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EDITED BY
Michael Wink,
Heidelberg University, Germany

REVIEWED BY
Ute Wittstock,
Technical University of Braunschweig, Germany
Christopher S. Jeffrey,
University of Nevada, Reno, United States

*CORRESPONDENCE
Paola Rubiano-Buitrago
pbuitrago@ice.mpg.de
Hannah M. Rowland
hrowland@ice.mpg.de

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Differential accumulation of cardenolides from *Asclepias curassavica* by large milkweed bugs does not correspond to availability in seeds or biological activity on the bug Na^+/K^+-ATPase

Paola Rubiano-Buitrago[1,2]*, Shrikant Pradhan [1], Veit Grabe[3], Alfonso Aceves-Aparicio [1], Christian Paetz[2] and Hannah M. Rowland[1]*

[1]Research Group Predators and Toxic Prey, Max Planck Institute for Chemical Ecology, Jena, Germany, [2]Research Group Biosynthesis/NMR, Max Planck Institute for Chemical Ecology, Jena, Germany, [3]Microscopic Imaging Service Group, Max Planck Institute for Chemical Ecology, Jena, Germany

Milkweed–herbivore systems are characterized by cardenolide chemical defenses and specialized herbivore adaptations such as physiological target site insensitivity. Cardenolide defenses in milkweeds can vary in terms of the total concentration, differences in the polarity of individual cardenolides, and the substitution of the steroidal structures that can contribute to the molecule's reactivity. The variability in cardenolide defenses could represent the plant's response to natural selection and adaptation of resistant herbivores and is a characteristic of phenotype-matching between defensive and offensive traits resulting from coevolution. Here, we test the phenotypic match of the cardenolide composition of seeds of *Asclepias curassavica* and those sequestered by nymphs and adults of the specialized seed herbivore *Oncopeltus fasciatus*, combined with tests of the inhibitory capacity of a subset of seed cardenolides against the Na^+/K^+-ATPase of *O. fasciatus* and a non-adapted insect (*Drosophila melanogaster*). We compare this with the inhibitory capacity against the highly sensitive porcine Na^+/K^+-ATPase. Among the five most abundant cardenolides present in milkweed seeds, glucopyranosyl frugoside, glucopyranosyl gofruside, and glucopyranosyl calotropin were significantly more abundant in the seeds than in the adults and nymphs; the bugs contained higher concentrations of the deglucosylated compounds. The most abundant compound, glucopyranosyl frugoside, was also the most inhibitory for *O. fasciatus*, but *O. fasciatus* was significantly more tolerant to all compounds compared to *D. melanogaster* and the highly sensitive porcine enzyme. Our results add to the evidence that *O. fasciatus* sequesters specific individual cardenolides from its *Asclepias* host plants that are not directly linked to the concentration and inhibitory potency.

KEYWORDS

***Oncopeltus fasciatus*, cardiac glycoside, phytochemical diversity, structure–activity relationship, toxin–receptor interactions, resistance**

1. Introduction

Insects can impose natural selection for chemical defenses in the plant tissues on which they feed, leading to phenotype-matching between defensive and offensive traits that may have resulted from coevolution (Futuyma and Agrawal, 2009). Such biochemical coevolutionary arms races (Ehrlich and Raven, 1964) can drive evolutionary innovation and diversification (Mauricio and Rausher, 1997; Berenbaum and Zangerl, 1998; Zangerl and Berenbaum, 2003; Agrawal, 2005; Benderoth et al., 2006; Prasad et al., 2012). For example, the evolution of novel genes for glucosinolate detoxification in Pierid butterflies has been accompanied by duplication and neofunctionalization of defensive glucosinolate genes in Brassicaceae host plant species (Wheat et al., 2007; Sønderby et al., 2010; Edger et al., 2015; BlaŽević et al., 2020). Explaining the diversity of plant secondary metabolites and insect herbivore tolerance mechanisms observed in nature is of interest to evolutionary and chemical ecologists who seek to understand the origins of phytochemical diversity and the scope for coevolution in a community context (Berenbaum et al., 1991; Jones and Firn, 1991; Berenbaum and Zangerl, 1996; Richards et al., 2015).

Many plants simultaneously produce multiple secondary metabolites of the same class (Dyer et al., 2018). One explanation for this is that across populations, different herbivores can select for specific defensive traits (Ayres et al., 1997; Züst et al., 2012), and individual plant compounds are targeted at distinct herbivores (i.e., compound selectivity). For example, multiple specialized herbivores of milkweed plants (*Asclepias* spp.) each have well-characterized genetically based physiological differences in tolerance to the overall concentration of the plant's toxic cardenolides (Dobler et al., 2012; Karageorgi et al., 2019). Cardenolides are a structurally diverse group of compounds that are found in at least 12 plant families (Agrawal et al., 2012). They consist of a steroid backbone, an unsaturated lactone, and glycoside moieties (Malcolm, 1991). Single species of *Asclepias* can contain up to 21 different cardenolides, and both compound diversity and concentration are variable among tissues eaten by insects (Abe et al., 1992; Warashina et al., 2008; Opitz and Müller, 2009; Zhang et al., 2014). These host plant cardenolides are selectively sequestered by insect herbivores. For example, the seed bug *Oncopeltus fasciatus* often sequesters intermediate and more polar cardenolides, even if reared on different host plants that have distinct chemical profiles (Moore and Scudder, 1985). The bug's cardenolide profiles often differ from the cardenolide profile of the host plants (Duffey and Scudder, 1974; Isman et al., 1977; Scudder et al., 1986), which can be explained by the metabolism of some cardenolides into distinct products (Agrawal et al., 2022). Studies that investigate the functional link between the specific defense compounds and their impacts on herbivores will advance our understanding of how the diversity of the same secondary metabolite compound arises, and why plants repeatedly derivatize the same class of compounds (Whitehead et al., 2022).

