

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. These relationships were originally classified as Batesian, or parasitic, but were later reclassified as Müllerian, or mutualistic, based on predator bioassays. The Müllerian reclassification implies that 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 nonvolatile defensive compounds from its larval host–plant, the Carolina willow (*Salix caroliniana*, Salicaceae), but also secretes volatile defensive compounds when disturbed. 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. The phenolics we found are known to deter predator attack in other prey systems, including other willow-feeding insect species. Because these compounds have a generalized defensive function at

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the concentrations we described, our results are consistent with the Müllerian reclassification put forth by other researchers based on 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 warranting future study.

Keywords Chemical defense · Chemical mimicry · *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 nonrewarding (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, b). In these behavioral experiments, monarch and queen butterflies were 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, b), 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, viceroys were 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, 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 is 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 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 antiherbivore 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 *Phratora vitellinae* and *Chrysomela* spp. (Coleoptera: Chrysomelidae) (Pasteels et al. 1988; Köpf 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). By using high-performance liquid chromatography–mass spectrometry (LC/MS), and gas chromatography–mass spectrometry (GC/MS), we investigated whether (1) viceroy butterflies sequester nonvolatile defensive phenolic glycosides from their larval host-plants; (2) viceroy butterflies emit volatile defensive phenolic compounds when disturbed; and (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 *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 diverged from this ancestral pattern with an orange or brown ground color with black markings. Viceroy butterflies (*Limenitis archippus*) are widely distributed over North America, and they exhibit regional variation in adult color pattern (Scott 1986) that correlates with 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 (*Limenitis archippus archippus*, *Limenitis archippus 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 (*Limenitis archippus floridensis* and *Limenitis archippus obsolata*). Viceroy larvae primarily feed on *Salix* and *Populus* (Salicaceae), unlike monarch and queen larvae that feed on members of the Asclepiadaceae (Scott 1986). In Florida, where the biological collections for this study were done, the viceroy butterfly (*L. archippus 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, N26°31.69', W–81°40.67' and Newnan's Lake, Gainesville, Alachua County, N29°38.19', W–82°12.00'. These sites are 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), whereas a willow sample consisted of 16 young leaves, 2 leaves from 8 different plants (~10 g dry weight). All samples were weighed, then air-dried at room temperature for 1 wk, and re-weighed. For the GC/MS analysis, live butterflies were individually sampled by using both solid phase microextraction (SPME) 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 Apin 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; and 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; and female viceroy 2, 1.17 g. Whole butterflies including their wings were used in the extraction. 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 min, and then allowed to soak overnight. The mixture was partitioned with an equal volume of hexane, $\times 3$. The methanol fraction was concentrated to dryness, weighed, and 1 mg samples were resuspended in 1 ml of high-performance liquid chromatography (HPLC) grade methanol, then filtered through a 0.45- μm PTFE filter (Whatman) for analysis.

Identification of Nonvolatile Defensive Compounds Chromatograms were obtained by 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 \times 20 mm) and Waters Nova Pak reverse phase C18 column (4.6 \times 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 with an Agilent 1100 HPLC system tandem with Agilent MSD-Trap-SL ion trap mass spectrometer. LC parameters were the same as above. The MS acquisition parameters were positive electrospray ionization 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 –500 V, capillary current 24.4 nA, current end plate 1,138.6 nA, RF amplitude capillary exit 158.5 V, and skimmer 40.0 V.

Table 1 LC 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

Compounds were identified by comparing their retention times and mass spectra (MS and MS/MS) with those of authentic standards.

Quantification of Nonvolatile 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 by using 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 with 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. Concentrations were considered consistent if run 1 and run 2 were within 10% of each other. If not, then the sample was reinjected 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 Inc. 2002) with 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 that a predator would encounter when it disturbs a viceroy, StableFlex PDMS/DVB-coated SPME fibers (Supelco, USA) were used to sample the volatiles in the headspace above the abdomen

Table 2 Compound-specific LC/MS and LC/MS/MS identification and quantification results of viceroy butterfly and its larval host–plant

Compound	Retention Time (min)	Diagnostic Ions (m/z) Parent/Daughters	Willow Conc.±SD (mg/g dry wt.) ($N=4$)	Viceroy Conc.±SD (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

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+H₂O] from 8.0–10 min; and for tremulacin 546 m/z [M+H₂O] from 18.0–20.0 min.

