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Compound-Specific Behavioral and Enzymatic Resistance to Toxic Milkweed Cardenolides in a Generalist Bumblebee Pollinator

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Abstract
Plant secondary metabolites that defend leaves from herbivores also occur in floral nectar. While specialist herbivores often have adaptations providing resistance to these compounds in leaves, many social insect pollinators are generalists, and therefore are not expected to be as resistant to such compounds. The milkweeds, *Asclepias* spp., contain toxic cardenolides in all tissues including floral nectar. We compared the concentrations and identities of cardenolides between tissues of the North American common milkweed *Asclepias syriaca*, and then studied the effect of the predominant cardenolide in nectar, glycosylated aspecioside, on an abundant pollinator. We show that a generalist bumblebee, *Bombus impatiens*, a common pollinator in eastern North America, consumes less nectar with experimental addition of ouabain (a standard cardenolide derived from Apocynacid plants native to east Africa) but not with addition of glycosylated aspecioside from milkweeds. At a concentration matching that of the maximum in the natural range, both cardenolides reduced activity levels of bees after four days of consumption, demonstrating toxicity despite variation in behavioral deterrence (i.e., consumption). *In vitro* enzymatic assays of Na^+/K^+-ATPase, the target site of cardenolides, showed lower toxicity of the milkweed cardenolide than ouabain for *B. impatiens*, indicating that the lower deterrence may be due to greater tolerance to glycosylated aspecioside. In contrast, there was no difference between the two cardenolides in toxicity to the Na^+/K^+-ATPase from a control insect, the fruit fly *Drosophila melanogaster*. Accordingly, this work reveals that even generalist pollinators such as *B. impatiens* may have adaptations to reduce the toxicity of specific plant secondary metabolites that occur in nectar, despite visiting flowers from a wide variety of plants over the colony's lifespan.

Keywords Activity · *Asclepias* · *Bombus* · Cardenolide · Cardiac glycoside · Consumption · Enzyme · Na^+/K^+-ATPase · Sodium–potassium pump

Introduction

The presence of toxic plant secondary metabolites in floral nectar is enigmatic, as nectar is considered to function primarily as a reward for visiting pollinators (Adler 2000; Rhoades and Bergdahl 1981; Stevenson 2020; Stevenson et al. 2017). While it is possible that the presence of these secondary metabolites in nectar is passively influenced by levels in leaves for defense from herbivores (Adler et al. 2006; Jacobsen and Raguso 2018; Kessler and Halitschke 2009), adaptive explanations for their presence in nectar have also been proposed (Adler 2000). These explanations include that secondary metabolites may function to filter out less efficient pollinators (Cane et al. 2020; Janzen 1977; Kessler and Baldwin 2007), prevent microbial degradation of nectar (Schmitt et al. 2021), change pollinator behavior in beneficial ways (Kessler et al. 2008; Wright et al. 2013), or modulate relationships with species that are herbivores as larvae and nectarivores as adults (Jones and Agrawal 2016). Herbivores of toxic plants are often specialists, with adaptations to cope with secondary metabolites in leaves (Bennett and Wallsgrove 1994; Petschenka et al. 2017), but social insect pollinators are often generalists (Fontaine et al. 2009), and therefore are likely sensitive to the toxic effects of specific metabolites (Cane et al. 2020).

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The milkweeds, *Asclepias* spp., have a community of specialist herbivorous insects that are resistant to and sequester the cardenolide compounds that make milkweeds toxic (Brower et al. 1972; Petschenka et al. 2017). Milkweed floral nectar, however, also contains cardenolides (Manson et al. 2012; Villalona et al. 2020), with qualitative and quantitative differences in concentration from leaf tissues (Manson et al. 2012). While some specialists with tolerance to cardenolides such as monarch butterflies, *Danaus plexippus*, drink nectar from milkweed flowers and move pollinia (Ivey et al. 2003; Kephart 1983), the most important milkweed pollinators are large bodied Hymenopterans, including bumblebees *Bombus* spp. (Fishbein and Venable 1996; Jennersten and Morse 1991; Kephart 1983; Morse and Fritz 1983; Villalona et al. 2020). Most bumblebees are generalists, although they show species variation in traits such as tongue length that may enable partitioning of floral resources (Heinrich 1976; Inouye 1978; Ranta and Lundberg 1980), and some bumblebees specialize on particular plant species such as *Bombus consobrinus* on the toxic monkshood, *Aconitum septentrionale* (Gosselin et al. 2013; Laverty and Plowright 1988; Thøstesen and Olesen 1996).

A recent study used a combination of field observations and analysis of iNaturalist photographs from the eastern US to assess bumblebee populations and visits to *Asclepias syriaca* versus other plant species (Villalona et al. 2020). *Bombus impatiens* was the most abundant bumblebee, comprising 49% of the bumblebee observations according to iNaturalist records, but *B. impatiens* were infrequent visitors to *A. syriaca*, comprising significantly less of the visitors to *A. syriaca* than would be expected given their abundance (Villalona et al. 2020). In contrast, *Bombus griseocollis* was more often recorded on *A. syriaca* flowers than would be expected given its relative abundance, and lab experiments demonstrated that *B. griseocollis* showed greater avoidance of, and tolerance to, the commercially available cardenolide ouabain than *B. impatiens* (Villalona et al. 2020). This result indicates that *B. griseocollis* may have coevolved to be better able to detect cardenolides and more resistant to their toxic effects than other bumblebee species, including *B. impatiens*, and that *B. impatiens* appears to avoid visiting milkweed flowers. Deterrence of the most abundant potential pollinator in the community should be costly for *A. syriaca* pollination in the Northeast. We therefore decided to investigate the effects of milkweed-specific compounds on this abundant and likely senstive pollinator species.

Cardenolides are toxic due to their inhibition of the cellular animal enzyme Na^+/K^+-ATPase. Cardenolides as secondary metabolites in leaves and seeds have independently evolved in at least 12 angiosperm families (Agrawal et al. 2012; Malcolm 1991). The three commercially available cardenolides predominantly used in ecological experiments are digitoxin and digoxin, both derived from foxglove, *Digitalis purpurea,* native to Europe, and ouabain, derived from two plants in the Apocynaceae native to east Africa. Different cardenolides vary in water solubility and membrane permeability (Malcolm 1991), as well as in structural elements of the genin and glycoside groups that have been shown to impact binding affinity to the Na^+/K^+-ATPase 4 to 94 fold in experiments with monarchs (Agrawal et al. 2012; Petschenka et al. 2018). In particular, cardenolides in the milkweeds, (*Asclepias* spp. and other Asclepiadoideae) typically have a *trans* configuration of rings A and B in the steroidal skeleton, in contrast to the *cis* configuration typical of cardenolides from other plant groups (Agrawal et al. 2012; Malcolm 1991). As toxicity may be affected by whether cardenolides have *cis* or *trans* configurations (Hoch 1961; Petschenka et al. 2018) in addition to other structural attributes, studying the impacts of specific *Asclepias* spp. cardenolides on milkweed pollinators is an important next step.

