry relationship.

Using the viceroy-queen system, I studied: 1) can the viceroy exist in populations without its co-model, the queen?; 2) if so, is there geographic variation in viceroy chemical defense in relation to absence of the queen?; and 3) how does this spatial

pattern in chemical defense relate to viceroy palatability and the functional benefits of mimicry such as predator aversion learning and memory retention? Using a combination of field sampling, chemical analysis, and predator bioassay, this study demonstrates the importance of explaining geographic variation in relation to understanding how mimicry forms and functions through time.

Methods and Materials

Geographic locations and survey techniques

I surveyed and collected viceroy and queen butterflies three times (June, July, and September) in each of two years (2003 and 2004) at eight locations in Florida, USA. From south to north, the locations were Immokalee, Collier County (N 26 21.96, W -81 31.28); Lehigh Acres, Lee County (N 26 31.69, W -81 40.67); Lake Istokpoga, Highlands County (N 27 17.77, W -81 17.64); Cornwell, Highlands Country (N 27 23.81, W -81 07.16); Leesburg, Lake County (N 28 47.28, W -81 53.68); Cedar Key, Levy County (N 29 12.82, W -83 02.39); Newnan's Lake, Gainesville, Alachua County (N 29 38.19, W -82 12.00); and Jena, Dixie County (N 29 40.01, W -83 11.09). These collection sites were in close proximity to the viceroy collection sites used in previous studies (Brower 1958; Ritland and Brower 1991). Relative abundance of each species was measured by calculating the rate of capture per species per min per person. Two field surveyors sampled each site and the same individuals surveyed all the sites. One surveyor sampled queens and the other sampled viceroys, switching their target species after 1 hour. Each field site was sampled for 2 hours continuously along a 400 meter transect on sunny days

between 900 and 1600 hours. Data were analyzed using repeated-measures, linear regression with latitude as the predictor variable (JMP-In 2002).

Chemical analyses

Identification and quantification of defensive phenolic glycosides in viceroys and their larval host plants (LC/MS)

For the LC/MS analysis, I evaluated if the defensive phenolic glycosides in the viceroy butterfly and/or its larval host plant, the Carolina willow (*Salix caroliniana*) varied geographically between the sites. Four samples of butterflies and three samples of willow were collected from each of the eight population locations in July 2004. A butterfly sample consisted of 5 adults (either all male or all female) (~ 0.5 g dry weight), while a willow sample consisted of 16 young leaves, two leaves from eight different plants (~ 10 g dry weight). All specimens were weighed, then air-dried at room temperature for one week and re-weighed. Whole butterflies including their wings were used in the extraction. The extraction and identification protocol is described in detail in a previously published study (Prudic *et al.* 2007). Analyses of each sample were conducted using an Agilent 1100 HPLC system tandem with Agilent MSD-Trap-SL ion trap mass spectrometer. Calibration curves were constructed for the three phenolic compounds using the LC-MS protocol described above. For each compound, a characteristic product ion was chosen from its MS/MS as its quantification ion. Peak integration and quantification were performed automatically using Agilent Chemstation software (version A.10.01). The same samples were run twice for quantification to

ensure consistency within a sample. The concentrations were considered consistent if run 1 and run 2 were within 10% of each other. If not, then the sample was re-injected until the two runs reached the consistency criteria. However, only the concentration of the first run was used for reporting and statistical analyses (N = 24 willow samples; N = 32 viceroy samples). Concentration data were log transformed and then checked for normalcy and homoscedasticity. Transformed data were analyzed using repeated-measures, linear regression with queen abundance in a population as the predictor variable (JMP-In 2002).

Identification and quantification of volatile defensive phenolics in viceroys (GC/MS)

For the GC/MS analysis, I evaluated if the chemical profile of the viceroy defensive secretion changed based on geographic location. Butterflies were caught at the eight field sites in July 2004, stored live in the refrigerator, and analyzed within the next three days before being fed. The defensive secretion was sampled directly from the abdomen of the butterfly using a glass capillary (Prudic *et al.* 2007). There were three males and three females per site, and each individual butterfly secretion was analyzed separately (N = 48). Volatile compounds were quantified by the external standard method using a six-point standard curve with standards ranging from 0.005-5.0 µl/ml. Calibration curves from triplicate injections of 2.0µl were obtained using the GC-MS protocol above. Peak integration and quantification were performed automatically using Saturn 2100 Workstation software. Two µls of the secretion was collected from a disturbed butterfly and dissolved in 2.0 µl of ethyl acetate with 1.0 µl of 0.25 M p-chlorotoluene as the internal standard. Then 2.0 µl of this solution was injected directly

into the GC column. Each butterfly sample was run on the GC twice for consistency. The concentrations were considered consistent if run 1 and run 2 were within 5% of each other. If not, then the individual butterfly was re-sampled until the two runs reached the consistency criterion. However, only the concentration of the first run was used for reporting and statistical analyses (N = 48). Concentration data were log transformed and then checked for normalcy and homoscedasticity. Transformed data were analyzed using repeated-measures, linear regression with queen abundance in a population as the predictor variable (JMP-In 2002).

Behavioral experiments

Predator and experimental arena

Laboratory-reared adult Chinese praying mantids (Mantidae: *Tenodera aridifolia sinensis*) served as the experimental predator. Mantids are a known predator of butterflies and have been observed preying on viceroys in the field (Prudic, pers. obs.). Egg cases were purchased from Carolina Biological Supply Company and reared to adults on two separate occasions: February 2003-May 2003 and February 2004-May 2004. On both occasions, mantids were reared in individual cages on a diet of fruit flies, houseflies, true bugs, and crickets. Mantids did not have access to butterfly or distasteful prey before the experimental feeding trials. Each mantid was fed two adult crickets every night throughout the period of experimentation.

All behavioral experiments were conducted in a laboratory arena consisting of three components: a rectangular perch for the predator, a square floor, and a cylindrical

wall. The entire arena was painted a dark uniform gray. The arena was illuminated by three full-spectrum halogen lamps (Solux-Eiko, 50W, 4700^{o}K, 36^{o} field of illumination). Each lamp was positioned 23 cm above the highest point of the perch and 20 cm from the other lamps. In all experiments, a trial began by placing a single mantid at the top of the perch inside the arena wall, such that the mantid's longitudinal axis was perpendicular to the long axis of the perch. Each mantid was allowed to acclimate for five minutes before trials began. All mantids remained at the top of the perch for all experiments and trials. Viceroys collected in July 2003 and July 2004 were used in this experiment. Abdomens rather than the entire insect were used for consistency with previous experiments involving viceroys and avian predators (e.g.: Ritland and Brower 1991; Ritland 1995). Mantids attacked and rejected viceroy abdomens at similar rates to live butterflies (Prudic, unpublished data). The viceroy abdomens have black with white stripes, while the other butterfly abdomens used in the experiment were uniformly either white or light brown. A single butterfly abdomen was introduced to the arena by attaching one end of a string to a dowel then slowly dropping the attached abdomen from above and to the side of the mantid.

Learning assay

This experiment compared predator aversion learning rates between sites to evaluate the relative palatability of viceroy butterflies to predators. According to research on predator behavior and prey toxicity, as palatability increases the rate of aversion learning should decrease, or the predator should require more experiences to learn to avoid the prey (Lindström *et al.* 1999; Skelhorn and Rowe 2006). Naïve mantids

(n=16, 8 per treatment) were assigned randomly to a site so that a single mantid experienced viceroys from one locale. A trial ended either 5 minutes after a mantid attacked and ate the abdomen, or, if the mantid did not attack the abdomen, 5 minutes after the abdomen was presented to the mantid. After the trial ended, the butterfly abdomen or its remains were removed. If the mantid attacked the abdomen, it was returned to its holding cage after 2.5 minutes. If the mantid did not attack the abdomen, it was presented with a known palatable butterfly abdomen of similar size (*Vanessa cardui* or *Pieris rapae*) after 2.5 minutes to evaluate its hunger status. If the mantid attacked the palatable abdomen, it was evaluated as hungry. The mantid was not allowed to feed on the palatable abdomen because the abdomen. This protocol prevented the mantid from associating its response to the viceroy abdomen with a palatable reward. A mantid was considered to show an aversion to the viceroy abdomen when it oriented but failed to attack, and subsequently attacked a palatable abdomen, in three consecutive trials. To evaluate if there was a geographic difference between prey palatability and predator aversion learning rate, the number of trials until mantids reached aversion criterion was compared between geographic locations. Data were log transformed for normalization and then checked for normalcy and homoscedasticity. Transformed data were analyzed using a linear regression with queen abundance in a population as the predictor variable (JMP-In 2002).