Recently, the interactions between particular toxic cardenolide heart poisons and insect target sites have begun to be identified (Petschenka et al., 2018; Agrawal et al., 2022; López-Goldar et al., 2022). Agrawal et al. (2022) found that the dominant seed cardenolide in *Asclepias syriaca* (glycosylated aspecioside) is more inhibitory to the target site of the seed herbivore than its sequestered conversion product (aspecioside A) and that the subdominant seed cardenolides—diglycosylated syriogenin and labriformin—were significantly more inhibitory than the more abundant compounds. The differential accumulation of the compounds could be evidence that they are under selection, especially if this is correlated to geographical areas that vary in insect diversity and herbivory. Further studies on the specificity in chemical defense toward particular resistant enzymes will increase our understanding of milkweed–herbivore coevolution and more broadly phytochemical diversity.

Here, we study the specific interaction of cardenolide toxins from *Asclepias curassavica* plants and insect Na^+/K^+-ATPases. We compare the cardenolide composition of seeds of *Asclepias curassavica* to those sequestered by nymphs and adults of the specialized seed herbivore *Oncopeltus fasciatus*. We combine this with tests of the inhibitory capacity of a subset of the seed cardenolides against the Na^+/K^+-ATPase of *Oncopeltus* and a non-adapted insect (*Drosophila melanogaster*). The cardenolides can be differentiated by the presence of a hydroxyl group (coroglaucigenin) or a carbonyl group (corotoxigenin/calotropagenin) at the C-19 position of the steroidal structure (see Figure 1 and Rubiano-Buitrago et al., 2023). The native range of *A. curassavica* is South America and Mexico, and it has spread to California, Florida, Tennessee, and Texas. *A. curassavica* is eaten by several specialist herbivores including true bugs, from the family Lygaeidae. *A. curassavica* is present in the migration range of milkweed bugs (Miller and Dingle, 1982; United States Department of Agriculture, 2020). The bugs feed on the seeds (Burdfield-Steel and Shuker, 2014) and sequester cardenolides present in the seeds for their own defense (Scudder and Meredith, 1982; Moore and Scudder, 1985). *O. fasciatus* is adapted to feed on *Asclepias* due to specific amino acid substitutions in the physiological target—the Na^+/K^+-ATPases—which reduce the insect's sensitivity (Bramer et al., 2015). We aimed to answer the following question in the context of milkweed-herbivore interactions: Do milkweed bugs sequester cardenolides according to the availability in the seeds or according to the inhibitory capacity of the cardenolides on the bugs' Na^+/K^+-ATPase?

2. Materials and methods

2.1. Cardenolides from *Asclepias curassavica* seeds and sequestration

Here, we compared the quantification of 10 seed cardenolides sequestered by nymphs and adults of the large milkweed bug *Oncopeltus fasciatus* (see methods in Rubiano-Buitrago et al., 2023). These cardenolides are: 3-O-β-allopyranosyl coroglaucigenin, 3-[4′-O-β-glucopyranosyl-β-allopyranosyl] coroglaucigenin, 3′-O-β-glucopyranosyl-15- β-hydroxycalotropin, 3-O-β-glucopyranosyl-12-β-hydroxyl coroglaucigenin, 4′-O-β-glucopyranosyl frugoside, 4′-O-β-glucopyranosyl gofruside, 3′-O-β-glucopyranosyl calotropin, frugoside, gofruside, and 16α-hydroxycalotropin.