We recently isolated the milkweed cardenolide glycosylated aspecioside from seeds of common milkweed, *Asclepias syriaca,* via fractionation (Agrawal et al. 2022). Here we show that this compound is one of the most abundant cardenolides in *A. syriaca* nectar. To assess its impact on the abundant *Bombus impatiens*, we evaluated consumption of nectar with this milkweed cardenolide compared to the standard ouabain over four days. After four days we recorded activity levels of bees to assess how cardenolide consumption was affecting behavior. Finally, we used an *in vitro* Na^+/K^+-ATPase assay to evaluate the relative toxicity of the milkweed cardenolide and ouabain on the bumblebee Na^+/K^+-ATPase in contrast to Na^+/K^+-ATPase from unadapted fruit flies, *Drosophila melanogaster* (Karageorgi et al. 2019).

Methods and Materials

Plant Chemistry

Samples of each of four tissue types (leaves, latex, flower buds, and floral nectar) were collected from flowering common milkweed plants at two field sites in Ithaca, NY, in early July 2021. All samples were collected into a cooler with ice packs. For each plant sample, we collected the following from a single ramet: 1) youngest fully expanded leaf (undamaged), 2) 25 μL latex from cut leaf petiole (cutting more if needed to reach 25 μL) into 0.5 mL Millipore water, 3) 5 unopened buds that were close to opening, and 4) 20 opened flowers, collected individually (upside down) in 1.5 ml Eppendorf tubes. For leaves, buds and flowers we carefully wicked latex away from the stalk (petiole or pedicel) with a kimwipe (then resnipped and wicked) in an attempt to exclude latex from the sample. In the lab, leaf and

bud samples were immediately frozen at -80 °C, while nectar was collected by centrifuging the flower tubes at 5000 rpm for 1 min, and then pooled across the 20 flower samples per plant. A measured volume (65–125 µL) of nectar was transferred to a pre-weighed 2 mL screw-cap tube and frozen at -80 °C. Leaf, bud and nectar samples were then freeze-dried for subsequent extraction, and nectar tubes were re-weighed to determine nectar dry mass (range 3–19 mg).

Latex samples were extracted immediately (without freezing) in 0.5 mL Millipore water by sonicating twice for 5 min, centrifuging at 14,000 rpm and 4 °C for 12 min and transferring the supernatant to a new tube. Residual latex was then extracted in 0.5 mL methanol, using a FastPrep twice for 45 s each at 6.5 m/s. Extracts were centrifuged, as above, and standardized portions of the water and methanol extract were pooled for each sample, for a final concentration of 57% methanol in water (0.7 mL, 17.5 µL latex equivalent). From previous work we estimated the dry mass to be 23 mg on average for 25 µL of *A. syriaca* latex. Latex extracts were filtered with 0.2 µM syringe filters and loaded into LCMS vials. After freeze-drying, leaf and bud samples were ground to a fine powder, and then 50 mg of this powder was extracted with 1 mL of methanol using the FastPrep method described above, followed by centrifugation. From each sample, 0.7 mL supernatant (35 mg dry mass equivalent) was transferred to a new tube, dried down, and resuspended in 200 µL methanol before filtration and loading into vials for liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS) analysis.

For LC-HRMS we used a reversed-phase chromatography Dionex 3000 LC coupled to an Orbitrap Q-Exactive mass spectrometer controlled by Xcalibur software (ThermoFisher Scientific). Methanolic extracts were separated on an Agilent Zorbax Eclipse XDB-C18 column (150 mm × 2.1 mm, particle size 1.8 µm) maintained at 40 °C with a flow rate of 0.5 mL/min. Solvent A: 0.1% formic acid (FA) in water; solvent B: 0.1% formic acid in acetonitrile. A/B gradient started at 5% B for 2 min after injection and increased linearly to 98% B at 11 min, followed by 3 min at 98% B, then back to 5% B over 0.1 min and finally at 5% B held for an additional 2.9 min to re-equilibrate the column. Mass spectrometer settings were: spray voltage (-3.0 kV, + 3.5 kV), capillary temperature 380 °C, probe heater temperature 400 °C; sheath, auxiliary, and sweep gas 60, 20, and 2 AU, respectively. S-Lens RF level: 50, resolution 240,000 at *m/z* 200, AGC target 3e6. Each sample was analyzed in positive electrospray ionization mode with *m/z* ranges 70–1000. Parameters for data-dependent MS/MS (dd-MS2): MS1 resolution: 60,000, AGC Target: 1e6. MS2 resolution: 30,000, AGC Target: 2e5, maximum injection time: 50 ms, isolation window 1.0 m/*z*, stepped normalized collision energy (NCE) 10, 30; dynamic exclusion: 1.5 s, top 5 masses selected for MS2 per scan. LC-HRMS data were analyzed using MZmine software (see below). MS1 spectra were acquired for each sample and MS1 and MS2 spectra were obtained from each QC sample.

The acquired LC-HRMS data files were converted to mzXML files using the ProteoWizard MSconvert tool before being preprocessed with the open-source MZmine 2 software (Pluskal et al. 2010). Data processing consisted of peak detection, deconvolution, removal of isotopes, peak alignment, filtering, and peak filling. Quality control (QC) metabolites with a coefficient of variation (CV) greater than 30% were removed from the whole data matrix. We mined the generated feature table to retrieve the 17 cardenolide ion adducts known to be present in *A. syriaca* (Agrawal et al. 2012) and confirmed by comparing MS2 fragmentation spectra. The list of cardenolides is: aspecioside A, desglucosyrioside, diglycosylated digitoxigenin A and B, diglycosylated oxidized syriogenin A and B, diglycosylated syriogenin, glycosylated aspecioside, glycosylated syriobioside, glycosylated syriogenin A and B, labriformin, oxidized labriformin, syriobioside A and B, syrioside A and B. After normalization using a standard, we calculated the relative concentration based on ion counts for all cardenolides. A calibration curve of aspecioside A was used to quantify aspecioside A and glycosylated aspecioside in all tissue samples on a dry mass basis. We compared the cardenolide identities and concentrations across milkweed tissues using a PERMANOVA with the package vegan (Oksanen et al. 2013) in R version 4.2.1.