Retention assay

This experiment evaluated the number of days until the mantid re-attacked a viceroy abdomen after reaching aversion criterion. I compared predator memory retention

between sites to evaluate the relative palatability of viceroy butterflies to predators. According to research on predator behavior and prey toxicity, as palatability increases the rate of memory retention should decrease, or the predator should re-attack the prey sooner (Skelhorn and Rowe 2006). In order to test this hypothesis, the same mantids (n=16) used in the learning experiment were tested two days after the day they met the aversion criterion (see above) and retested every second day thereafter. A trial ended either when the mantid attacked and consumed a viceroy, or when the mantid oriented to a viceroy, failed to attack, but subsequently attacked a palatable butterfly abdomen. A mantid was considered to have lost its aversive response when it attacked and consumed a viceroy abdomen. Data were log transformed for normalization and then checked for normalcy and homoscedasticity. Transformed data were analyzed using a linear regression with queen abundance in a population as the predictor variable (JMP-In 2002).

Results

Geographic survey and relative butterfly abundance

Across seasons and between years, latitude positively affected viceroy abundance (r^2=0.65, p=0.016). More northern Florida populations had more viceroys on average, than southern Florida populations (Fig. 1). The same trends were found when only July 2003 and July 2004 records were analyzed, those months being the collection periods for the chemical and behavioral experiments (r^2=0.71, p=0.009). In contrast to the viceroy pattern, latitude negatively affected queen abundance (r^2=0.84, p=0.001). There were more queens in populations in southern Florida than in populations in northern Florida

(Fig. 1). The same trends held when only July 2003 and July 2004 records were analyzed (r^2=0.82, $p<0.001$).

Chemical defenses of viceroy butterflies and their larval host plant, Carolina willow

There were no marked differences in the type or concentration of phenolic glycosides across populations of Carolina willow (Fig. 2a). There were also no differences between the type of defensive compounds in the viceroy across populations; salicin, salicortin, and tremulacin were always detected. However, there were differences in concentrations of some compounds across populations. For the non-volatile compounds, tremulacin and salicortin were both in higher concentrations in viceroys populations with low queen abundance compared to populations with high queen abundance (Table 1) (Fig. 2b); salicin did not different between populations (Table 1) (Fig. 2b). The differences in salicortin and tremulacin were responsible for the observed differences in total non-volatile phenolics between locations (Table 1). For the volatile compounds, viceroys from populations with low queen abundance had a greater concentration of benzaldehyde and salicylaldehyde than viceroys from populations with high queen abundance (Table 1) (Fig. 2c). These two compounds were responsible for the observed differences in total volatile phenolics between populations (Table 1) (Fig. 2c).

Predator behavioral responses and viceroy palatability

All Chinese mantids ate and consumed a viceroy butterfly abdomen at least once. Collection year (2003 or 2004) did not affect the rate of learning (p=0.16). However, the mantids learned to avoid viceroys faster when they were trained to viceroys from populations with low queen abundance as compared to viceroys with high queen abundance (r^2=0.71, p=0.001) (Fig. 3a). All mantids retained their aversion response to viceroy abdomens for at least 3 days and as long as 25 days. Mantids trained to avoid viceroy abdomens collected from locations with low queen abundance remembered to avoid these abdomens longer, on average, than mantids trained to avoid viceroy abdomens from high queen abundance locations (r^2=0.62, p=0.01) (Fig. 3b).

Discussion

This integrative approach combining prey and predator perspectives resulted in a new understanding of prey chemical defense in a mimicry system. The relative abundance of the co-model, the queen butterfly, at a given location was associated with differences in the chemical defense and resultant palatability of the other model species, the viceroy butterfly. In populations where queens were less abundant, viceroys had greater concentrations of chemical defenses and decreased palatability compared to viceroy populations where the queen was more abundant (Fig. 2 and 3, Table 1). However, this geographic variation in viceroy chemical defense and palatability was not correlated with concentration differences in the predominant viceroy larval host plant, the Carolina willow (Fig. 2, Table 1). Concentrations of foliar phenolic glycosides are highly variable in *Salix* spp. There is generally little spatial variation among populations from

similar environmental conditions (Lower and Orians 2003) or among genetically similar populations (Orians *et al.* 1996). There are numerous examples of warningly colored insects varying geographically in chemical defenses (e.g., Smiley *et al.* 1985; Moranz and Brower 1998), but it has always thought to be in relation to chemical differences between different host species, not in relation to the relative abundance of other prey.

This observed pattern may also explain why the viceroy-queen system has been described as both Batesian (Brower 1958) and Müllerian (Ritland and Brower 1991; Prudic *et al.* 2007). The experiments that resulted in the Batesian classification were performed with viceroys and queens collected from the same population, while the experiments that resulted in the Müllerian classification were performed with viceroys collected both from locations with a high abundance of queens and from locations with a low abundance of queens. The viceroy-queen system fits best with the Müllerian classification since the viceroy is sharing the cost of predator education with the queen even though this cost is distributed unequally between populations (Mallet 1999).

This observed pattern of geographic variation in viceroy chemical defense and resultant palatability could arise via different mechanisms. Although there were no observed quantitative differences in host plant chemical defense between populations, undetected variation in foliar defensive chemistry may have contributed to the observed pattern in viceroy palatability. A more likely explanation, there may be physiological costs to acquiring, storing, and emitting phenolic defensive compounds. There is no information currently known about these potential costs in either the viceroy, or any of its congeners. However, consuming host plants high in phenolic glycosides negatively

affects growth and development in the butterfly, *Papilio canadiensis* (Matsuki and MacLean 1994) and the willow feeding leaf beetle, *Phratora vulgatissima* (Kelly and Curry 1991). The complex transport system that mediates sequestration of phenolic glycosides in *P. vitellinae* from its larval host plant has been shown at the molecular level to be potentially metabolically costly (Kuhn *et al.* 2004). Possibly when the queen is more abundant in a location, the viceroy can reduce sequestration costs with relatively little increased risk of predation.

To further complicate our understanding of the potential mechanism, the viceroy-queen system has two different chemical defenses involved in predator education, cardiac glycosides and phenolic glycosides. It has been shown that two different chemical defenses at lower concentrations increases the rate of predator aversion learning and memory retention than a single chemical defense at a higher concentration (Skelhorn and Rowe 2005). Thus, where the queen is relatively more abundant, a relatively lower amount of chemical defenses in viceroys may be sufficient for predator education.

Mimicry involves multiple concurrent interactions between prey and their predators, so geographic variation could be particularly important in generating different ecological and evolutionary pathways. The viceroy exhibits chemical and palatability polymorphism across populations depending on the presence or absence of its model, the queen. This chemical polymorphism probably evolved as a response to increased predation on the viceroys in populations where the queen was not present or at very low frequencies. Wing coloration polymorphisms are well documented in some butterfly mimicry systems such as *Heliconius erato* (Mallet and Joron 1999) and *Papilio dardanus*

(Nijhout 2003). Wing coloration polymorphisms are thought to arise and be maintained by local adaptation to the environment (Mallet and Joron 1999). However, as shown in this study, changes in the relative frequencies of the mimicry participants could also generate these wing coloration changes.

These geographic changes in relative abundance may facilitate the evolution of warning coloration and new mimicry systems in previously undescribed ways. The viceroy is more chemically defended and is carrying almost the entire cost of predator education in some locations. Based on current understanding of *Limenitis* phylogenetics and Florida biogeography, the viceroy probably evolved its warning coloration signal first as a Batesian mimic of the queen and only later acquired the ability to sequester noxious compounds from its larval host (Mullen 2006; Prudic and Oliver, App. D). In the future, the viceroy could conceivably become a model itself for an entirely new Batesisan mimicry species in locations where it occurs without the queen. The geographic mosaic of mimicry relationships may therefore facilitate the progression from palatable, cryptic avoider to palatable, false signaler to unpalatable, honest signaler.

In conclusion, incorporating predator and prey perspectives with geographic variation has provided new insight into the study of mimicry and its dynamics. This study illustrates that it may be inappropriate to characterize an entire mimicry system on the basis of limited geographical sampling. Across a geographic area, the signals and roles of the co-models are affected by their relative abundance in each location. Chemical defense and palatability of one prey species varies geographically depending on the relative abundance of the other participant. In populations with a lower abundance

of the co-model, a signaler has greater chemical defenses and is more unpalatable than in populations where the co-model is at a higher frequency. This pattern is probably related to the costs of producing, acquiring, or storing the defense. These empirical findings emphasize the importance of studying mimicry across multiple locations to understand the function and evolution of mimicry. This type of geographic variation could result in additional mechanisms to generate aposematic coloration and new mimicry relationships. These results beg the question of how many other mimicry systems vary geographically in relative abundance and palatability of the participants and if this pattern is a general one. If this is a general pattern, new mathematical models of mimicry need to incorporate relative abundance and palatability with geographic information.