LC–MS–MS = liquid chromatography–mass spectrometry–mass spectrometry

^aQuantification ion

of both stressed and normal butterflies (Borg-Karolson 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 1 hr of sampling of 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; 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 ($N=20$).

Data were collected with a Varian Saturn 2100T GC/MS and a Chrompack capillary column (CP Sil 8 CB; 30 m \times 0.25 mm; 0.5 μ m film thickness). The initial column oven temperature was set for 80°C, increasing to 210°C at 10°C/min; the injector, transfer line, and trap temperatures were 230, 250, 200°C, respectively, and the electron voltage was set at 70 eV. Ultra high purity 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 μ l/ml. Calibration curves from triplicate injections of 2.0 μ l were obtained by using the GC/MS protocol above. Peak integration and quantification were performed automatically with Saturn 2100 Workstation software. The same insects used for compound identification were reanalyzed for compound quantification; however, for this analysis, the secretion was sampled directly from the abdomen of the butterfly with a glass capillary (Supplemental Material Fig. 2). Two microliters of the secretion were collected from a disturbed butterfly and dissolved in 2.0 μ l of ethyl acetate with 1.0 μ l of 0.25 M *p*-chlorotoluene as the internal standard. Then, 2.0 μ l of this solution were injected directly into the GC column. Each butterfly sample was run twice on the GC 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 resampled 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 Inc. 2002) using one-way ANOVAs.

Results

In the LC/MS assays, we characterized the tissue chemical profiles of the Florida viceroy (*L. archippus floridensis*) and its larval host-plant Carolina willow (*S. caroliniana*). Of the five peaks shared between the viceroy and willow extracts, three 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. 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 viceroi during a predation simulation and compared it to the volatile profile of a viceroi at rest. During these collection bouts, an unstressed viceroi never emitted a secretion, but the stressed butterflies always did. By 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 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 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.

Fig. 1 Extracted ion chromatograms of shared phenolic glycosides of Carolina willow and viceroi 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 viceroi butterfly sample 1 (10 males) from Green Meadow Road site. Specific site information is provided in the methods and materials section

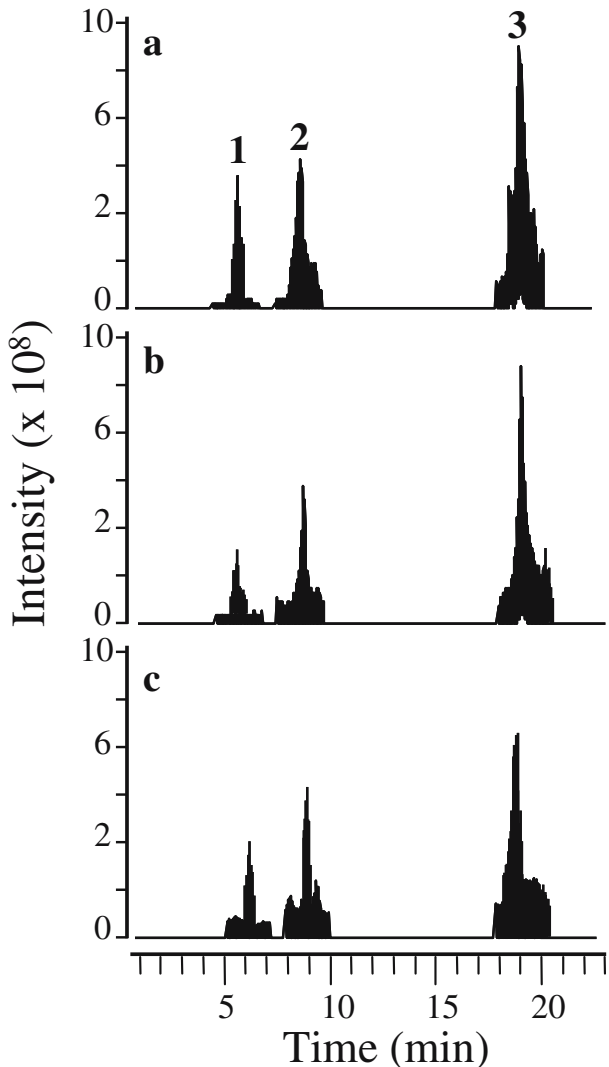


Table 3 Concentration averages of nonvolatile phenolic glycosides in willow and viceroy butterfly (total, geographic location, and sex) (LC/MS) and concentration averages of volatile phenolics released from stressed butterflies (total, geographic location, and sex) (GC/MS)