Consumption Assay

We tested the effect of ouabain octahydrate (CAS# 11,018–89-6, Sigma-Aldrich, ≥ 95%) and glycosylated aspecioside on nectar consumption behavior by individual bumblebees, by measuring the consumption of artificial nectar (0.7 M aqueous sucrose) spiked with each compound at two different concentrations and a control sucrose nectar. We used cardenolide concentrations of 13.7 nmol/mL (low) and 137 nmol/mL (high) for molecular mass of 712 g/mol for glycosylated aspecioside which were approximately equivalent to the endpoints of the previously reported range of cardenolides in milkweed nectar of 10 and 100 ng/µL (Manson et al. 2012). Colonies of bumblebees, *Bombus impatiens*, were obtained from Koppert Biological Systems (Howell MI USA). These bees were commercially raised with no prior experience foraging outside their colony. All bees were therefore naïve to cardenolides in nectar. A total of 106 individual bees were selected from seven different *Bombus impatiens* colonies at random (21 bee replicates per treatment, 3 from each colony, except the low ouabain treatment which had an extra replicate at 22) and placed in isolation within 18 mL polypropylene vials. Vial caps

were equipped with a 2 mL microcentrifuge tube feeder filled with 1 mL of nectar solution. Feeders were made by piercing the conical bottom of the microcentrifuge tube with a 0.8 mm diameter sewing needle, allowing bees to access the nectar solution ad libitum. Vials were placed on their sides at a 5° angle and incubated in total darkness at 27 °C and 50% relative humidity. We measured bee consumption of nectar every 24 h for the duration of the assay (96 h). Feeder tubes were weighed at the beginning and end of each 24 h period for the 96 h duration of the assay, with solutions refreshed daily, to estimate the mass of nectar consumed by each bee per day. The average 24 h mass loss of nectar-solution control tubes was subtracted from the estimated mass consumed by each bee to control for nectar evaporation and leakage each day to calculate the corrected mass. Control vials were incubated in identical conditions as experimental vials except without bees. At the end of the experiment, bee fresh mass was calculated to normalize consumption against individual bee mass. We analyzed the corrected mass per gram of bee for each day using a linear mixed-effect model (LMM) with the lme4 package (Bates et al. 2014) in R with fixed effects of colony, and interaction between day and nectar treatment, and a random effect of individual bee. We compared nectar treatments in Tukey adjusted post-hoc tests using the emmeans package (Lenth 2020).

Activity Level Assays

As a means to test for toxicity of cardenolides, we examined activity levels of bees after consumption of cardenolide and control nectars in petri dish arenas. Bees were removed from their feeding vials after 96 or 120 h of nectar consumption, a range over which we expected to see behavioral effects but when the majority of bees were still alive, and placed in petri dish arenas (100 mm × 15 mm polystyrene) for activity level video analysis. We conducted activity level trials with bees from 5 colonies and 7 to 11 bees per treatment. Bees in each treatment were distributed between groups tested after 96 h and after 120 h. Bee movement was video recorded using a Gopro HERO7 Silver video camera (1080P resolution, 29.97 FPS). Movement was auto-tracked (settings: evolve 30%, tether 5%, automark = 2) using Tracker software (version 6.0.3; http://physlets.org/tracker), which logged X and Y coordinates, path length (cm), and velocity (cm/s) frame by frame for a total of 5 min. We analyzed cumulative path length at the end of the 5-min trial using a two-way ANOVA with factors of nectar treatment and whether the trails occurred after 96 or 120 h of consumption. Treatments were compared using Tukey adjusted post-hoc tests with the emmeans package.

Na^+/K^+-ATPase Assay

Using an *in vitro* assay, we tested the Na^+/K^+-ATPases of *Bombus impatiens* and fruit flies, *Drosophila melanogaster*, as a control species for tolerance to glycosylated aspecioside and ouabain. Brains were dissected from *Bombus impatiens*, and whole heads removed from *Drosophila melanogaster* (wild type, not genetically modified) specimens that had been frozen alive and stored at -80 °C. Individual bee brains and each batch of fly heads was homogenized in 0.5 mL Millipore water using a Wheaton all-glass grinder, frozen, freeze dried, and stored at -80 °C until use in assays. Just prior to running the assays, each prep was brought up in cold Millipore water, using sonication, to reach a concentration of 1 *B. impatiens* brain per mL or 12.5 *D. melanogaster* heads per 450 µL. These concentrations were chosen, based on preliminary analyses, to fall within the range of ATPase activity for which a linear relationship between (cleaved) phosphate concentration and spectrophotometric absorbance is achieved.

Na^+/K^+-ATPases were analyzed for activity across a 6-point dilution series of glycosylated aspecioside (10^{-4} M to 10^{-9} M) and ouabain (10^{-3} M to 10^{-8} M), in 20% DMSO. Assays were run as in previous studies (Petschenka et al. 2013), except that enzyme prep, reaction buffer and ATP were combined in a mastermix, on ice, and added together to the cardenolide solution in each sample well. For each dilution series, we included a full reaction well (no cardenolide) and a background well (fully inhibitive ouabain solution and a reaction buffer lacking K^+). Assay plates were incubated at 37 °C for 20 min on a Bioshake IQ (Quantifoil instruments), after which enzymatic activity was interrupted by addition of 100 µL sodium dodecyl sulfate (SDS) to each well. Inorganic phosphate released from enzymatically hydrolyzed ATP was stained using 100 µL of a Taussky-Shorr color reagent and quantified photometrically at 700 nm. The absorbance for each reaction was corrected for non-specific activity by subtracting the absorbance of the background reaction. Three to four technical replicates for each enzyme and cardenolide were included.

For each cardenolide curve, we estimated the amount of cardenolide required for 50% inhibition of the enzyme activity (IC50) by fitting a logistic function to the absorbance data and extracting the molar concentration at the inflection point. We used the nlme function with SSfpl from the nlme package (Pinheiro et al. 2007) in R to perform a 4-parameter (upper asymptote, lower asymptote, xmid, scal) vs. a 3-parameter logistic function (upper asymptote, xmid (inflection point), scal). We found the best fit (lowest AIC) with the 3-parameter function (which estimated the upper asymptote of the dose–response curve but fixed the lower asymptote at 0). We examined effects of cardenolides (ouabain or glycosylated aspecioside), enzyme source (*B.*

impatiens or *D. melanogaster*), and their interaction on the IC50 using two-way ANOVA; technical replicate was also included in the model.