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Table, Figures, and Appendices

Table 1. Summary of linear regression analyses of the effects of co-model abundance (the queen butterfly) on concentrations of willow (*Salix caroliniana*) foliar chemical defense, viceroy butterfly (*Limenitis archippus*) non-volatile chemical defense and viceroy volatile chemical defense. Significant effects labeled in bold. Slope is change from low to high queen abundance and is only reported for significant results.

Source	Trait	N	df	F	P	R^2 (Slope)
Queen Abundance	Willow phenolic glycosides					
	Salicin	24	1, 23	1.33	0.37	0.18
	Salicortin	24	1, 23	0.84	0.53	0.03
	Tremulacin	24	1, 23	0.20	0.56	0.04
	Total phenolic glycosides	24	1, 23	3.24	0.21	0.35
	Viceroy phenolic glycosides					
	Salicin	32	1, 31	4.20	0.16	0.22
	Salicortin	**32**	**1, 31**	**21.45**	**0.001**	**0.58 (Negative)**
	Tremulacin	**32**	**1, 31**	**25.72**	**0.002**	**0.62 (Negative)**
	Total phenolics	**32**	**1, 31**	**23.22**	**0.002**	**0.60 (Negative)**
	Viceroy volatile phenolics					
	Benzaldehyde	**48**	**1, 47**	**23.27**	**0.003**	**0.76 (Negative)**
	Benzoic acid	48	1, 47	2.48	0.11	0.29
	Methyl salicylate	48	1, 47	0.27	0.60	0.15
	Salicylaldehyde	**48**	**1, 47**	**28.88**	**0.001**	**0.83 (Negative)**
	Total volatile phenolics	**48**	**1, 47**	**26.53**	**0.002**	**0.79 (Negative)**

Figure Text

Figure 1: Average relative abundance differences of viceroy and queen butterflies across latitudes. Each data point represents the average abundance across collections at a given site. ● represents viceroy butterflies, ■ represents queen butterflies.

Figure 2: Average concentration differences (± S.E.) in defensive compounds between sites in (A) the Carolina willow the larval host plant of the viceroy; (B) the viceroy butterfly; and (C) the viceroy butterfly's defensive secretion. Category of low queens includes populations where the queen collection rate was less than 1 queen / person / 30 min. Category of high queens includes populations where the queen collection rate ranged between 8 – 13.33 queens / person / 30 min. These categories were not part of the statistical analyses but are presented for illustrative purposes.

Figure 3: Results from palatability experiments with mantid predators (A) average number of encounters until mantid reached aversion learning criterion based on viceroy population origin (B) average days until mantid forgot aversion response and attacked the viceroy. Category of low queens includes populations where the queen collection rate was less than 1 queen / person / 30 min. Category of high queens includes populations where the queen collection rate ranged between 8 – 13.33 queens / person / 30 min. These categories were not part of the statistical analyses but are presented for illustrative purposes.

Figure 1

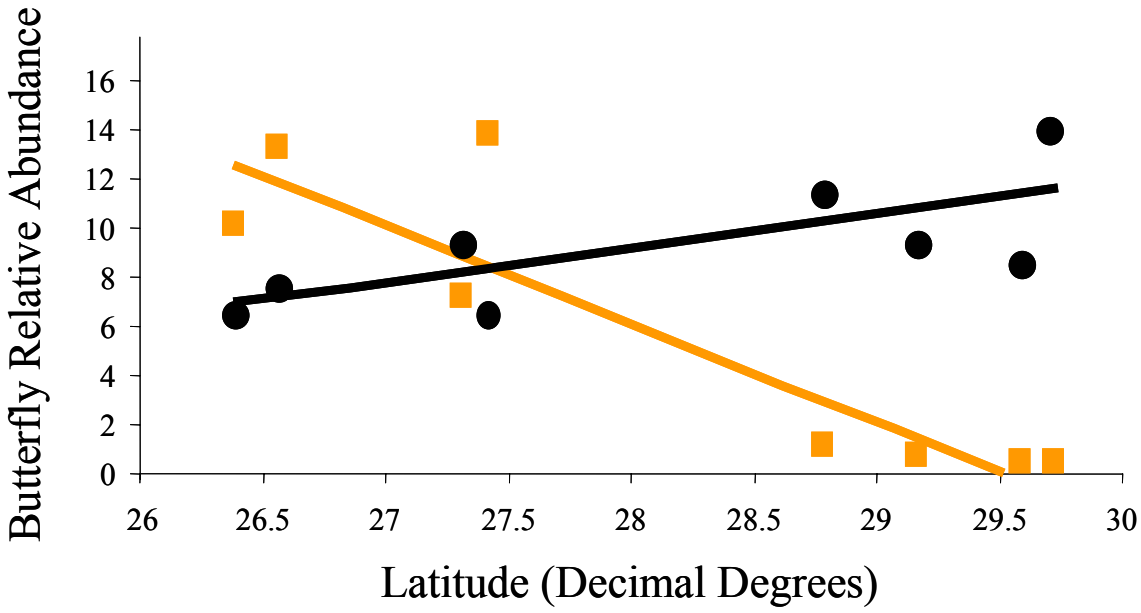

Butterfly Relative Abundance
16
14
12
10
8
6
4
2
0
26
26.5
27
27.5
28
28.5
29
29.5
30
Latitude (Decimal Degrees)