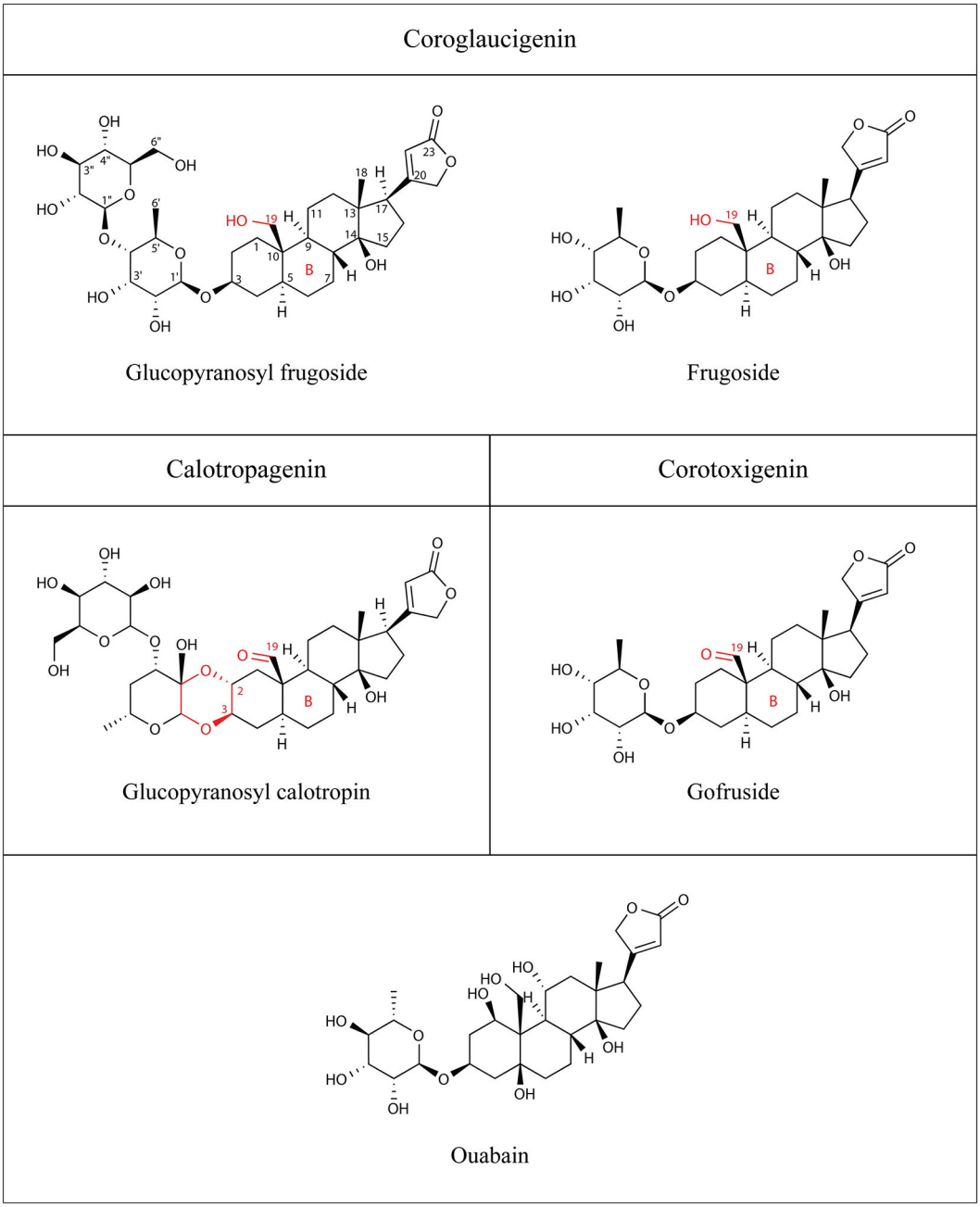

FIGURE 1
Structures of the cardiac glycosides used in this study: glucopyranosyl frugoside, glucopyranosyl calotropin, frugoside, gofruside, and ouabain. Structural features that differentiate the three types of steroidal structure are drawn in red and include functional groups at C-19, a dioxane ring in C-2, C-3, and the ring B of the steroidal core.

To assess the sequestration behavior of *O. fasciatus*, we used bugs that originated from a long-term laboratory colony (originally from the United States) maintained on sunflower seeds. We reared the bugs in terrarium boxes (37 cm × 22 cm × 25 cm) lined with tissue paper and provided them with *ad libitum* water in Eppendorf tubes plugged with dental cotton, and *ad libitum* *Asclepias curassavica* seeds as a food source. The colonies of *O. fasciatus* were kept in an incubator (Polyklima PK 520-LED) at 70% humidity with a day/night cycle of (18:6) and temperatures of 28°C during the day and 18°C at night. *O. fasciatus* nymphs develop through five instars (N1–N5), after which they molt into adults. We collected three groups of adults and three groups of N5 nymphs from the experimental colonies and froze them at −80°C.

We extracted cardenolides from the three pools of adults and three pools of N5 nymphs. Each pool weighed ∼700 mg. We ground the frozen material and exhaustively extracted cardenolides using a 1:1 MeOH/H_2O three times (MeOH ROTISOLV 99.9%, Carl Roth GmbH, Karlsruhe, Germany, water was HPLC grade,

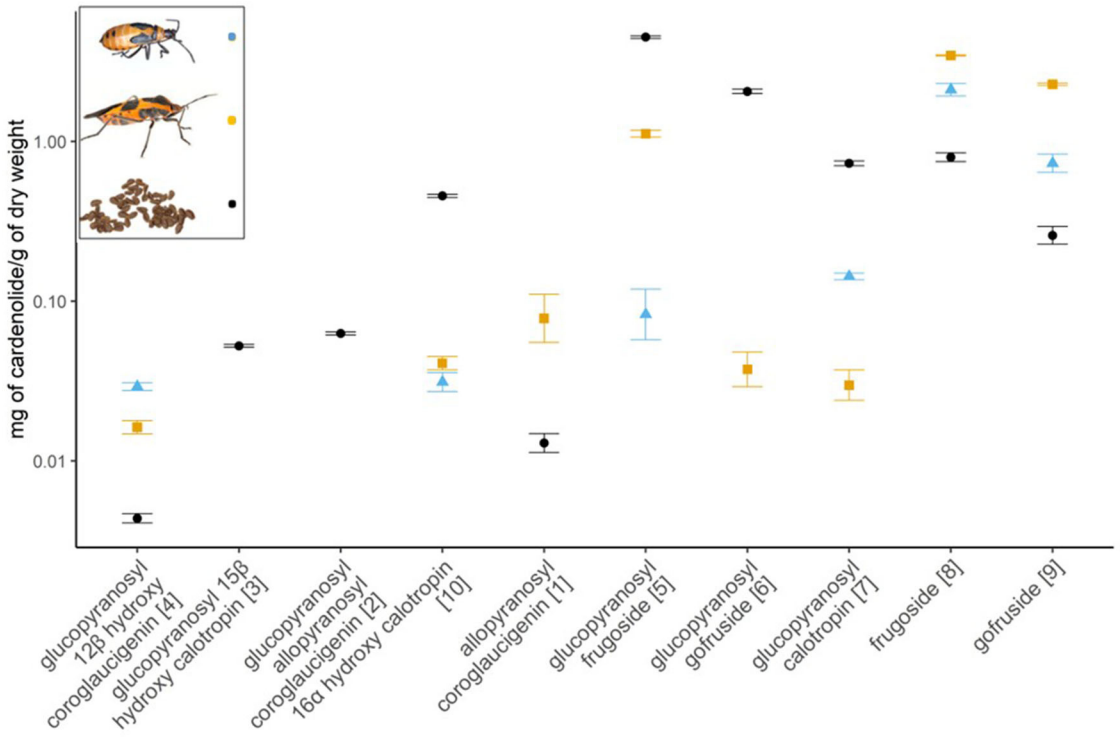

FIGURE 2
Concentration of cardenolides in *A. curassavica* seeds (black) and sequestered by large milkweed bugs (*O. fasciatus*; whole adults extracted, orange; nymphs, blue; N = 9 per compound), shown as means ± SE quantified by LC-HRMS. The compounds are arranged from most polar on the left to non-polar on the right. The numbers in square brackets correspond with compound identification from Rubiano-Buitrago et al. (2023). Note the *y*-axis is on a log scale. Images: *Oncopeltus* nymph (Veit Grabe), *Oncopeltus* adult (Jena Johnson; The Scientist), seeds (Francesca Protti-Sánchez).