Compound	Willow Conc.±SD		Viceroy Conc.±SD		
	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) ^b	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) ^b	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 ^c	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 ^c	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) ^b	6.86+6.18 (M) vs. 5.38+3.26 (F)
Tremulacin ^c	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) ^b	24.46+18.83 (M) vs. 28.67+19.60 (F)

^a Measured in micrograms per microliter (total, $N=20$; geography, $N=10$; sex, $N=10$)

^b Significant difference (one-way ANOVA, $P<0.05$)

^c Measured in micrograms per gram dry weight (total, $N=4$; geography, $N=2$; sex, $N=2$)

F = Female, GMR = Green Meadow Road, M = male, NL = Newnan's Lake, NS = not sampled

Discussion

Originally, the viceroy butterfly was widely regarded as a palatable, Batesian mimic without any form of chemical defense (Brower 1958a, b). However, subsequent predator bioassays suggest that it is unpalatable and chemically defended against predation. These results led to a re-classification of the system as Müllerian (Ritland and Brower 1991). Our results are consistent with the Müllerian, not the Batesian, classification. We have shown that adult viceroys possess compounds that function as defensive compounds in other willow-feeding insects. We found three phenolic glycosides (salicin, salicortin, and tremulacin) in the larval host-plant, the Carolina willow, and the adult viceroy (Fig. 1; Table 2). It is 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 volatile 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). Future sampling that focuses 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 (*S. caroliniana*) has been chemically characterized for phenolic glycosides, and the three phenolic glycosides we documented resemble the chemical profiles of other willow species (Palo 1984; Nyman and Julkunen-Tiitto 2005) 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). In this case, they are 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 other bioassay experiments 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 at concentrations similar to the viceroy, they are deterrent and toxic, and they also 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 that 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 Sztajnkrzyer 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 are known to occur in the foliar scent of willows (Dötterl et al. 2005), and thus 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.

Our chemical results are consistent with other researchers' reclassification 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 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, a comparison of these two types of Müllerian systems will provide a 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 sequesters defensive phenolic glycosides from its larval host-plant, whereas 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 differences could actually facilitate Müllerian mimicry evolution by increasing rates of predator aversion learning and remembering (Skelhorn and Rowe 2005). Our findings provide a likely mechanism that explains previously reported viceroy unpalatability. These results are consistent with the Müllerian reclassification of this classic mimicry system.