Figure 2

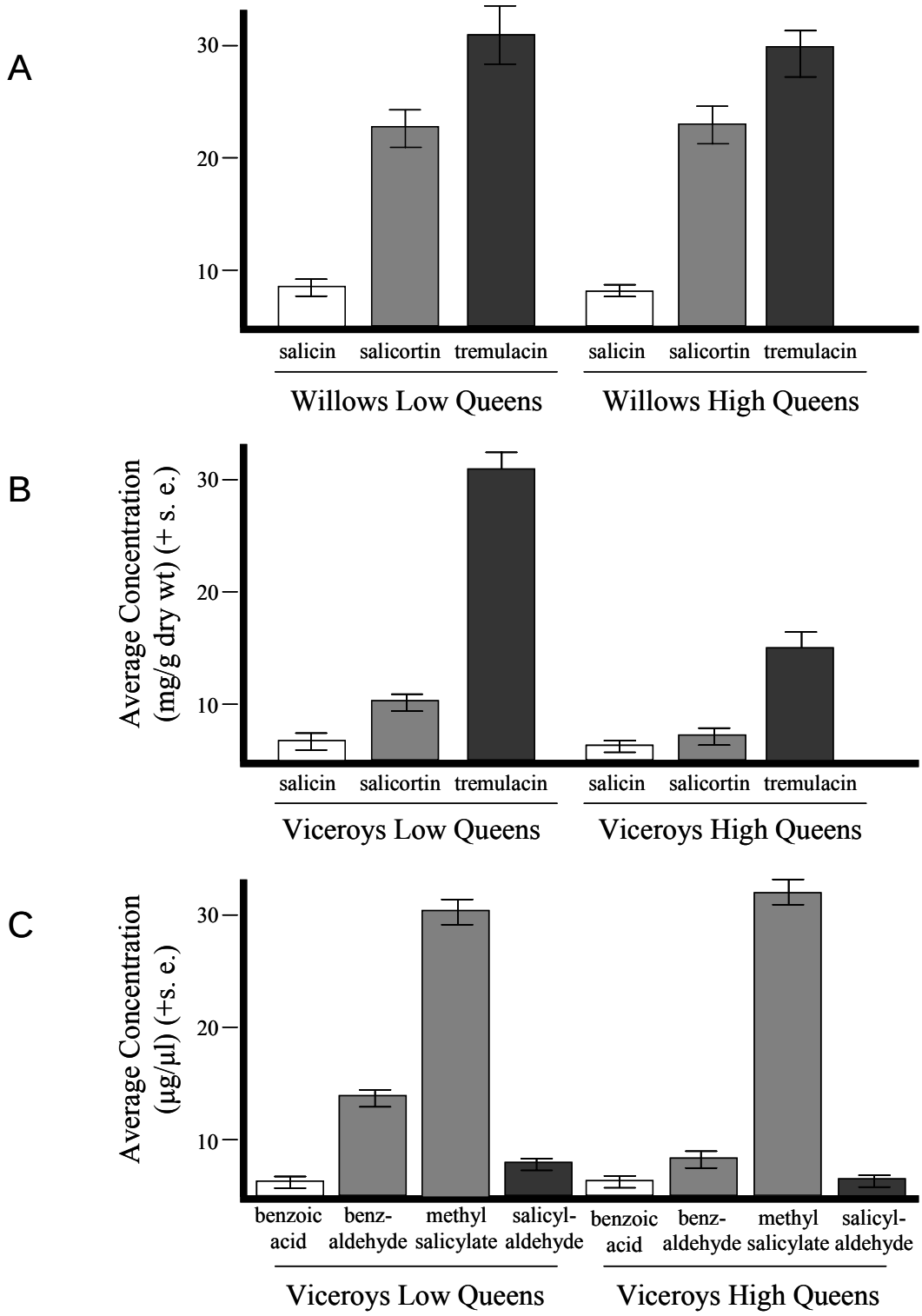
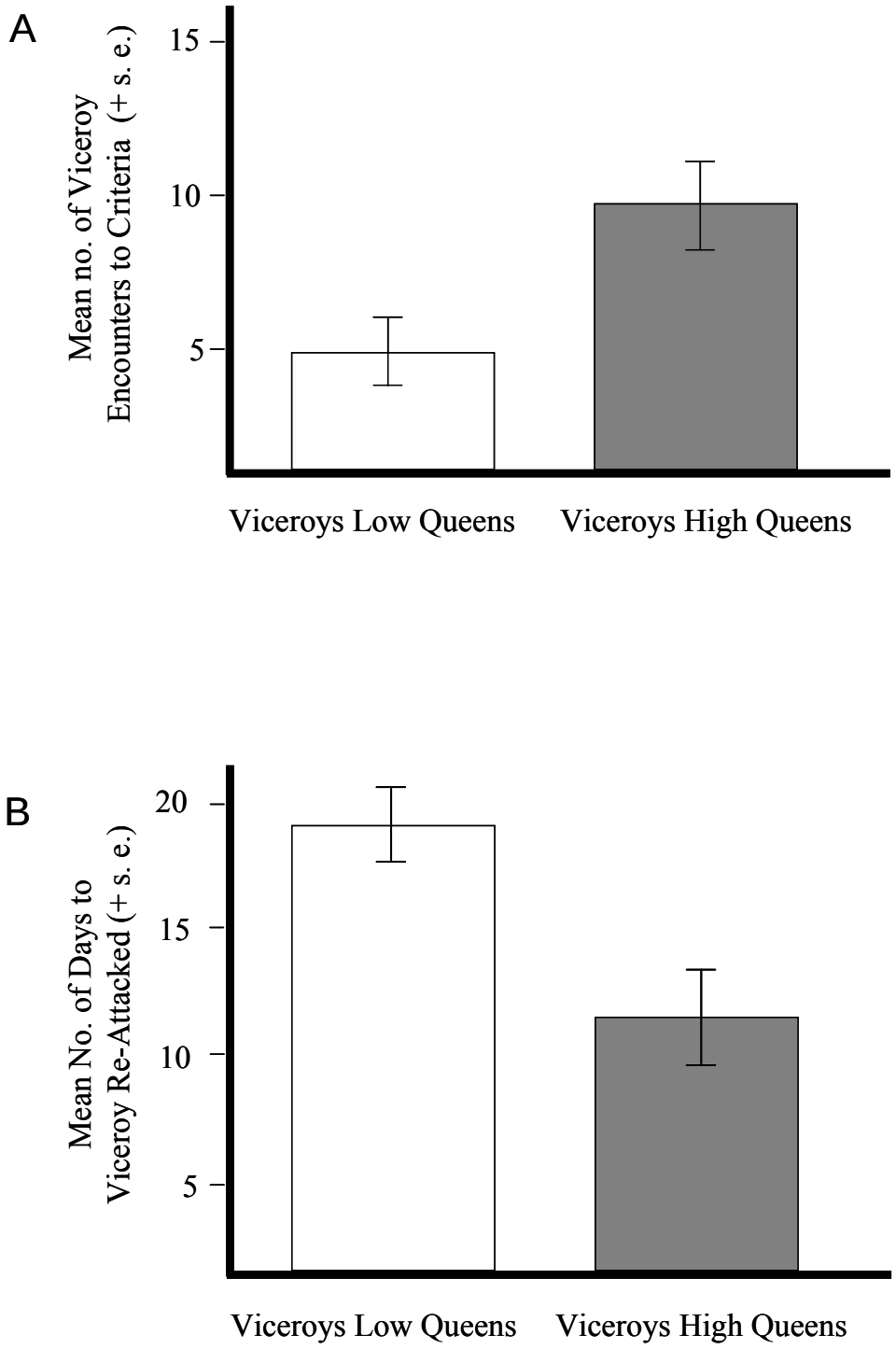

A
30
20
10
salicin
salicortin
tremulacin
salicin
salicortin
tremulacin
Willows Low Queens
Willows High Queens
B
Average Concentration
(mg/g dry wt) (+ s. e.)
30
20
10
salicin
salicortin
tremulacin
salicin
salicortin
tremulacin
Viceroys Low Queens
Viceroys High Queens
C
Average Concentration
(µg/µl) (+s. e.)
30
20
10
benzoic acid
benz-aldehyde
methyl salicylate
salicyl-aldehyde
benzoic acid
benz-aldehyde
methyl salicylate
salicyl-aldehyde
Viceroys Low Queens
Viceroys High Queens

Figure 3


A
Mean no. of Viceroy Encounters to Criteria (+ s. e.)
15
10
5
Viceroys Low Queens
Viceroys High Queens
B
Mean No. of Days to Viceroy Re-Attacked (+ s. e.)
20
15
10
5
Viceroys Low Queens
Viceroys High Queens

ADDENDIX D

ONCE A BATESIAN MIMIC, NOT ALWAYS A BATESIAN MIMIC: MIMIC REVERTS BACK TO ANCESTRAL PHENOTYPE WHEN MODEL IS ABSENT

Once a Batesian mimic, not always a Batesian mimic: mimic reverts back to ancestral phenotype when the model is absent

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Abstract

Batesian mimics gain protection from predation through the evolution of physical similarities to a model species that possesses anti-predator defenses. This protection should not be effective in the absence of the model since the predator does not identify the mimic as potentially dangerous and both the model and the mimic are highly conspicuous. Thus, it is presumed that the Batesian mimic always occurs within the geographic range of the model even though suitable habitat may exist outside of that range. There are several documented examples of Batesian mimics occurring in locations without their models, but the evolutionary responses remain largely unidentified. A mimetic species has four alternative responses to the loss of model presence. If predation is weak, it could maintain its mimetic signal. If predation is intense, it is widely presumed the mimic will go extinct. However, the mimic could also evolve a new color pattern to mimic another model species or it could revert back to its ancestral, non-mimetic and presumably less conspicuous, phenotype. We used molecular phylogenetic approaches to reconstruct and test the evolution of mimicry in the North American admiral butterflies (*Limenitis*: Nymphalidae). We confirmed that the non-mimetic, white-banded form is the ancestral phenotype within all admiral butterflies. However, one species, *L. arthemis*, evolved the black, pipevine swallowtail mimetic form but later reverted to the white-banded ancestral form. This character reversion is strongly correlated with the geographic absence of the model species and its host plant. Our results support the prediction that a Batesian mimic does not persist in locations without

its model, but they do not go extinct either. The mimic can revert back to its ancestral, less conspicuous form.

Keywords: character evolution, Lepidoptera, parametric bootstrap, wing pattern evolution

Introduction

Many organisms have evolved defensive color patterns to mitigate the persistent risk of predation. Aposematic, or warning, coloration is one such defensive strategy used by noxious organisms to visually communicate their toxicity or distastefulness to potential predators (Cott 1940; Guilford 1990). An aposematic pattern confers survival benefits to the prey because it is both easier for the predator to learn and less likely to be forgotten (Gittleman & Harvey 1980; Roper & Redstone 1987; Roper 1990; Alatalo & Mappes 1996; Prudic *et al.* 2007). These benefits are believed to have facilitated the evolution of aposematic coloration from ancestrally cryptic patterns (Fisher 1930; Sherratt & Beatty 2003). Closely related to aposematic coloration is visual defensive mimicry. Visual defensive mimicry is often a deceptive signal, defined as a close physical resemblance among unrelated species (Bates 1862). Batesian mimicry is a specialized signal with complex interactions between the honest signaler, the cheater and the predators (Ruxton *et al.* 2004 and references therein). In this mimicry system, an unprotected species (mimic) evolves a physical resemblance to an aposematic species (model), and the mimic gains protection from predation by deceiving the predators. This relationship is considered parasitic because the unprotected species undermines the effectiveness of the aposematic signal.