Results

Plant Chemistry

The chemical composition of *A. syriaca* is different depending on the tissues, whether considering all the metabolites detected (Fig. S1) or only the 17 identified cardenolides present (Fig. 1A) (PERMANOVA; $R^2=0.56$, $F_{3,44}=17.68$, $P=0.001$). Post-hoc tests on the PERMANOVA showed all tissues were different from each other (adjusted $P<0.006$) except buds and leaves (adjusted $P=0.312$). Glycosylated aspecioside and aspecioside A, which have the same genin but differ by one carbohydrate unit, are the most abundant cardenolides in bud, leaf, nectar, but not in latex where the concentrations were very low as explained by PC1 in the principal component analysis (Figs. 1, S2, S3). Concentrations of glycosylated aspecioside and aspecioside A appear to be 1.6- to 3.2-fold lower in nectar compared to bud and leaf (Fig. 1B). In nectar, except for these two compounds, all other cardenolides have very low concentrations or are not detected at all, whereas bud and leaf tissues have more cardenolides diversity similarity, results illustrated by PC2 in the principal component analysis (Fig. 1A).

Consumption Assay

Nectar consumption was affected by day and nectar treatment (interaction term: LMM; $\chi^2=22.07$, $df=4$, $P<0.001$) with a decline in consumption over time in the high ouabain treatment in comparison to the other treatments (Fig. 2). We also found an effect of nectar treatment alone ($\chi^2=20.01$, $df=4$, $P<0.001$), day alone ($\chi^2=11.22$, $df=1$, $P<0.001$) and colonies ($\chi^2=12.20$, $df=4$, $P=0.016$). Post-hoc tests showed significantly more consumption of the sucrose control than the high ouabain treatment ($t=-4.20$, $P<0.001$) and more consumption of the high glycosylated aspecioside than the high ouabain ($t=3.57$, $P=0.005$).

Activity Level Assay

Cumulative movement of individual bees over 5 min was affected by nectar treatment (ANOVA; $F_{4,43}=3.64$, $P=0.012$; Fig. 3) but not by whether bees were tested after 96 or 120 h of consumption ($F_{1,43}=2.12$, $P=0.15$). Post-hoc tests showed reduced activity levels compared to the sucrose control for both the high glycosylated aspecioside ($t=-3.09$, $P=0.027$) and the high ouabain concentration ($t=-3.34$, $P=0.014$). Although intermediate in magnitude, the low ouabain and low glycosylated aspecioside cardenolide treatments did not significantly reduce activity compared to the sucrose control.

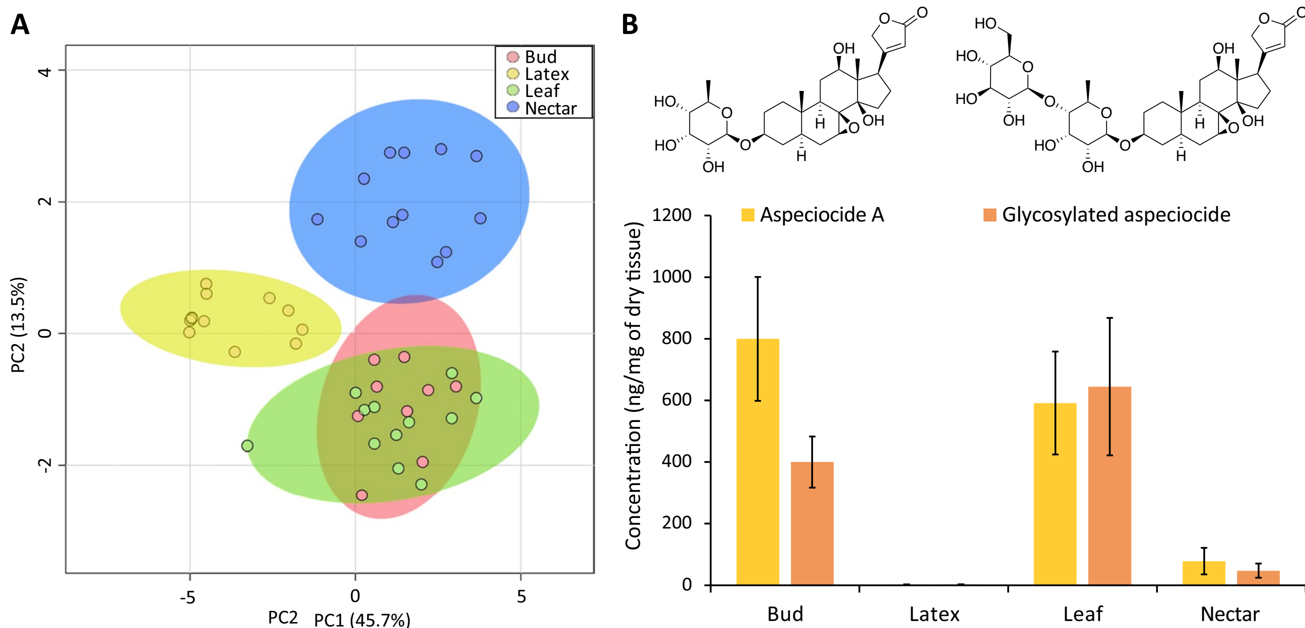

Fig. 1 Chemical composition of *A. syriaca* based on LC-HRMS: (**A**) Principal component analysis showing the differences in cardenolide composition across different tissues ($N=12$ per group). Eclipse represents the region of 95% confidence, (**B**) Concentrations of the two most abundant cardenolides in *A. syriaca* across plant tissue types