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References

- ACKERY, P. R. and VANE-WRIGHT, R. I. 1984. Milkweed Butterflies. Cornell University Press, NY.
- BAIRLEIN, F. 1997. Food choice in birds and insect chemical defenses. *Entomol. Gener.* 21:205–216.
- BATES, H. W. 1862. Contributions to an insect fauna of the Amazon valley. Lepidoptera: Heliconidae. *Trans. Linn. Soc. London* 23:495–566.
- BERENBAUM, M. R. and MILICZKY, E. 1984. Mantids and milkweed bugs: efficacy of aposematic coloration against invertebrate predators. *Am. Midl. Nat.* 111:64–68.
- BORG-KARLSON, A. K. and MOZURAITIS, R. 1996. Solid phase micro extraction technique used for collecting semio chemicals: identification of volatiles released by individual signaling *Phyllonorycter sylvella* moths. *Z. Naturforsch.* 51c:599–602.
- BROWER, J. V. Z. 1958a. Experimental studies of mimicry in some North American butterflies. I. The monarch, *Danaus plexippus*, and viceroy, *Limenitis archippus*. *Evolution* 12:32–47.
- BROWER, J. V. Z. 1958b. Experimental studies of mimicry in some North American butterflies. III. *Danaus gilippus berenice* and *Limenitis archippus floridensis*. *Evolution* 12:273–285.
- BROWER, L. P., SEIBER, J. N., NELSON, C. J., LYNCH, S. P., and HOLLAND, M. M. 1984. Plant-determined variation in the cardenolide content, thin-layer chromatography profiles, and emetic potency of monarch butterflies, *Danaus plexippus* L (Lepidoptera, Danaidae) reared on milkweed plants in California. 2. *Asclepias speciosa* (Apocynales, Asclepiadaceae). *J. Chem. Ecol.* 10:601–639.
- CHARLESWORTH, D. and CHARLESWORTH, B. 1975. Theoretical genetics of Batesian mimicry. II. Evolution of supergenes. *J. Theor. Biol.* 55:305–324.
- DAVIDSON, C., ZIMMERMAN, E. F., and SMITH, P. K. 1961. Metabolism and toxicity of methyl salicylate. *J. Pharmacol. Exp. Ther.* 312:207–209.
- DENNO, R. F., LARSSON, S., and OLMSTEAD, K. L. 1990. Role of enemy free space and plant quality in host-plant selection by willow beetles. *Ecology* 71:124–137.
- DÖTTERL, S., FÜSSEL, U., JÜRGENS, A., and AAS, G. 2005. 1,4-Dimethoxybenzene, a floral scent compound in willows that attracts an oligolectic bee. *J. Chem. Ecol.* 31:2993–2998.
- FISHER, R. A. 1958. The Genetical Theory of Natural Selection, 2nd ed. Dover Publications, NY.
- GLENDINNING, J. I. 1992. The effectiveness of cardenolides as feeding deterrents to *Peromyscus* mice. *J. Chem. Ecol.* 18:1559–1575.
- GUILFORD, T. 1991. Is the viceroy a Batesian mimic? *Nature* 351:611.
- JULKUNEN-TIITTO, R. 1989. Phenolic constituents of *Salix*: a chemotaxonomic survey of further Finnish species. *Phytochemistry* 28:2115–2125.
- JULKUNEN-TIITTO, R. and SORSA, S. 2001. Testing the effects of drying methods on willow flavonoids, tannins, and salicylates. *J. Chem. Ecol.* 27:779–789.
- KÖPE, A., RANK, N. E., ROININEN, H., JULKUNEN-TIITTO, R., PASTEELS, J. M., and TAHVANAINEN, J. 1998. The evolution of host-plant use and sequestration in the leaf beetle genus *Phratora* (Coleoptera: Chrysomelidae). *Evolution* 52:517–528.
- LINDROTH, R. L. and HEMMING, J. D. C. 1990. Responses of the gypsy moth (Lepidoptera: Lymantriidae) to tremulacin, an aspen phenolic glycoside. *Environ. Entomol.* 19:842847.