Much theoretical and empirical research has focused on the conditions necessary for Batesian mimicry to arise and how it functions once it exists, but much less is known about how these relationships change through time, especially when the model is no longer present (Ruxton *et al.* 2004; Mappes *et al.* 2005). It is presumed that the

protection afforded to the mimic should break down in locations where the model is absent because the predators would not recognize either the model or mimic as unprofitable prey and the mimic has a highly conspicuous phenotype (Pfennig *et al.* 2001; Ruxton *et al.* 2004; Pfennig *et al.* 2007). The geographic range of the mimic is predicted to be limited by the geographic range of the model even though suitable habitat for the mimic may exist outside that range. Yet, there are examples where a mimic has been documented occurring in locations without its model (Ritland 1995; Pfennig *et al.* 2001; Prudic *et al.* 2002). In response to this change in model presence, the mimic species has several potential responses. This species could remain a Batesian mimic in locations without its model, especially when predation is weak (Pfennig *et al.* 2007). A mimic could also go extinct in these locations due to intense predation (Pfennig *et al.* 2001). Less explored are the expectations that the mimic could evolve a new color pattern to mimic another model species or revert back to its ancestral, non-mimetic phenotype (Ruxton *et al.* 2004). The latter two scenarios are considered the least likely and have the least empirical support because it is assumed Batesian mimicry and its considerable phenotypic change is underlain by considerable genetic change (Fisher 1930; Ruxton *et al.* 2004; Pfennig *et al.* 2007).

The North American admiral butterflies (Nymphalidae: *Limenitis*) provide a unique opportunity to investigate the evolution and maintenance of Batesian mimicry relationships. The ancestral wing pattern commonly found in Eurasian congeners is a disruptive pattern with a black ground color and dorsal-lateral white band (Platt 1983; Mullen 2006). However, in North America, three of the four species are mimics, each of

a different model species, and all with very specialized divergent wing morphologies (Prudic *et al.* 2002 and references therein). Unlike other well-studied butterfly mimicry systems such as *Heliconius*, these three species also have different larval host plant requirements than their respective models (Opler *et al.* 2004), and may exist in locations without their respective models. Of particular interest is the red-spotted purple (*L. arthemis astyanax* and *L. a. arizonensis*), a Batesian mimic of the pipevine swallowtail (Papilionidae: *Battus philenor*) (Platt *et al.* 1971). The white admiral (*L. a. arthemis* and *L. a. rubrofasciata*) is a close, non-mimetic relative of the red-spotted purple that exhibits the putative ancestral white-banded form of the Eurasian congeners. Based on wing color information, it has been hypothesized that the white admiral spread east from Eurasia across Beringia and down to its current range. Then the red-spotted purple arose in sympatry with the model, the pipevine swallowtail (Remington 1958; Platt & Brower 1968; Platt 1983). However, recent phylogenetic evidence indicates that this assumption of evolutionary history may not be the case. The white admiral may have arisen from the red-spotted purple lineage, but this hypothesis warrants further investigation (Mullen 2006).

Accurate phylogenies are powerful frameworks for comparative biology and the study of adaptation. However, this historical perspective is not generally used in the study of aposematism and mimicry (Härlin & Härlin 2003). The addition of phylogenetic information provides understanding of the evolution of aposematism that has not otherwise emerged from mathematical models or behavioral experiments (Ruxton *et al.* 2004). Here we reconstructed the evolutionary history of North American *Limenitis*

using molecular phylogenetic methods. We documented the direction of the evolutionary transitions from non-mimetic to mimetic signalers and vise versa. Using parametric bootstrapping we evaluated the likelihood that a mimetic form changed back to the ancestral, cryptic form. We then investigated if Batesian mimicry transitions from mimetic to non-mimetic were correlated with a loss of the model species, based on the geographic distribution of the model and its larval host plant.

Methods and Materials

Taxon sampling

We sampled all the North American members of the genus *Limenitis* and 3 species of Palearctic *Limenitis* (Table 1). We sampled all subspecies of *L. arthemis* and most subspecies of *L. archippus* and *L. weidemeyerii* (Hodges *et al.* 1983). We did not include any specimens from overlap or hybrid zones, as classification of hybrid individuals is difficult and may hinder phylogenetic inference (Grant & Grant 1998). Additionally, we included samples from two closely related genera, *Neptis* and *Adelpha*, as outgroups (Willmott 2003).

DNA extraction and sequencing

To evaluate the evolution of mimetic phenotypes, we first inferred the evolutionary relationships among North American *Limenitis* using one mitochondrial gene, cytochrome oxidase subunit II (*COII*), and one nuclear gene, Elongation factor 1 alpha (*EF1a*). We extracted total genomic DNA from leg or thoracic tissue using

DNeasy Tissue Extraction (Qiagen Inc., CA, USA) per the manufacturer's protocol. PCR reactions (50µl) were performed on a Mastercycler (Eppendorf, NY, USA), in 1X Eppendorf Hotmaster Taq Buffer containing 0.2 µM of each primer, 0.1 mM dNTPs, 2.5 mM MgCl2, and 1 unit of Eppendorf Hotmaster™ Taq DNA Polymerase. We amplified *COII* using the amplification primers Pierre and Eva (Caterino & Sperling 1999). The thermal cycle profile was an initial 1.5 min denaturation at 94°C; 32 cycles of 40 sec at 94°C, 40 sec at 45°C, and 45 sec at 72°C; a 7 min final extension at 72°C. To amplify *EF1a*, we used the amplification primers ef44f and efrcM4r (Monteiro & Pierce 2001) with the profile: initial 2 min denaturation at 94°C; 35 cycles of 20 sec at 94°C, 15 sec at 50°C, and 60 sec at 65°C; a 5 min final extension at 65°C. PCR products were purified with a Millipore Size Exclusion filtration (Millipore, MA, USA) prior to direct sequencing. Both strands were sequenced using an Applied Biosystems BigDye™ Terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, USA) and run on an Applied Biosystems 3730XL DNA Analyzer (Applied Biosystems, CA, USA). We generated consensus sequences from the two strands and aligned these sequences by eye with the aid of the program BioEdit (Hall 1999).

Phylogenetic analyses

We performed maximum parsimony and Bayesian MCMC on both the *COII* data and the *EF1a* data separately. For each gene, we began by removing redundant sequences from the alignment. Maximum parsimony analyses were performed in PAUP*4.0b10 (Swofford 2001). We initially performed a heuristic tree search with 1000

random-addition replicates, saving all the most parsimonious trees per replicate. To assess node support, we performed 1000 non-parametric bootstrap pseudoreplicates, each with 10 stepwise-addition, nearest-neighbor interchange heuristic search replicates, saving the first 1000 most parsimonious trees per heuristic search replicate.

For the Bayesian analysis, we used a hierarchical Likelihood Ratio Test (Huelsenbeck & Rannala 1997) to estimate the model of evolution. Both *COII* and *EF1a* best fit a GTR + Γ model, with empirical base frequencies. Using this model in Mr.Bayes 3.01 (Huelsenbeck & Ronquist 2001), we analyzed each gene separately; in each analysis, we ran four chains (three hot and one cold) for 2,000,000 generations, sampled trees every 100 generations, and discarded the trees sampled in the first 1,000,000 generations (burnin). To estimate the posterior probability of clades, we used the post-burnin sampled trees to generate a 50% majority rule consensus tree using the 'sumt' command in MrBayes.

To infer the relationships among North American *Limenitis*, we used the two gene trees to generate a species tree in Mesquite (Maddison & Maddison 2006). Briefly, because the two genes are evolving independently, and may not always accurately reflect the history of the species' relationships, we used the gene trees to search for the 'best fit' species tree that minimizes the number of deep coalescences of the gene trees (Maddison 1997). Gene trees of recently diverged taxa may be monophyletic due to incomplete lineage sorting (Hudson & Coyne 2002; Funk & Omland 2003), although a significant phylogenetic signal may still be present (Maddison & Knowles 2006). By accounting for the possibility of incomplete lineage sorting, this approach infers of species' relationships

without requiring the gene trees to be monophyletic. We used the heuristic search function in Mesquite to find the optimal species tree that minimizes the number of deep coalescences of the gene trees, treating gene trees as unrooted and allowing polytomies to be automatically resolved before the coalescence cost is assessed (Maddison & Maddison 2006)

Character evolution

We coded wing morphology into four states: 0=White-Banded (*Limenitis arthemis arthemis*, *L. arthemis rubrofasciata*, *L. weidemeyerii*), 1=White-Banded with Orange Tip (*L. lorquini*), 2=*Danaus* mimic (*L. archippus*), 3=Black (*L. arthemis astayanx*, *L. arthemis arizonensis*). Ancestral character states were reconstructed on the species tree in Mesquite (Maddison & Maddison 2006), using a parsimony model of unordered states.