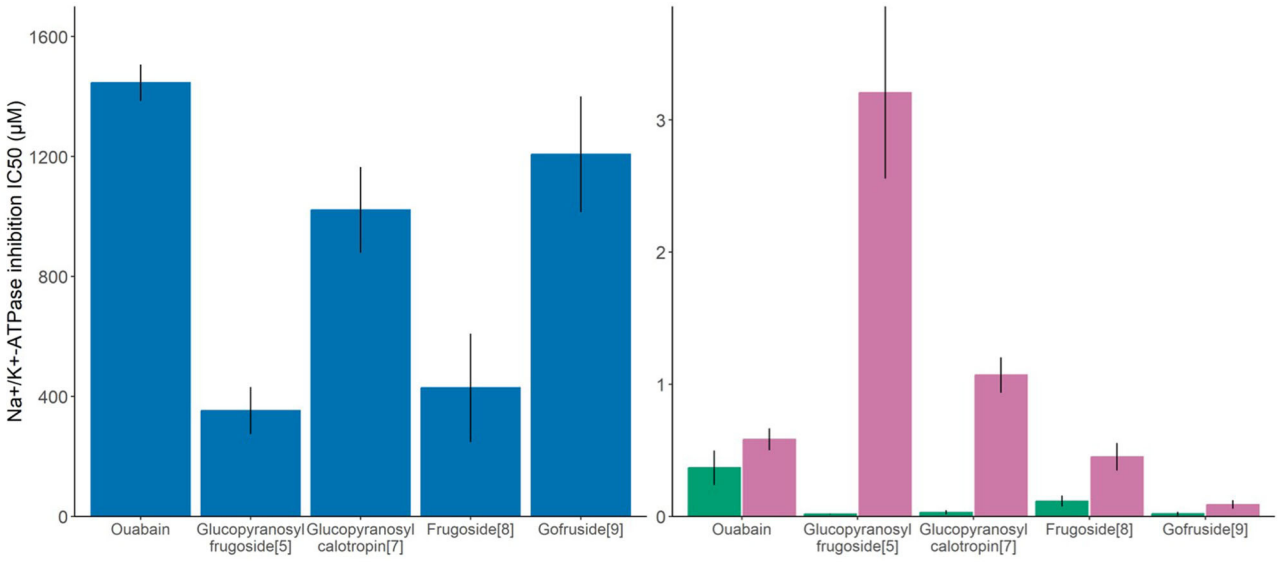

FIGURE 3
Molar concentration of plant toxin necessary to cause 50% inhibition of the *O. fasciatus* enzyme (blue), non-adapted insect *(D.melanogaster*, green), and a vertebrate reference (*S. domesticus,* purple) Na^+/K^+-ATPases, or IC_{50} of the dominant seed cardenolide (glucopyranosyl frugoside) and the subdominant compounds, all of which are sequestered, shown as means ± SE, *N* = 4–6 per compound). Higher values on the *y*-axis indicate that the enzyme is more tolerant to the cardenolide.

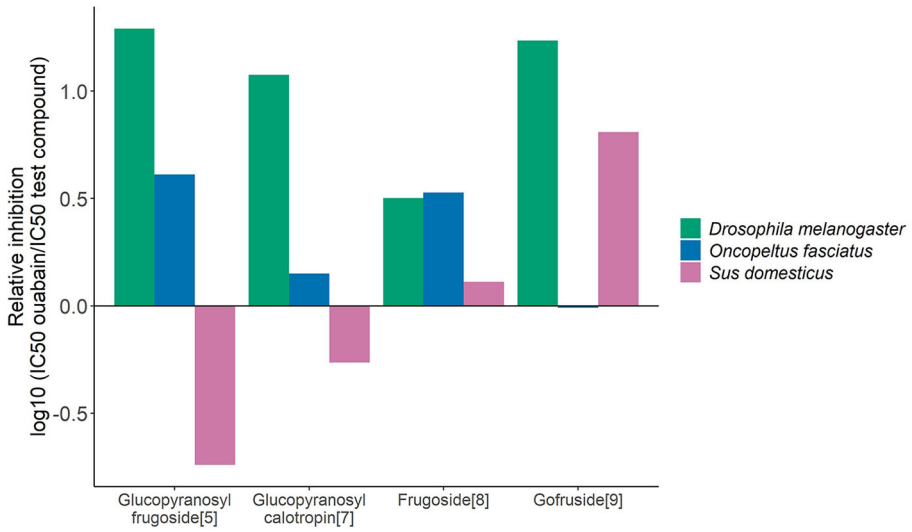

FIGURE 4
Selective effects of individual cardiac glycosides on different forms of Na^+/K^+-ATPase (*O. fasciatus*, blue; non-adapted *D. melanogaster*, green, and *S. domesticus*, pink). Relative inhibition values are based on IC_{50} values compared with the standard ouabain (computed as the log10 of the ratio (IC_{50} reference compound)/(IC_{50} test compound). Inhibition weaker than ouabain is indicated by negative values, while inhibition greater than ouabain is indicated by positive values.

deionized with a Merck Millipore Milli-Q A10, Merck KgA, Darmstadt, Germany), followed by two more times with 100% MeOH. The extracts were pooled and filtered through an MN HR-X 500 mg cartridge and washed with MeOH to remove lipophilic substances. The samples were dried using N_2 gas at 36°C.