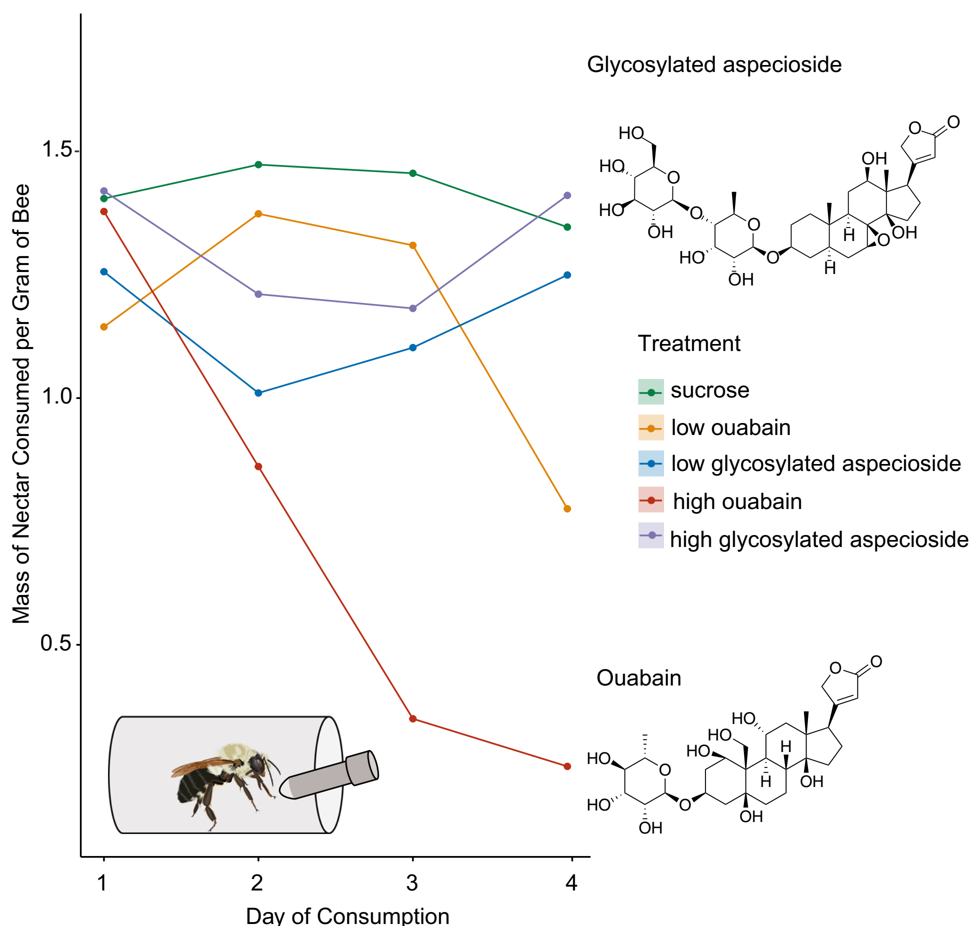

Fig. 2 Nectar mass consumed over four days, controlling for bee mass, with nectars spiked with different cardenolides. Points are means for individual bees on each day ($N=21$ bees per nectar treatment). Low concentrations of cardenolides were 13.7 nmol/mL and high concentrations were 137 nmol/mL

Na^+/K^+-ATPase Assay

The amount of cardenolide required for 50% inhibition of enzyme activity (IC50) was predicted by an interaction between the cardenolide identity (ouabain or glycosylated aspecioside) and the enzyme source (*B. impatiens* or *D. melanogaster*) ($F_{1,11}=0.84$, $P=0.011$). While Drosophila was equally inhibited by the two cardenolides, *B. impatiens* was more tolerant of glycosylated aspecioside isolated from milkweed (Fig. 4). In addition to the interaction, there were main effects of cardenolide identity (glycosylated aspecioside or ouabain; $F_{1,11}=1.01$, $P=0.007$) and enzyme source (*B. impatiens* or *D. melanogaster*; $F_{1,11}=2.87$, $P=0.0015$).

Discussion

The presence of toxic compounds in flower nectar presents the opportunity for testing hypotheses about how plants cope with the evolutionary trade-off of deterring herbivores and attracting pollinators (Stevenson 2020). Our data on plant chemistry indicates that the common milkweed, *Asclepias syriaca*, may cope with this tradeoff by maintaining different cocktails and concentrations of cardenolides in different plant parts, similarly to how cardenolides are differentially distributed between parts of *A. currassavica* that are consumed by different herbivores (López-Goldar et al. 2022). One of the two most common cardenolides in *A. syriaca* nectar, glycosylated aspecioside, was less deterrent to the bumblebee *B. impatiens* in terms of both nectar consumption and produced less enzyme inhibition, than the standard cardenolide ouabain, which is derived from an African confamilial plant. *B. impatiens* are generalists, visiting a wide range of different flowering plant species. For example, from one study in Québec, Canada, *B. impatiens* workers visited 41 different species of plants, with individual workers collecting pollen from a mean of 6 plant species per foraging trip (Gervais et al. 2020). Given their degree of generalism, and a previous study showing they appear to avoid visiting milkweeds (Villalona et al. 2020), it was unanticipated that *B. impatiens* would show enzymatic tolerance to a milkweed-specific cardenolide. The tolerance we observe to glycosylated aspecioside in *B. impatiens* is approximately two-fold the tolerance in *D. melanogaster.* In contrast, monarch butterflies, *Danaus plexippus*, have a tolerance to glycosylated aspecioside 500 times that of

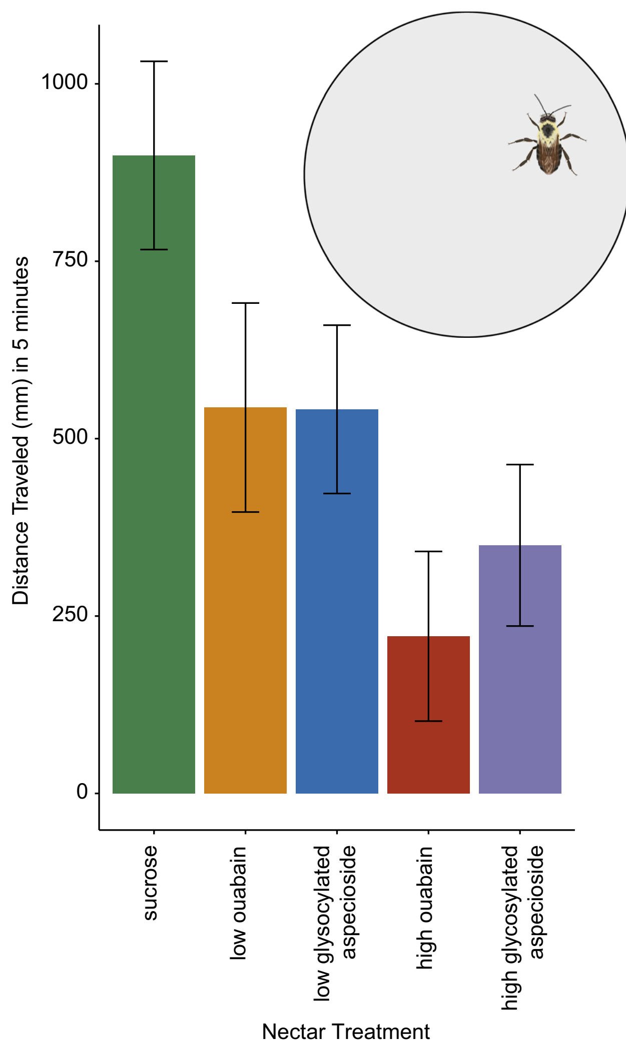

Fig. 3 Mean and 1 ± SE in mm of the total distance travelled by individual bees over 5 min in the petri-dish activity level assay for each nectar treatment (N = 7–11 bees per treatment). Low concentrations of cardenolides were 13.7 nmol/mL and high concentrations were 137 nmol/mL