Parametric bootstrapping

To determine if our reconstructions were significantly better than other reconstructions, we performed phylogenetic parametric bootstrapping (Huelsenbeck *et al.* 1996) on the *COII* gene tree. Briefly, we compared parsimony treelengths of an unconstrained search to treelengths of a search constrained to retain only those trees in which the black-winged taxa formed an exclusive clade. This difference in treelengths represented our observed test statistic (δ_{obs}). From the most parsimonious constrained trees, we used the tree with the highest likelihood to estimate the model of evolution

using hierarchical Likelihood Ratio Tests (Huelsenbeck & Rannala 1997). This model (GTR+Γ+I) was then implemented in Mesquite v.1.12 (Maddison & Maddison 2006) to simulate 1000 character matricies on the most parsimonious reconstruction with an exclusive black-winged clade. For each matrix, we performed two parsimony treesearches, one unconstrained and one constrained as above, and the difference in treelengths were used to generate a distribution of the test statistic, δ. We determined the significance of our results by comparing δ_{obs} with the simulated distribution of δ. A significant value of δ_{obs} leads to rejection of the hypothesis that the clade tested is monophyletic.

Host plant distribution and geographic range of L. arthemis mimetic forms

We used published information regarding butterfly and host plant county records to test if the geographic distribution of *L. arthemis* black (mimetic) form was correlated with the geographic distribution of the model, the pipevine swallowtail and/or the pipevine's larval host plants (*Aristolochia* spp.). We evaluated this question at two scales: at the level of our taxonomic sampling and at the level of the entire geographic range of the *L. arthemis*. For the butterfly information, we used two sources of county records (Opler *et al.* 2004; Landberry *et al.* 1998) and for the plant information, we used one source of county records (USDA 2007). From this information, we were able to document presence or absence of *L. arthemis*' larval host plant (*Salix* spp.), the model (*Battus philenor*) and its larval host plant (*Aristolochia* spp.) at both the exact locations where we sampled and across the entire range of *L. arthemis*. The model or the plant

species was categorized as present if it had ever been recorded in a given county where *L. arthemis* occurred. For our collection data, seventeen different counties were examined, 6 with the white-banded form and 11 with the black form. For the larger geographic study, 1509 out of 3135 counties in the United States (excluding Hawaii) had records of L. arthemis: 144 counties with the white-banded form only, 1166 counties with the black form only, and 199 counties with both forms together. We used linear regression in JMP-IN (JMP 2002) to evaluate factors predicting the geographic distributions of the mimetic and non-mimetic forms of *L. arthemis*.

Results

Phylogenetics & character evolution

Parsimony and Bayesian reconstructions for each gene were topologically congruent, although Bayesian reconstructions showed support for some clades that were not supported in the parsimony analyses (Figure 1). For *COII*, there were no shared haplotypes among the species of *Limenitis*. Within *L. arthemis*, the white-banded form and the black form also shared no haplotypes. There were no shared haplotypes between the two black form subspecies (*L. a. astyanax and L. a. arizonensis*). In contrast, many *EF1a* haplotypes were found in more than one species, and only *L. archippus* was recovered as monophyletic. The best fit species tree, based minimizing deep coalescences of the *COII* and *EF1a* Bayesian gene trees, is shown in Figure 2. Our results confirm that the white-banded form is the ancestral phenotype in the group (Mullen 2006). Character reconstructions on this species tree suggest a reversion from a

black, mimetic phenotype in *L. arthemis* to the white-banded, non-mimetic phenotype (Figure 2).

The most parsimonious trees in unconstrained searches of the *COII* data were 276 steps and the most parsimonious trees in constrained searches were 284 steps (δ_{obs} = 284-276=8). This falls well outside of our simulated distribution of δ (mean δ = 0.242, 95% CI = 0-3), so we reject an exclusive black-winged clade at $p<0.001$.

Host plant distribution and geographic range of L. arthemis mimetic forms

For the specimens used in the molecular study, the presence of willow, the major *L. arthemis* larval host plant, was found in every county in which *L. arthemis* was found so the presence of the host plant did not explain the different geographic distributions of the two *L. arthemis* forms (N=17). However, the presence of the white-banded form of *L. arthemis* was negatively correlated with the presence of the model, *Battus philenor*, in a county (N=6, r^2=0.45, p=0.004), whereas the black form was positively correlated with the presence of the model (N=11, r^2=0.75, $p<0.001$) (Table 2). In addition, the presence of the host plants of *Battus philenor*, *Aristolochia* spp., were positively correlated with the presence of black form of *L. arthemis* (N=11, r^2=0.33, p=0.024), but were negatively correlated with the presence of the white-banded form (N=6, r^2=0.31, p=0.025)(Table 2). For the entire geographic range of *L. arthemis*, willow always occurred in the same county as any *L. arthemis* and did not explain the different geographic distributions of the *L. arthemis* forms (N=1509). The presence of the white-banded form was again negatively correlated with the presence of the model, *Battus philenor* (N=346, r^2=0.15,

$p<0.001$), and the black form was positively correlated with the model (N=1362, $r^2=0.75$, $p<0.001$)(Table 2). These patterns were also explained by the distribution of the model's larval host plant (white-banded form: N=346, $r^2=0.22$, $p<0.001$, slope negative; black form: N=1362, $r^2=0.27$, $p<0.001$, slope positive).

Based on the results from both datasets, the presence of the black, mimetic form of *L. arthemis* is positively correlated with the presence of its model, *B. philenor* and its model's larval host plants, *Aristolochia* spp. Also, the presence of the white-banded, non-mimetic form of *L. arthemis* is negatively correlated with the presence of the model and its larval host plants. This difference in geographic distribution of the two forms of *L. arthemis* is not predicted by the presence of the larval host plants, *Salix* spp. There is suitable host for *L. arthemis* in locations both with *B. philenor* and without.

Discussion

Batesian mimicry is a highly specialized interaction between the honest signaler, the cheater, and the predator. The cheater or mimic gains protection from predation by resembling a dangerous or unpalatable species. Because it is dependant on this false signal, the Batesian mimic should not occur in locations without its model. The predators will not recognize the mimic as potentially dangerous, and they will also predate on the mimics more intensely because they are highly conspicuous (Ruxton *et al.* 2004; Pfennig *et al.* 2007). Yet, there are documented examples of a Batesian mimic occurring in locations without its model in ecological time (Ritland 1995; Pfennig *et al.* 2001; Prudic *et al.* 2002). Here we examined if a Batesian mimic could persist outside the range of its

model from an evolutionary perspective. There are four potential evolutionary outcomes outlined in the literature. A Batesian mimetic species could go extinct if predation was intense or it could persist as a Batesian mimic if predation was weak (Pfennig *et al.* 2007). Although thought to be more unlikely because of genetic constraints, a mimic could also evolve a new color pattern to mimic another model species; or it could revert back to its ancestral, non-mimetic phenotype (Fisher 1930; Ruxton *et al.* 2004). The data presented here support the hypothesis that a Batesian mimic does not persist in locations without its model, but it does not go extinct either (Figure 1; Table 2). Instead, in locations were the model and larval host plants do not occur, the mimic reverted back to its ancestral, non-mimetic form that is less conspicuous. Character reconstructions on the species tree suggest a reversion from a black, mimetic wing phenotype in *L. arthemis* to the white-banded, non-mimetic phenotype in *L. arthemis* (Figure 2). This evolutionary pattern is in contrast to other hypotheses of wing evolution in this group which purport the black form evolved from the white-banded for in this species (Remington 1958; Platt & Brower 1968; Platt 1983).

The reversion to a non-mimetic form is also consistent with the geographical distributions of the model and the model's larval host plants. The geographic distribution of the non-mimetic form (white-banded form) of *L. arthemis* is negatively correlated with the geographic range of *Battus philenor*; however, the mimetic form is positively correlated (Table 2). The white-banded form of *L. arthemis* is thought to be disruptive, making the butterfly difficult to track through riparian habitat and is most likely a better defensive strategy when the Batesian model is absent (Platt 1983). A similar geographic

pattern is found in another Batesian mimic of *Battus philenor*, the eastern tiger swallowtail (*Papilio glaucus*: Papilionidae), which is a female limited Batesian mimic of *B. philenor* (Brower 1958). Both mimetic and non-mimetic female forms of *P. glaucus* are present in locations where *B. philenor* occurs. However, in the same geographic region that *L. arthemis* changes from the black form to the white-banded form and *B. philenor* becomes increasingly rarer, *P. glaucus* females occur only as non-mimetic forms (Brower & Brower 1962).