We obtained three nymph extracts of 85.2, 115.1, and 121.0 mg, and three adult extracts of 86.0, 92.0, and 81.9 mg. From each replicate, we prepared a 2.6 mg/mL solution for high-resolution mass spectrometry (HPLC-HRMS). We injected 5 μL of each replicate three times, resulting in nine data points per compound. The high-performance liquid chromatography coupled with high-resolution mass spectrometry (HPLC-HRMS) was achieved using an Agilent 1,260 Infinity, with a reversed-phase Agilent Poroshell 120 column (2.7 μm particle size, 4.6 × 50 mm). The mobile phase consisted of acetonitrile (ACN, supplied with 0.1% formic acid, FA, Carl Roth GmbH, Karlsruhe, Germany) and water (HPLC grade, 0.1% FA, deionized with a Merck Millipore Milli-Q A10, Merck KgA, Darmstadt, Germany). The elution gradient started with ACN/H_2O (5:95) for 1 min, then to ACN/H_2O (95:5) for 8 min, which was maintained for 2 min. Later, it was set back to ACN/H_2O (5:95) for 1 min. High-resolution mass spectra were recorded on a Bruker Compact OTOF spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Electrospray ionization (ESI) in positive ion mode was used for the analysis in full scan and auto MS/MS modes, scanning masses from m/z 50–1,300. Sodium formate adducts were used for internal calibration HPC mode. Bruker Compass ver.1.9 (OTOF Control ver.5.1.107 and HyStar 4.1.31.1) was used for data acquisition and instrument control, and Bruker DataAnalysis ver. 5.1.201 was used for data processing.

We processed the data by extracting the ion chromatogram per compound and measuring the corresponding peak area. The concentration per injection was calculated as mg of cardenolide per g of dry weight.

2.2. Cardenolide toxicity tested by functional Na^+/K^+-ATPase assays

Na^+/K^+-ATPase extractions from the cardenolide-resistant seed bug (*Oncopeltus fasciatus;* Bramer et al., 2015; Agrawal et al., 2022; López-Goldar et al., 2022) and one non-adapted insect (*Drosophila melanogaster* wild type Canton S) (Dalla and Dobler, 2016; Dobler et al., 2019) were prepared according to the methods described in Petschenka et al. (2013, 2018, 2022). Fruit flies were reared under controlled conditions, and *O. fasciatus* were reared in terrarium boxes as described in section Cardenolides from *Asclepias curassavica* seeds and sequestration except that for this analysis, *O. fasciatus* adults were reared on sunflower seeds (*Helianthus annuus*).

Briefly, the brains and thoracic ganglia of individual *O. fasciatus* (killed and stored at −80°C) were dissected under deionized water. We pooled the brains of six individuals (following Bramer et al., 2015) and homogenized them in 500 μL of deionized water using an all glass 1 mL Wheaton grinder. For *D. melanogaster*, we dissected the heads of 90 individuals and homogenized them in 900 μL of deionized water and then split them into three 300 μL aliquots (following Taverner et al., 2019). All extracts were frozen, lyophilized, and stored at −80°C until use. Prior to the assays, the insect lyophilisates were reconstituted by adding 200 μL water (*O. fasciatus)* or 600 μL (*D. melanogaster*) followed by vortex and incubation for 10 min in a chilled ultrasonic bath. Porcine lyophilized Na^+/K^+-ATPase was dissolved in water to a concentration of 1 U/ml, stored at −80°C in single-use aliquots, and diluted with H_2O to 0.05 U/ml for use in the *in vitro* assay (final concentration in the assay, 0.01 U/ml).

All cardiac glycosides (Figure 1) were tested with at least two biological replicates of Na^+/K^+-ATPase (i.e., Na^+/K^+-ATPase

from genetically different specimens or pools of specimens). Reactions were performed in 96-well microplates to which the Na^+/K^+-ATPase protein was added in an eight-well row containing stabilizing buffers (see formulas in Petschenka et al., 2023). The first six wells in the eight-well row were exposed to exponentially decreasing concentrations of cardenolide (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M). The seventh well was 2% DMSO (as an experimental control), and the eighth well was exposed to a combination of inhibition buffers lacking KCl but supplemented with 10^{-3} M cardenolide. This allows the measurement of background ATP hydrolysis. The proteins were incubated on a BioShake Iq microplate shaker (Quantifoil Instruments) at 200 rpm and 37°C for 10 min after which ATP (Sigma Aldrich, A9062-1G) was added to each well and then incubated at 200 rpm and 37°C for further 20 min. The Na^+/K^+-ATPase activity was measured as the amount of inorganic phosphate enzymatically released from ATP in the presence of K^+ (Na^+/K^+-ATPase active) minus the amount of phosphate released in the absence of K^+ (Na^+/K^+-ATPase inactive). The activity was quantified photometrically at 700 nm with a microplate reader (BMG Clariostar) following (Taussky and Shorr, 1953). Background phosphate absorbance levels from the reactions with inhibiting factors were used to calibrate phosphate absorbance in wells measuring cardenolide inhibition and in the control wells (Petschenka et al., 2013). For each cardenolide, we carried out two technical replicates, making each data point an average of four to six measurements (Petschenka et al., 2012, 2013; Taverner et al., 2019).

2.3. Data evaluation

To compare the concentration of individual cardenolides available in the seeds to those sequestered by adults and nymphs we log-transformed the concentration data and analyzed the transformed data using analysis of variance. For glucopyranosyl frugoside and glucopyranosyl calotropin, the transformation did not improve the fit of the data for parametric tests, thus we compared them using the Wilcoxon rank test with a continuity correction. We did not analyze data where the bugs had not sequestered compounds (glucopyranosyl hydroxycalotropin and glucopyranosyl allopyranosyl coroglaucigenin) or where only the adult or the nymph (not both) had sequestered (allopyranosyl coroglaucigenin and glucopyranosyl gofruside).