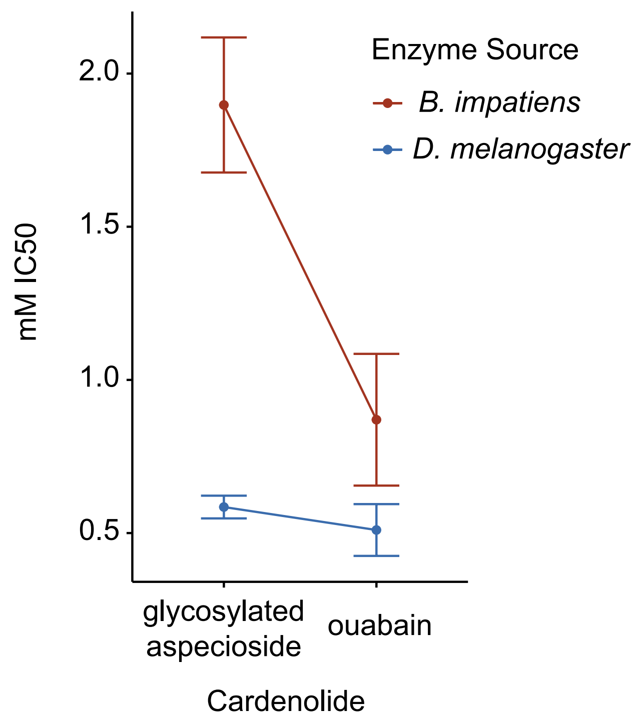

Fig. 4 Mean and 1 ± SE of the mM concentration of cardenolide required for 50% inhibition of Na^+/K^+-ATPase enzymes from *Bombus impatiens* and *Drosophila melanogaster* for glycosylated aspecioside from *A. syriaca* and the commercially available ouabain

the porcine Na^+/K^+-ATPase, which is typically used as a control for a non-adapted ancestral enzyme (Agrawal et al. 2022; Karageorgi et al. 2019). The tolerance that we record, therefore, while likely to be ecologically relevant, is much less than that of a milkweed specialist herbivore. Although as our results show, cardenolide concentrations and diversity are higher in leaves than in flower nectar.

The concetrations of cardenolides that we used in our experiments were higher than we and others have found in *A. syriaca* nectar (Villalona et al. 2020), but within the range reported for other milkweeds (Manson et al. 2012). The presence of toxic compounds in nectar deter flower visitors in other cases, for example with nicotine in wild tobaccos (Kessler and Baldwin 2007), and the steroidal alkaloid zygacine in death-camas (Cane et al. 2020). The generalist bumblebee native to Europe, *Bombus terrestris*, in contrast, is not deterred by the nectar toxins quinine, caffeine, nicotine, amygdalin, and grayanotoxin at ecologically relevant concentrations (Tiedeken et al. 2014). In this case the authors suggest that bees may not be able to detect these toxins at the concentrations that occur in flower nectar (Tiedeken et al. 2014). Lack of deterrence to toxic nectar compounds has been proposed to occur because natural selection operates at the colony level, where toxins may be diluted and where individual behaviors contribute to the collective. Additionally, toxic secondary metabolites in nectar may not be deterrent to bees if they are medicinal, which may be the case for bees coping with infections of the gut pathogen *Crithidia bombi* (Baracchi et al. 2015; Manson et al. 2010; Richardson et al. 2015; Richardson et al. 2016).

A lack of behavior deterrence, however, does not necessarily require physiological tolerance to toxic effects. Of particular relevance, another North American bumblebee, *Bombus griseocollis*, is a more common visitor to *Asclepias syriaca* than would be expected given its abundance. Furthermore, this bumblebee has a lower detection threshold for, and is a more tolerant consumer of high concentrations of ouabain than *B. impatiens* or *B. bimaculatus* (Villalona et al. 2020). Although the IC50 for *B. griseocollis* Na^+/K^+-ATPase has not been tested, it would certainly be interesting

to compare enzymatic tolerance to glycosylated aspecioside in *B. griseocollis* and *B. impatiens*. In addition, it would be interesting to compare the efficiency of these two bumblebees as pollinators of *A. syriaca* by comparing pollinia removal not just visitation rate. For example, one study showed *Bombus pennsylvanicus* to be a common visitor to *A. syriaca* but this species was rarely observed removing pollinia (Betz et al. 1994). It has been proposed that the evolution of tolerance to plant toxins in pollinators may enable plants to increase their leaf defenses if plants cannot prevent spread of secondary metabolites from other parts into flower nectar (Cane et al. 2020). Our data, however, shows that *A. syriaca* does appear to partition cardenolides between different tissues. It would be interesting to test whether the cardenolides that are most common in nectar: aspecioside A and glycosylated aspecioside, are the least toxic compounds to bees in the suite that *A. syriaca* possesses.

Different cardenolides have been shown to vary in their toxicity to bees (Detzel and Wink 1993). Cardenolides vary in their glycoside side chains, which have different binding affinities with the Na^+/K^+-ATPase and in general higher binding affinity results in greater inhibition of the enzyme (with some exceptions) (Paula et al. 2005). The two most common cardenolides we found in *A. syriaca* nectar contain the same genin (the steroid and lactone backbone of a cardenolide) and have *trans* configurations of steroidal skeleton rings A and B. Both of these compounds are relatively polar, as is ouabain, which may not only allow them to dissolve in nectar but also has been proposed to make them less toxic than apolar cardenolides which more easily cross insect membrane barriers (Rasmann and Agrawal 2011). Our enzyme assays did not indicate a difference in toxicity between glycosylated aspecioside and ouabain for the control *Drosophila melanogaster* enzymes, indicating that the difference in toxicity of these two compounds for *B. impatiens* is likely due to adaptations by the bee enzyme rather than structural differences in the compounds.

Eleven species of insects that feed on plants containing cardenolides including the monarch butterfly, *Danaus plexippus*, have an amino acid substitutions in their Na^+/K^+-ATPase at positions 111, 119, and 122, which increase tolerance to ouabain (Dobler et al. 2012). The use of CRISPR-Cas9 has shown that the monarch Na^+/K^+-ATPase, when edited into *D. melanogaster,* confers insensitivity to cardenolides (Karageorgi et al. 2019), highlighting the importance of amino acid substitutions in resistance. Like *D. melanogaster* (Dobler et al. 2012), *B. impatiens* does not have a substitution at position 111, 199 or 122 (Villalona et al. 2020), indicating that these amino acid substitutions do not explain tolerance in *B. impatiens,* although there may be other substitutions involved that are not yet described in *B. impatiens*. There are other pathways to resistance, such as membrane-partitioning of consumed cardenolides away from sensitive organs in arctiid moths (Petschenka et al. 2012), or an excretion mechanism via the Malpighian tubules for example in the cases of *Chrysolina coerulans* and *Spodoptera littoralis* when fed the pyrrolizidine alkaloid senecionine (Hartmann et al. 1999). The mechanism of tolerance to glycosylated aspecioside but not ouabain remains unknown in *B. impatiens*, and is worthy of further investigation.