This reversion to a non-mimetic wing phenotype in *L. arthemis* is not unexpected given our knowledge of the genetic architecture underlying wing pattern variation. Even though butterfly wing patterns are a complex trait, the genetic mechanisms may be quite simple and relatively easy to change (Nijhout 1991). In butterfly Batesian mimicry, the mimic's wing pattern is thought to be produced by a single gene of major effect with a suite of modifier genes that refine the wing pattern (Turner 1977). This mechanism has not been demonstrated definitively in *Limenitis* (but see Platt 1983); although, it has been shown in other Batesian mimics such as *Papilio dardanus* (Nijhout 2003). Also, in *Heliconius* butterflies, a homologous gene or complex of genes regulates wing pattern diversity in three different species with different phenotypes (Joron *et al.* 2006). Rather than a constraining role, this locus seems to provide wing phenotype flexibility across species and environments. It presumably functions by responding to a wide range of selection pressures including predation to produce radically divergent, locally adapted wing patterns from a very similar genetic mechanism. The genes regulating butterfly wing patterns do not appear to become canalized when these species become mimics.

Our results also relate to a previously unreported phenomenon regarding butterfly distribution and range**.** Butterfly species, like many phytophagous insects, are limited by the distribution of their larval host plants. Host acceptance and selection is determined by a wide variety of ecological and physiological factors (e.g. Futuyma & Moreno 1988; Bernays 1989; Thompson 2005). However, all of these factors relate to the interaction between insect species and host plant species. Our results suggest that the distribution of the black form of *L. arthemis* is determined not by the distribution of its own larval host plant, but by the distribution of its model's larval host plant (Table 2). Thus, the broad geographic distributions of phytophagous insect species and subspecies also depend on other indirect interactions in their communities other than the relationship with its own larval host plant. This distributional pattern has been shown multiple times at a community scale (e.g. Bernays 1989) but not across the larger geographic range of a species or subspecies.

Conclusions

Overall, our results document that a Batesian mimic can not persist in locations without its model. This is pattern in correlated to the presence of the model and the model's host plants. Batesian mimicry is a specialized signal with complex interactions between the honest signaler, the cheater, and the predators in a community. The cheater's distribution may be limited by the distribution of the honest signaler in many cases. However, our findings also demonstrate that a mimic does not necessarily go extinct either without its model. When the aposematic model is absent, the mimic can

revert to its ancestral, non-mimetic form which is less conspicuous to predators and persist without its Batesian model.

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Tables, Figures, and Appendices

Table 1. Specimen information for molecular phylogeny

Taxon	N	Locality	Wing Morphology
Adelpha bredowii	1	Plumas Co. California, USA	White-banded with orange tip
Limenitis archippus	1	Graham Co. Arizona, USA	Orange, *Danaus* mimic
	2	Santa Cruz Co. Arizona, USA	Orange, *Danaus* mimic
	1	Collier Co. Florida, USA	Orange, *Danaus* mimic
	1	Levy Co. Florida, USA	Orange, *Danaus* mimic
	1	Thomas Co. Georgia, USA	Orange, *Danaus* mimic
	1	Jones Co. Iowa, USA	Orange, *Danaus* mimic
	1	Hampshire Co. Massachusetts, USA	Orange, *Danaus* mimic
	3	Chambers Co. Texas, USA	*Danaus* mimic
	1	Dane Co. Wisconsin, USA	Orange, *Danaus* mimic
L. arthemis arthemis	1	Hennepin Co. Minnesota, USA	White-banded, non-mimetic
	1	Forest Co. Wisconsin, USA	White-banded, non-mimetic
	1	Dane Co. Wisconsin, USA	White-banded, non-mimetic
L. arthemis arizonensis	5	Santa Cruz Co. Arizona, USA	Black, *B. philenor* mimic
	3	Cochise Co. Arizona, USA	Black, *B. philenor* mimic
	1	Catron Co. New Mexico, USA	Black, *B. philenor* mimic
L. arthemis astyanax	1	Alachua Co. Florida, USA	Black, *B. philenor* mimic
	1	Brooks Co. Georgia, USA	Black, *B. philenor* mimic
	1	Lowndes Co. Georgia, USA	Black, *B. philenor* mimic
	1	Muron Co. Indiana, USA	Black, *B. philenor* mimic

	1	Calvert Co. Maryland, USA	Black, *B. philenor* mimic
	1	Hampshire Co. Massachusetts, USA	Black, *B. philenor* mimic
	1	Co. Mississippi, USA	Black, *B. philenor* mimic
	2	Abbeville Co. South Carolina, USA	Black, *B. philenor* mimic
L. arthemis rubrofasciata	1	Edmonton, Alberta, Canada	White-banded, non-mimetic
	1	Calgary, Alberta, Canada	White-banded, non-mimetic
	4	Fairbanks North Star Co. Alaska, USA	White-banded, non-mimetic
L. camilla	1	Friuli, Italy	White-banded, non-mimetic
	2	Yamanashi, Japan	White-banded, non-mimetic
	1	Lerida, Spain	White-banded, non-mimetic
L. lorquini	1	Colusa Co. California, USA	White-banded with orange tip, *Adelpha* mimic
	1	Inyo Co. California, USA	White-banded with orange tip, *Adelpha* mimic
	1	Modoc Co. California, USA	White-banded with orange tip, *Adelpha* mimic
	2	Mono Co. California, USA	White-banded with orange tip, *Adelpha* mimic
	1	Sierra Co. California, USA	White-banded with orange tip, *Adelpha* mimic
	1	Solano Co. California, USA	White-banded with orange tip, *Adelpha* mimic
	2	Douglas Co. Nevada, USA	White-banded with orange tip, *Adelpha* mimic
	1	Carson City, Nevada, USA	White-banded with orange tip, *Adelpha* mimic

L. populi	1	Fracto-Drama, Greece	White-banded, non-mimetic
	1	Nacka Erstavik, Sweden	White-banded, non-mimetic
L. reducta	2	Katerini, Greece	White-banded, non-mimetic
	1	Lerida, Spain	White-banded, non-mimetic
L. weidemeyerii	1	Las Animas Co. Colorado, USA	White-banded, non-mimetic
	2	Catron Co. New Mexico, USA	White-banded, non-mimetic
	3	Elko Co. Nevada, USA	White-banded, non-mimetic
	1	Eureka Co. Nevada, USA	White-banded, non-mimetic
	1	Taos Co. New Mexico, USA	White-banded, non-mimetic
	1	Sublette Co. Wyoming	White-banded, non-mimetic
Neptis rivularis	1	Kahaeibar-Azarbaiyan, Iran	White-banded, non-mimetic

Table 2. Presence and absence county records of the model *Battus philenor* and its larval host plant, *Aristolochia* spp., in relation to county records of the mimetic and non-mimetic forms of *L. arthemis*. a) is the data from the specimens used in the molecular phylogeny; b) is the data across the entire geographic range of *L. arthemis*

a)

		County presence of *L. a. astyanax* & *L. a. arizonensis* (mimetic, black form)	**County presence of *L. a. arthemis* & *L. a. rubrofasicata* (non-mimetic, white-banded form)**
***Battus philenor* Batesian model**	Present	10	0
	Absent	1	6
***Aristolochia* spp. (*Battus* host)**	Present	8	0
	Absent	3	6

b)

		County presence of *L. a. astyanax* & *L. a. arizonensis* (mimetic, black form)	**County presence of *L. a. arthemis* & *L. a. rubrofasicata* (non-mimetic, white-banded form)**
***Battus philenor* Batesian model**	Present	897	90
	Absent	465	256
***Aristolochia* spp. (*Battus* host)**	Present	788	35
	Absent	574	311

Figure Legends

Figure 1

Consensus trees from Bayesian inference of (a) *COII* and (b) *EF1a*. Branch labels show Maximum Parsimony Bootstrap support/Bayesian posterior probability. Wing morphology character states for terminal taxa are shown.

Figure 2

Species tree of North American *Limenitis* inferred from minimizing deep coalescences of gene trees. Branch colors show maximum parsimony character reconstructions of ancestral states (orange=*Danaus* mimic, black=*Battus* mimic, hatched pattern=*Adelpha* mimic, white=non-mimetic, disruptive coloration).