For cardenolide sensitivity measurements, calibrated absorbance values were converted to the percentage of non-inhibited Na^+/K^+-ATPases activity based on measurements from the control wells. These data were plotted, and $\log_{10}$ of the IC_{50} values was calculated for each biological replicate from non-linear fitting using a four-parameter logistic curve, with the top and bottom asymptotes set to 100 and zero, respectively. We fitted the curves using the nlsLM function of the minipack.lm library in R (R Core Team, 2022) (Supplementary Figure 3 and Supplementary Table 1). We compared the $\log_{10}$ IC_{50} values of individual cardenolides for each Na^+/K^+-ATPase enzyme using the analysis of variance, with Tukey's HSD pairwise contrasts. We calculated the fold differences between the IC_{50} values of individual cardenolides for non-adapted and adapted insect Na^+/K^+-ATPase and the porcine Na^+/K^+-ATPase. To calculate the effects of individual cardiac glycosides on different forms of Na^+/K^+-ATPase (adapted or non-adapted), we compared the inhibition to a reference cardenolide by calculating the log10 of the ratio (IC_{50} reference compound)/(IC_{50} test compound). We used ouabain (Sigma Aldrich, O3125-1G) as a reference as it is the most widely used cardenolide in research on Na^+/K^+-ATPase (Petschenka et al., 2018). Here, negative values represent compounds that inhibit the Na^+/K^+-ATPase more weakly than ouabain, and positive values represent compounds that inhibit the Na^+/K^+-ATPasemore strongly than ouabain. We also calculated this ratio using glucopyranosyl frugoside as the reference, because it is the most abundant cardenolide in the seeds (Rubiano-Buitrago et al., 2023).

All analyses were conducted in R (version 1.4.1717).

3. Results

3.1. Seed cardenolides and sequestration

We found significant differences among the concentration of cardenolides available in the seeds of *A. curassavica* and those sequestered by nymphs and adults of *O. fasciatus* (see Supplementary Table 2). Among the five most abundant cardenolides present in milkweed seeds (accounting for 91% of the total), glucopyranosyl frugoside, glucopyranosyl gofruside, and glucopyranosyl calotropin were significantly more abundant in the seeds than the adults and nymphs. Frugoside and gofruside were significantly more dominant in the adults and nymphs than in the seeds (Figure 2 and Supplementary Figures 1, 2). Of the minor compounds, the adults and nymphs sequestered significantly more glucopyranosyl-12β-hydroxyl coroglaucigenin than was present in the seeds, but significantly less 16α-hydroxycalotropin. Neither adults nor nymphs sequestered glucopyranosyl-15β-hydroxycalotropin or glucopyranosyl-allopyranosyl coroglaucigenin that were present in the seeds. Adult bugs, but not nymphs, sequestered allopyranosyl coroglaucigenin and glucopyranosyl gofruside from the seeds. There were eight other cardenolides in the seeds that we did not identify and that the bugs did not sequester. There were also 14 cardenolides in the LC-HRMS data of *O. fasciatus* that did not have the retention time or the fragmentation pattern of our purified cardenolide reference obtained from the seeds, and that were also absent from the spectrometric data of the seeds. Of these 14 compounds, seven were present in both adults and nymphs, five were only found in the nymphs, and two were only found in the adults (see Supplementary Table 3).

3.2. Cardenolide toxicity

The compounds tested (glucopyranosyl frugoside, glucopyranosyl calotropin, frugoside, gofruside, and ouabain, Figure 3) had significantly different effects on the *O. fasciatus* Na^+/K^+-ATPase enzyme (ANOVA: $F_{(4,17)} = 9.70$, $p = 0.0003$). Glucopyranosyl frugoside was four times more inhibitory than ouabain (Tukey's HSD: estimate $= -0.68 \pm 1.19$, $p = 0.002$). Frugoside was 3.5 times more inhibitory than ouabain (Tukey's HSD: estimate $= -0.64 \pm 1.13$, $p = 0.007$). Glucopyranosyl

calotropin and gofruside did not significantly differ from ouabain in inhibitory capacity (Tukey's HSD: estimate = −0.165 ± 0.65, $p = 0.84$; estimate = −0.01 ± 0.51, $p = 0.99$).

For the sensitive invertebrate *D. melanogaster*, we also found a significant difference between the compounds on the IC_{50} values (ANOVA: $F_{(4,23)} = 8.04$, $p = 0.0003$). Glucopyranosyl frugoside was 20 times more inhibitory than ouabain (Tukey's HSD: estimate = −1.21 ± 2.14, $p = 0.007$). Gofruside was 17 times more inhibitory than ouabain (Tukey's HSD: estimate = −1.43 ± 2.36, $p = 0.001$), and glucocalotropin was 12 times more inhibitory than ouabain (Tukey's HSD: estimate = −1.40 ± 2.33, $p = 0.002$). Frugoside did not differ significantly from ouabain (fold difference = 3; Tukey's HSD: estimate = −0.51 ± 1.46, $p = 0.5$). We also found a significant variation in the inhibitory capacity of the compounds on the highly sensitive porcine enzyme (ANOVA: $F_{(4,25)} = 49.63$, $p < 0.0001$). Glucopyranosyl frugoside was five times less inhibitory than ouabain (Tukey's HSD: estimate = 0.72 ± 0.37, $p = 0.00002$), and glucopyranosyl calotropin was 1.5 times less inhibitory (Tukey's HSD: estimate = 0.27 ± 0.08, $p = 0.18$), whereas gofruside was 6.5 times more inhibitory (Tukey's HSD: estimate = −0.88 ± 1.23, $p = 0.00007$). Frugoside was not significantly different from ouabain in inhibitory activity (fold difference = 1.3, Tukey's HSD: estimate = −0.13 ± 0.48, $p = 0.78$).