We show differential distribution of 17 cardenolide compounds across *Asclepias syriaca* tissues. This differential distribution may be a mechanism for milkweeds to escape the trade-off between using toxic secondary metabolites to deter herbivores and poisoning pollinators. It remains unknown how plants modulate the presence of secondary metabolites in nectar (Stevenson 2020), but *A. syriaca* may have some mechanism allowing plants to control which cardenolides occur in nectar. Latex apparently transports some of the most non-polar cardenolides in milkweeds, and the lack of latex in nectar may explain part of its composition. It is unknown whether the two compounds we demonstrate to be the most abundant in nectar (aspecioside A and glycosylated aspecioside) have been under selection to occur in nectar because they are less inhibitory to the bee Na^+/K^+-ATPase or if bees have experienced selection to resist the toxic effects of these compounds. Milkweeds have large floral displays with abundant nectar; being able to utilize this large nectar source may have been advantageous enough over evolutionary time that even a generalist pollinator such as *B. impatiens* has evolved tolerance to milkweed nectar cardenolides.

We highlight the importance of studying specific compounds in plant–insect interactions and not only the commercially available relatives, as they may have very different effects. The most abundant cardenolides in nectar occur at higher concentrations in leaves indicating that milkweeds may be under selection to reduce their presence in nectar, and we demonstrate negative effects of a specific nectar cardenolide on bee acitivity levels. At the same time, bees may be under selection to evolve tolerance to these milkweed compounds in order to utilize milkweeds as a nectar source. This research demonstrates evidence for adaptations to reduce the cost of toxic compounds in nectar, on the sides of both the plant and pollinator, indicating the contrasting forces of selection on defensive compounds in plant survival and reproduction.

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Authors' contributions PLJ, AAA, and KRM designed the experiment. KRM conducted the bee consumption and activity level experiments. APH collected milkweed tissues. CD conducted the mass spectrometry. APH conducted the enzyme assays. SVP analyzed the behavioral

videos. PLJ wrote the main manuscript text. PLJ and KRM analyzed data and prepared Figs. 2, 3, 4. CD prepared Fig. 1 and supplementary figures. All authors reviewed the manuscript.

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Data availability All of the raw data is available as supplementary material.

Declarations

Competing interests The authors declare no competing interests.

References

Adler LS (2000) The ecological significance of toxic nectar. Oikos 91:409–420
Adler LS, Wink M, Distl M, Lentz AJ (2006) Leaf herbivory and nutrients increase nectar alkaloids. Ecol Lett 9:960–967
Agrawal AA et al (2022) Functional evidence supports adaptive plant chemical defense along a geographical cline. Proc Natl Acad Sci USA 119:e2205073119
Agrawal AA, Petschenka G, Bingham RA, Weber MG, Rasmann S (2012) Toxic cardenolides: chemical ecology and coevolution of specialized plant–herbivore interactions. New Phytol 194:28–45
Baracchi D, Brown MJ, Chittka L (2015) Behavioural evidence for self-medication in bumblebees? F1000Res 4:73
Bates D, Mächler M, Bolker B, Walker S (2014) Fitting linear mixed-effects models using lme4. arXiv:14065823
Bennett RN, Wallsgrove RM (1994) Secondary metabolites in plant defence mechanisms. New Phytol 127:617–633
Betz RF, Struven RD, Wall JE, Heitler FB (1994) Insect pollinators of 12 milkweed (Asclepias) species. In: Wickett RG, Lewis PD, Woodliffe A, Pratt P (eds) Proceedings of the Thirteenth North American Prairie Conference: Spirit of the Land, our Prairie Legacy. Department of Parks & Recreation, Windsor, pp 45–60
Brower LP, McEvoy PB, Williamson KL, Flannery MA (1972) Variation in cardiac glycoside content of monarch butterflies from natural populations in eastern North America. Science 177:426–429
Cane JH, Gardner DR, Weber M (2020) Neurotoxic alkaloid in pollen and nectar excludes generalist bees from foraging at death-camas, *Toxicoscordion paniculatum* (Melanthiaceae). Biol J Linn Soc 131:927–935
Detzel A, Wink M (1993) Attraction, deterrence or intoxication of bees (Apis mellifera) by plant allelochemicals. Chemoecology 4:8–18
Dobler S, Dalla S, Wagschal V, Agrawal AA (2012) Community-wide convergent evolution in insect adaptation to toxic cardenolides by substitutions in the Na, K-ATPase. Proc Natl Acad Sci USA 109:13040–13045
Fishbein M, Venable DL (1996) Diversity and temporal change in the effective pollinators of *Asclepias tuberosa*. Ecology 77:1061–1073
Fontaine C, Thébault E, Dajoz I (2009) Are insect pollinators more generalist than insect herbivores? Proc R Soc B: Biol Sci 276:3027–3033
Gervais A, Courtois È, Fournier V, Bélisle M (2020) Landscape composition and local floral resources influence foraging behavior but not the size of B*ombus impatiens* Cresson (Hymenoptera: Apidae) workers. PLoS ONE 15:e0234498
Gosselin M, Michez D, Vanderplanck M, Roelants D, Glauser G, Rasmont P (2013) Does *Aconitum septentrionale* chemically protect floral rewards to the advantage of specialist bumblebees? Ecol Entomol 38:400–407
Hartmann T, Theuring C, Schmidt J, Rahier M, Pasteels JM (1999) Biochemical strategy of sequestration of pyrrolizidine alkaloids by adults and larvae of chrysomelid leaf beetles. J Insect Physiol 45:1085–1095
Heinrich B (1976) Resource partitioning among some eusocial insects: bumblebees. Ecology 57:874–889
Hoch JH (1961) A survey of cardiac glycosides and genins. University of South Carolina Press, Columbia, SC USA
Inouye DW (1978) Resource partitioning in bumblebees: experimental studies of foraging behavior. Ecology 59:672–678
Ivey CT, Martinez P, Wyatt R (2003) Variation in pollinator effectiveness in swamp milkweed, *Asclepias incarnata* (Apocynaceae). Am J Bot 90:214–225
Jacobsen DJ, Raguso RA (2018) Lingering effects of herbivory and plant defenses on pollinators. Curr Biol 28:1164–1169
Janzen DH (1977) Why don't ants visit flowers. Biotropica 9:252
Jennersten O, Morse DH (1991) The quality of pollination by diurnal and nocturnal insects visiting common milkweed, *Asclepias syriaca*. Am Midl Nat 125:18–28
Jones PL, Agrawal AA (2016) Consequences of toxic secondary compounds in nectar for mutualist bees and antagonist butterflies. Ecology 97:2570–2579
Karageorgi M et al (2019) Genome editing retraces the evolution of toxin resistance in the monarch butterfly. Nature 574:409–412
Kephart SR (1983) The partitioning of pollinators among three species of *Asclepias*. Ecology 64:120–133
Kessler A, Halitschke R (2009) Testing the potential for conflicting selection on floral chemical traits by pollinators and herbivores: predictions and case study. Funct Ecol 23:901–912
Kessler D, Baldwin IT (2007) Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of *Nicotiana attenuata*. Plant J 49:840–854
Kessler D, Gase K, Baldwin IT (2008) Field experiments with transformed plants reveal the sense of floral scents. Science 321:1200–1202
Laverty TM, Plowright R (1988) Flower handling by bumblebees: a comparison of specialists and generalists. Anim Behav 36:733–740
Lenth R, Singmann H, Love J, Buerkner P, Herve M (2020) Emmeans: estimated marginal means. R package version 1.4. 4. Am Stat 34(4):216–221
López-Goldar X, Hastings A, Züst T, Agrawal A (2022) Evidence for tissue-specific defence-offence interactions between milkweed and its community of specialized herbivores. Mol Ecol 31:3254–3265
Malcolm SB (1991) Cardenolide-mediated interactions between plants and herbivores Herbivores: their interactions with secondary plant metabolites. In: Rosenthal GA, Berenbaum MR (eds) Herbivores Their Interactions with Secondary Plant Metabolites, 2nd edn. Academic Press Inc, San Deigo, pp 251–296
Manson JS, Otterstatter MC, Thomson JD (2010) Consumption of a nectar alkaloid reduces pathogen load in bumble bees. Oecologia 162:81–89
Manson JS, Rasmann S, Halitschke R, Thomson JD, Agrawal AA (2012) Cardenolides in nectar may be more than a consequence of allocation to other plant parts: a phylogenetic study of *Asclepias*. Funct Ecol 26:1100–1110
Morse DH, Fritz RS (1983) Contributions of diurnal and nocturnal insects to the pollination of common milkweed (*Asclepias syriaca* L.) in a pollen-limited system. Oecologia 60:190–197
Oksanen J, Simpson G, Blanchet F, Kindt R, Legendre P, Minchin P, O'Hara R, Solymos P, Stevens M, Szoecs E, Wagner H, Barbour M, Bedward M, Bolker B, Borcard D, Carvalho G, Chirico M, De Caceres M, Durand S, Evangelista H, FitzJohn R, Friendly M,