Figure 1

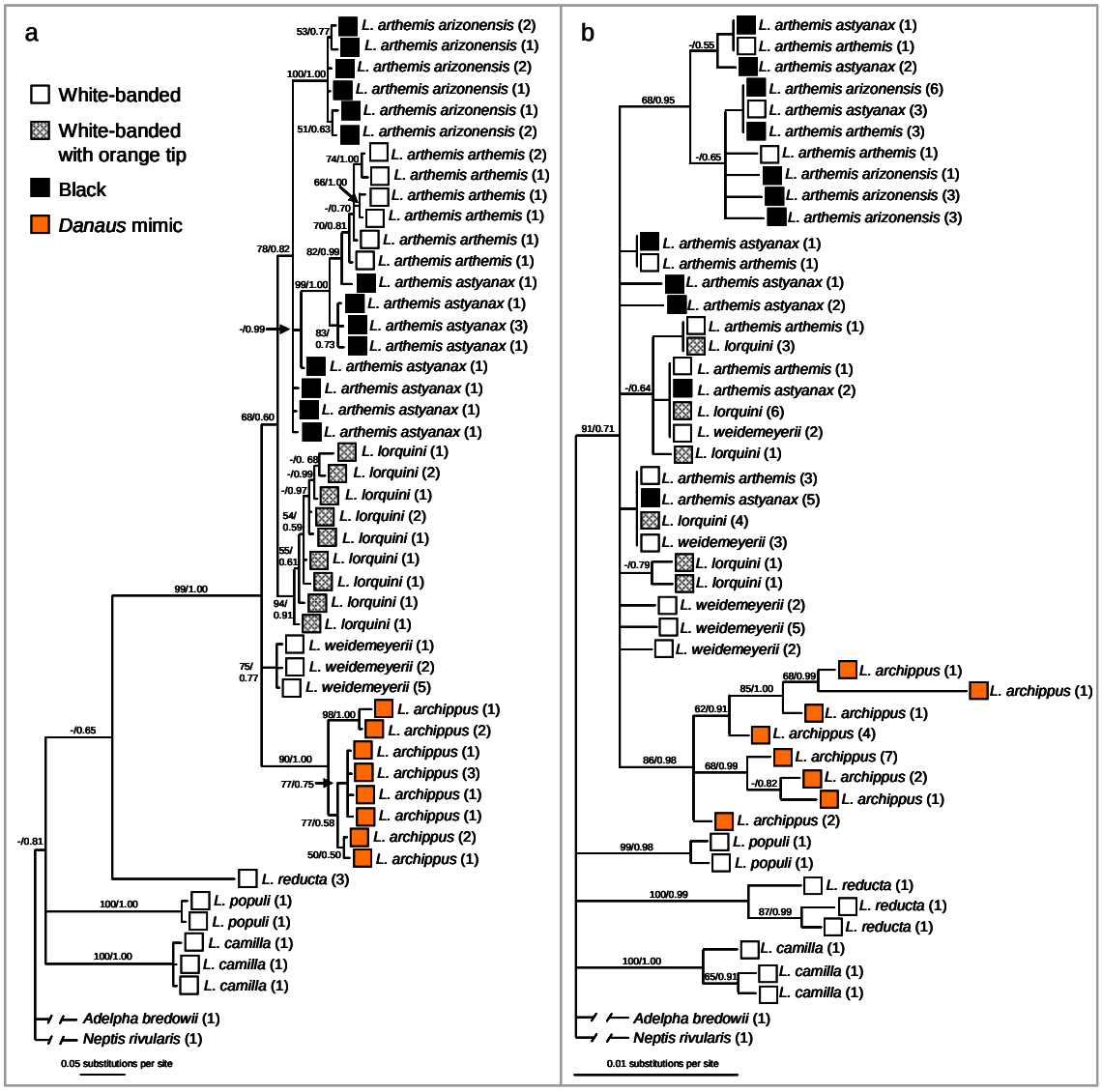
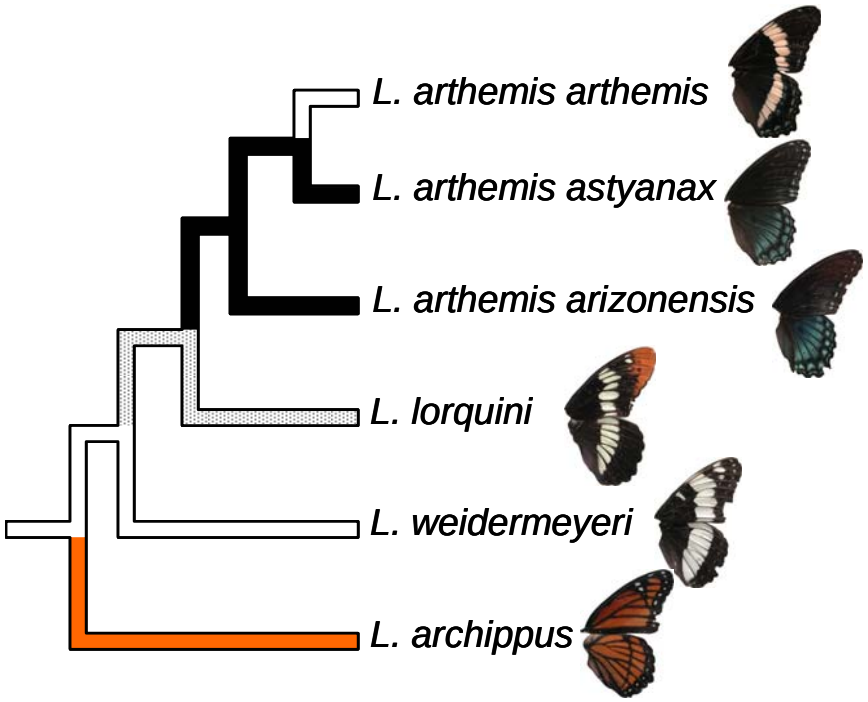

a
White-banded
White-banded with orange tip
Black
Danaus mimic
L. arthemis arizonensis (2)
L. arthemis arizonensis (1)
L. arthemis arizonensis (2)
L. arthemis arizonensis (1)
L. arthemis arizonensis (1)
L. arthemis arizonensis (2)
L. arthemis arthemis (2)
L. arthemis arthemis (1)
L. arthemis arthemis (1)
L. arthemis arthemis (1)
L. arthemis arthemis (1)
L. arthemis astyanax (1)
L. arthemis astyanax (1)
L. arthemis astyanax (3)
L. arthemis astyanax (1)
L. arthemis astyanax (1)
L. arthemis astyanax (1)
L. arthemis astyanax (1)
L. lorquini (1)
L. lorquini (2)
L. lorquini (1)
L. lorquini (2)
L. lorquini (1)
L. lorquini (1)
L. lorquini (1)
L. lorquini (1)
L. weidemeyerii (1)
L. weidemeyerii (2)
L. weidemeyerii (5)
L. archippus (1)
L. archippus (2)
L. archippus (1)
L. archippus (3)
L. archippus (1)
L. archippus (1)
L. archippus (2)
L. archippus (1)
L. reducta (3)
L. populi (1)
L. populi (1)
L. camilla (1)
L. camilla (1)
L. camilla (1)
Adelpha bredowii (1)
Neptis rivularis (1)
0.05 substitutions per site
b
L. arthemis astyanax (1)
L. arthemis arthemis (1)
L. arthemis astyanax (2)
L. arthemis arizonensis (6)
L. arthemis astyanax (3)
L. arthemis arthemis (3)
L. arthemis arthemis (1)
L. arthemis arizonensis (1)
L. arthemis arizonensis (3)
L. arthemis arizonensis (3)
L. arthemis astyanax (1)
L. arthemis arthemis (1)
L. arthemis astyanax (1)
L. arthemis astyanax (2)
L. arthemis arthemis (1)
L. lorquini (3)
L. arthemis arthemis (1)
L. arthemis astyanax (2)
L. lorquini (6)
L. weidemeyerii (2)
L. lorquini (1)
L. arthemis arthemis (3)
L. arthemis astyanax (5)
L. lorquini (4)
L. weidemeyerii (3)
L. lorquini (1)
L. lorquini (1)
L. weidemeyerii (2)
L. weidemeyerii (5)
L. weidemeyerii (2)
L. archippus (1)
L. archippus (1)
L. archippus (1)
L. archippus (4)
L. archippus (7)
L. archippus (2)
L. archippus (1)
L. archippus (2)
L. populi (1)
L. populi (1)
L. reducta (1)
L. reducta (1)
L. reducta (1)
L. camilla (1)
L. camilla (1)
L. camilla (1)
Adelpha bredowii (1)
Neptis rivularis (1)
0.01 substitutions per site

Figure 2


L. arthemis arthemis
L. arthemis astyanax
L. arthemis arizonensis
L. lorquini
L. weidermeyeri
L. archippus

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