Frugoside caused a unidirectional effect on the three enzymes compared to ouabain, whereas glucopyranosyl calotropin, glucopyranosyl frugoside, and gofruside produced countervailing effects (Figure 4). Glucopyranosyl calotropin and glucopyranosyl frugoside were stronger than ouabain on the insect enzymes but weaker than ouabain on the porcine Na^+/K^+-ATPases. Gofruside had almost equal to lower effect than ouabain for *O. fasciatus* but was more inhibitory for the sensitive species. Using glucopyranosyl frugoside as a reference in place of ouabain shifted the direction of the effects (Supplementary Figure 4).

We found no significant relationship between concentration in the seeds and inhibitory effects of cardenolides across the insect enzymes (Pearson's correlations: *D. melanogaster* $R = -0.32$, $p = 0.68$; *O. fasciatus* $R = -0.66$, $p = 0.34$), but there was a trend toward a decrease in inhibitory capacity with increasing concentration for the porcine enzyme ($R = 0.97$, $p = 0.026$) (Supplementary Figure 5).

4. Discussion

We quantified the cardenolides sequestered by the specialized milkweed herbivore *Oncopeltus fasciatus* across two stages of its life cycle when reared on seeds of *Asclepias curassavica*. We combined the identification and quantification of sequestered cardenolides with tests of their inhibitory capacity of the Na^+/K^+-ATPase. Among the five most abundant cardenolides present in milkweed seeds, frugoside and gofruside were more concentrated in the bugs than the seeds. These two compounds had contrasting potency toward the *O. fasciatus* enzyme–frugoside (and its glucosylated counterpart), which was the most inhibitory, whereas gofruside was among the weakest inhibitors. When comparing the inhibitory capacity of all the compounds to a non-adapted insect *Drosophila melanogaster* and the highly sensitive porcine Na^+/K^+-ATPase, we found that the compounds varied less in their inhibition of the *O. fasciatus* enzyme compared to the non-adapted insect enzyme. The finding that there is differential accumulation of *A. curassavica* seed cardenolides by *O. fasciatus* adds to the evidence that the bug sequesters particular, but not all, cardenolides from its *Asclepias* hostplants (Duffey et al., 1978; Scudder and Meredith, 1982; Meredith et al., 1984).

Milkweed bugs reared on seeds of *A. curassavica* sequestered two of the more non-polar cardenolides available in the seeds (frugoside and gofruside), which is in line with the preferential sequestration of the non-polar cardenolide digitoxin in the fat body of adult *O. fasciatus* reported by Duffey et al. (1978). The fat body often accumulates non-polar compounds owing to the lipophilic nature of such compounds (Kilby, 1963). *O. fasciatus* in our study did not sequester several compounds that are present in the seeds, which is also in line with the results of Moore and Scudder (1985) who showed that *O. fasciatus* sequestered most, but not all, of the cardenolides in seeds of *A. speciosa* (9 of 13 cardenolides sequestered) and *A. syriaca* (6 of 8 cardenolides sequestered). We found 14 compounds in the bugs that did not have the retention time or the fragmentation pattern of cardenolides from the seeds. A similar sequestration behavior has also been reported by Moore and Scudder (1985) who found that *O. fasciatus* contained 12 cardenolides that were not present in *A. speciosa* seeds, and 8 cardenolides that were not present in *A. syriaca* seeds. Our results could indicate that *O. fasciatus* concentrated some compounds that have such low abundance in the seeds that they were not detectable by spectrometric measurements or could indicate the metabolism of cardenolides, which is known to occur when *O. fasciatus* is reared on *A. syriaca* (Agrawal et al., 2022). The higher concentration of frugoside and gofruside in the bugs than in seeds could indicate that *O. fasciatus* cleaves the sugar from the glucosylated compounds during sequestration. To verify this, the insects could be reared on artificial diets containing these isolated cardenolides and the sequestration products could then be quantified (e.g., Pokharel et al., 2021; Agrawal et al., 2022).

Until recently the Na^+/K^+-ATPase of *O. fasciatus* has been studied almost exclusively with reference to ouabain (though see: Herbertz et al., 2020; Agrawal et al., 2022; López-Goldar et al., 2022). The main result from our tests of four compounds of *A. curassavica* is that glucopyranosyl frugoside and frugoside have potency against *O. fasciatus*, which has a highly adapted sodium pump characterized by gene duplications and substitutions (Bramer et al., 2015; Lohr et al., 2017). Phytochemical profiles of the aerial parts and roots of *A. curassavica* have no reports of glucopyranosyl frugoside being isolated, which could suggest it is a compound specific to the seeds and, given its inhibitory capacity, under selection by the seed bugs. Frugoside, on the other hand, is well distributed across the plant's tissues (Roy et al., 2005; Warashina and Noro, 2008; Warashina et al., 2008; Li et al., 2009; Al-Snafi, 2015; Ji et al., 2022). We did not test these compounds on other adapted herbivores, such as Monarch butterflies (*Danaus plexippus*), and so our data do not allow us to draw specific conclusions about *Oncopeltus*–milkweed coevolution (Berenbaum and Zangerl, 1996), but open up a path for future research on toxin–receptor interactions.