Furneaux B, Hannigan G, Hill M, Lahti L, McGlinn D, Ouellette M, Ribeiro Cunha E, Smith T, Stier A, Ter Braak C, Weedon J (2013) vegan: Community Ecology Package. R package version 2.6-2. https://cran.r-project.org/web/packages/vegan/index.html

Paula S, Tabet MR, Ball WJ (2005) Interactions between cardiac glycosides and sodium/potassium-ATPase: three-dimensional structure− activity relationship models for ligand binding to the E2-pi form of the enzyme versus activity inhibition. Biochemistry 44:498–510

Petschenka G, Fandrich S, Sander N, Wagschal V, Boppré M, Dobler S (2013) Stepwise evolution of resistance to toxic cardenolides via genetic substitutions in the Na+/K+-ATPase of milkweed butterflies (Lepidoptera: Danaini). Evolution 67:2753–2761

Petschenka G, Fei CS, Araya JJ, Schröder S, Timmermann BN, Agrawal AA (2018) Relative selectivity of plant cardenolides for Na+/K+-ATPases from the monarch butterfly and non-resistant insects. Front Plant Sci 9:1424

Petschenka G, Offe JK, Dobler S (2012) Physiological screening for target site insensitivity and localization of Na+/K+-ATPase in cardenolide-adapted Lepidoptera. J Insect Physiol 58:607–612

Petschenka G, Wagschal V, von Tschirnhaus M, Donath A, Dobler S (2017) Convergently evolved toxic secondary metabolites in plants drive the parallel molecular evolution of insect resistance. Am Nat 190:S29–S43

Pinheiro J, Bates D, DebRoy S, Sarkar D, Team RC (2007) Linear and nonlinear mixed effects models. R package version 3:1–89. https://cran.r-project.org/web/packages/nlme/index.html

Pluskal T, Castillo S, Villar-Briones A, Orešič M (2010) MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. BMC Bioinformatics 11:1–11

Ranta E, Lundberg H (1980) Resource partitioning in bumblebees: the significance of differences in proboscis length. Oikos 35:298–302

Rasmann S, Agrawal AA (2011) Latitudinal patterns in plant defense: evolution of cardenolides, their toxicity and induction following herbivory. Ecol Lett 14:476–483

Rhoades DF, Bergdahl JC (1981) Adaptive significance of toxic nectar. Am Nat 117:798–803

Richardson LL et al (2015) Secondary metabolites in floral nectar reduce parasite infections in bumblebees. Proc R Soc B: Biol Sci 282:20142471

Richardson LL, Bowers MD, Irwin RE (2016) Nectar chemistry mediates the behavior of parasitized bees: consequences for plant fitness. Ecology 97:325–337

Schmitt A, Roy R, Carter CJ (2021) Nectar antimicrobial compounds and their potential effects on pollinators. Curr Opin Insect Sci 44:55–63

Stevenson PC (2020) For antagonists and mutualists: the paradox of insect toxic secondary metabolites in nectar and pollen. Phytochem Rev 19:603–614

Stevenson PC, Nicolson SW, Wright GA (2017) Plant secondary metabolites in nectar: impacts on pollinators and ecological functions. Funct Ecol 31:65–75

Thøstesen AM, Olesen JM (1996) Pollen removal and deposition by specialist and generalist bumblebees in *Aconitum septentrionale*. Oikos 77:77–84

Tiedeken EJ, Stout JC, Stevenson PC, Wright GA (2014) Bumblebees are not deterred by ecologically relevant concentrations of nectar toxins. J Exp Biol 217:1620–1625

Villalona E, Ezray BD, Laveaga E, Agrawal AA, Ali JG, Hines HM (2020) The role of toxic nectar secondary compounds in driving differential bumble bee preferences for milkweed flowers. Oecologia 193:619–630

Wright G et al (2013) Caffeine in floral nectar enhances a pollinator's memory of reward. Science 339:1202–1204

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