- LINDROTH, R. L., HSIA, M. T. S., and SCRIBER, J. M. 1988a. Seasonal patterns in the phytochemistry of three *Populus* species. *Biochem. Syst. Ecol.* 15:681–686.
- LINDROTH, R. L., SCRIBER, J. M., and HSIA, M. T. S. 1988b. Chemical ecology of the tiger swallowtail: mediation of host use by phenolic glycosides. *Ecology* 69:814–822.
- MALLET, J. 1999. Causes and consequences of a lack of coevolution in Müllerian mimicry. *Evol. Ecol.* 13:777–806.
- MICHAEL, J. B. and SZTAJNKRYCER, M. D. 2004. Deadly pediatric poisons: nine common agents that kill at low doses. *Emerg. Med. Clin. North Am.* 22:1019–1050.
- MULLEN, S. P. 2006. Wing pattern evolution and the origins of mimicry among North American admiral butterflies (Nymphalidae: *Limenitis*). *Mol. Phylogenet. Evol.* 39:747–758.
- MÜLLER, M. S., MCWILLIAMS, S. R., PODELSAK, D., DONALDSON, J. R., BOTHWELL, H. M., and LINDROTH, R. L. 2006. Tri-trophic effects of plant defenses: chickadees consume caterpillars based host-plant defenses. *Oikos* 114:507–517.
- NISHIDA, R. 2002. Sequestration of defensive substances from plants by Lepidoptera. *Annu. Rev. Entomol.* 47:57–92.
- NYMAN, T. and JULKUNEN-TIITTO, R. 2005. Chemical variation within and among six northern willow species. *Phytochemistry* 66:2836–2843.
- PALO, R. T. 1984. Distribution of birch (*Betula* spp.), willow (*Salix* spp.), and poplar (*Populus* spp.) secondary metabolites and their potential role as chemical defense against herbivores. *J. Chem. Ecol.* 10:499–520.
- PASTEELS, J. M., DALOZE, D., and ROWELL-RAHIER, M. 1986. Chemical defense in chrysomelid eggs and neonate larvae. *Physiol. Entomol.* 11:29–37.
- PASTEELS, J. M., ROWELL-RAHIER, M., and RAUPP, M. J. 1988. Plant-derived defense in chrysomelid beetles, pp 235–272, in P. Barbosa and D. K. Letourneau (eds.). *Novel Aspects of Insect-Plant Interactions*. Wiley, New York, NY.
- PRUDIC, K. L., SHAPIRO, A. M., and CLAYTON, N. S. 2002. Evaluating a putative mimetic relationship between two butterflies, *Adelpha bredowii* and *Limenitis lorquini* (Lepidoptera: Nymphalidae). *Ecol. Entomol.* 27:68–75.
- PRUDIC, K. L., SKEMP, A. K., and PAPA, D. R. 2007. Aposematic coloration, luminance contrast, and the benefits of conspicuousness. *Behav. Ecol.* 18:41–46.
- RANK, N. E., SMILEY, J. T., and KOPE, A. 1996. Natural enemies and host-plant relationships for chrysomelid leaf beetles feeding on Salicaceae, pp 147–171, in P. H. Jolivet and M. L. Cox (eds.). *Chrysomelidae Biology*. SBE Publishing, Amsterdam, The Netherlands.
- RITLAND, D. B. and BROWER, L. P. 1991. The viceroy butterfly is not a Batesian mimic. *Nature* 350:497–498.
- RITLAND, D. B. and BROWER, L. P. 2002. Mimicry-related variation in wing color of viceroy butterflies (*Limenitis archippus*: Nymphalidae): a test of the model-switching hypothesis. *Holarctic Lepid.* 7:5–11.
- ROTHSCHILD, M. 1991. Is the viceroy a Batesian mimic? *Nature* 351:611–612.
- RUXTON, G. D., SHERRAT, T. N., and SPEED, M. P. 2004. *Avoiding Attack: The Evolutionary Ecology of Crypsis, Warning Signals and Mimicry*. Oxford University Press, Oxford, UK.
- SAS Institute Inc. 2002. JMP-IN Statistical Exploration Software, Version 5. SAS Institute Inc., Cary, NC.
- SCOTT, J. A. 1986. *The Butterflies of North America*. Stanford University Press, Stanford, CA.
- SKELHORN, J. and ROWE, C. 2005. Tasting the difference: do multiple defence chemicals interact in Müllerian mimicry? *Proc. R. Soc. Lond., B Biol Sci* 272:339–345.
- SMILEY, J. T., HORN, J. M., and RANK, N. E. 1985. Ecological effects of salicin at three trophic levels: new problems from old adaptations. *Science* 229:649–651.
- SOETENS, P., PASTEELS, J. M., DALOZE, D., and KAISIN, M. 1998. Host-plant influence on the composition of the defensive secretion of *Chrysomela vigintipunctata* larvae (Coleoptera: Chrysomelidae). *Biochem. Syst. Ecol.* 26:703–712.
- TAHVANAINEN, J., HELLE, E., JULKUNEN-TIITTO, R., and LAVOLA, A. 1985. Phenolic compounds of willow bark as deterrent against feeding by mountain hare. *Oecologia* 65:319–323.
- THOMPSON, J. N. 2005. *The Geographic Mosaic of Coevolution*. University of Chicago Press, Chicago, IL.