Our results are in contrast with those of Agrawal et al. (2022), who found that the dominant cardenolide in seeds of *Asclepias syriaca* was a less potent inhibitor of the *O. fasciatus* Na^+/K^+-ATPase than a minor compound, labriformin, that

was the most potent inhibitor. Labriformin is characterized by nitrogen- and sulfur-containing thiazolidine ring moiety, which was not present in any of the major cardenolides isolated in *A. curassavica* seeds, but is known to be present in the leaves (Agrawal et al., 2022). Cardenolides with a thiazolidine ring are predicted to interact with the binding site of *O. fasciatus* Na^+/K^+-ATPase in *in silico* molecular docking simulations (Ramos et al., 2021), which likely explains why *O. fasciatus* sequesters the less toxic nitrogen-containing oxidized labriformin (Agrawal et al., 2022). Of our isolated compounds, we might have expected gofruside (which contains an aldehyde moiety as part of their corotoxigenin steroidal structure, Figure 1) and glucopyranosyl calotropin (which contains the same aldehyde group as gofruside and also has a dioxane ring bond in C-2 and C-3, Figure 1) to have been more inhibitory to the *Oncopeltus* enzyme (Zhang et al., 2014). For example, corotoxigenin cardenolides, like gofruside, are predicted to form a strong hydrophobic pi-sigma interaction between amino acid residue 783 and the steroidal ring B, which is hypothesized to assist the cardenolide-sensitive Na^+/K^+-ATPase complex (see the porcine model in Meneses-Sagrero et al., 2022). However, gofruside and glucopyranosyl calotropin did not differ in their inhibitory properties compared to ouabain to the *O. fasciatus* Na^+/K^+-ATPase. Both cardenolides were less inhibitory than the major compound in the seeds, which have hydroxyl moiety at C-19 (Figure 1). Gofruside and glucopyranosyl calotropin were, however, highly inhibitory to the *D. melanogaster* enzyme.

Different degrees of oxidation in the C-19 position are predicted to generate a key hydrogen bond between the cardenolide and the enzyme amino acid residue 111, a site that is strongly implicated in cardenolide resistance (Bramer et al., 2015). The hydrogen bonds between amino acid residue 111 and the aldehyde or hydroxyl moieties are predicted to influence the stability of the cardenolide-protein complex (Meneses-Sagrero et al., 2022). Further *in silico* molecular docking simulations of these compounds against both resistant and sensitive enzymes are needed to clarify the importance of the oxidation in the steroidal C-19 in relation to the amino acid residues that have been implicated in resistance.

Glycosylated cardenolides are generally considered to be more toxic than corresponding genins in whole organism vertebrate assays (Hoch, 1961). Petschenka et al. (2018) found that glycosides were on average 6-fold more potent in their inhibition of insect Na^+/K^+-ATPases, including the specialized monarch butterfly Na^+/K^+-ATPase, compared to genins. Agrawal et al. (2022) also found that diglycosylated compounds were highly inhibitory. We found similar inhibition of glucopyranosyl frugoside and frugoside against *O. fasciatus,* but the deglycosylated version was more inhibitory for the porcine enzyme. Given that we have only one comparison of a glycoside vs. its aglycone, we can tentatively suggest that the effect of the cardiac glycoside sugar moiety on inhibition depends on the specific biochemical properties of the target, the Na^+/K^+-ATPase enzyme. It is also suggested that the toxicity of cardenolides can increase from polar to non-polar compounds (Petschenka and Dobler, 2009; Rasmann and Agrawal, 2011; Agrawal et al., 2012; López-Goldar et al., 2022). In our analysis of five cardenolides with different polarities and Na^+/K^+-ATPase pumps from three species, we do not observe a clear trend where polarity is a key factor for the inhibitory response.

4.1. Synthesis

Our study, along with Agrawal et al. (2022) and Petschenka et al. (2018), focuses on individual compounds, rather than natural mixtures in which those compounds are encountered in nature (though see López-Goldar et al., 2022). Mixtures of cardenolides may be maintained because of synergistic or additive interactions among compounds (Richards et al., 2016; Whitehead et al., 2021). Mixtures may also be maintained because of antagonism, where the effects of active constituents are masked by other compounds in a complex mixture (Caesar and Cech, 2019). Comparison of the IC_{50} values for single cardenolides vs. mixtures of cardenolides would be useful for uncovering the combined effects of the compounds and could provide a functional explanation for the repeated derivatization of cardenolides in milkweeds (Whitehead et al., 2021) and the differences in the abundance and inhibitory properties of cardenolides in different species of *Asclepias* (Agrawal et al., 2022; López-Goldar et al., 2022). Such research could also shed more light on whether there are costs associated with the biosynthesis of different cardenolides (e.g., those with extra glycosylation, or further modifications to the sugar moiety), and the cost-benefit of producing specific cardenolides in relation to differences in insect diversity, herbivore specialization, and the constraints on those herbivores. Although Agrawal et al. (2022) found no significant effects of the most potent inhibitors on *O. fasciatus* growth and performance, we have recently shown that sequestration can be costly in terms of oxidative state (Blount et al., 2023; Heyworth et al., 2023) which is visible in the wing colors that milkweed herbivores use as anti-predator defenses. More tests on the costs of sequestration will also be important for understanding the evolutionary and ecological interactions between the plant and the herbivore.

Data availability statement

The original contributions presented in the study are publicly available. The raw data and R scripts for statistical analysis and data visualisation can be found at: https://edmond.mpdl.mpg.de/dataset.xhtml?persistentId=doi:10.17617/3.SQGWHL.

Author contributions

Conceptualization: HMR, CP, and PR-B. Methodology: PR-B, SP, VG, CP, and HMR. Formal analysis and writing—original draft preparation: HMR and PR-B. Investigation: PR-B and SP. Resources and supervision: HMR and CP. Data curation: PR-B. Writing—review and editing: SP, CP, AA-A, and VG. Visualization: AA-A and PR-B. Project administration and funding acquisition: HMR. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1175205/full#supplementary-material

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