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LETTER

Testing the selective sequestration hypothesis: Monarch butterflies preferentially sequester plant defences that are less toxic to themselves while maintaining potency to others

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Abstract

Herbivores that sequester toxins are thought to have cracked the code of plant defences. Nonetheless, coevolutionary theory predicts that plants should evolve toxic variants that also negatively impact specialists. We propose and test the selective sequestration hypothesis, that specialists preferentially sequester compounds that are less toxic to themselves while maintaining toxicity to enemies. Using chemically distinct plants, we show that monarch butterflies sequester only a subset of cardenolides from milkweed leaves that are less potent against their target enzyme (Na^+/K^+-ATPase) compared to several dominant cardenolides from leaves. However, sequestered compounds remain highly potent against sensitive Na^+/K^+-ATPases found in most predators. We confirmed this differential toxicity with mixtures of purified cardenolides from leaves and butterflies. The genetic basis of monarch adaptation to sequestered cardenolides was also confirmed with transgenic *Drosophila* that were CRISPR-edited with the monarch's Na^+/K^+-ATPase. Thus, the monarch's selective sequestration appears to reduce self-harm while maintaining protection from enemies.

KEYWORDS
cardenolide cardiac glycoside, chemical ecology, coevolution, milkweed *Asclepias*, monarch butterfly *Danaus plexippus*, sequestration

INTRODUCTION

Highly specialized insect herbivores that sequester defence compounds from their host plants present a conundrum to coevolutionary ecologists. On the one hand, such specialists are often thought of as truly immune to plant toxins (Ali & Agrawal, 2012). On the other hand, if coevolution is ongoing, it is predicted that plants will produce effective novel toxins against even the most specialized herbivores (or alternatively, decline in chemical defences, and switch to other strategies such as tolerance; Agrawal & Fishbein, 2008; Cornell & Hawkins, 2003). Various forms of evidence, from measures of natural selection (Lankau, 2007), population variation (Zangerl & Berenbaum, 2003), species comparisons (Cacho et al., 2015), and following the fate of various defence chemicals (Fontanilla et al., 2022; Gonzales-Vigil et al., 2011) can be leveraged to address these issues.

We define sequestration as the accumulation of plant defence compounds in the insect body, typically following digestion, recognizing that compounds may be modified during the process (Beran & Petschenka, 2022; Dreisbach et al., 2023). Here we address the relative toxicity of plant extracts versus insect-sequestered compounds to a specialist herbivore to test the prediction that such insects reduce self-harm by preferentially sequestering compounds that are the least toxic to themselves. We hypothesized that the subset of compounds that are sequestered would have reduced toxicity due to chemical modification or because they are taken up selectively. These are not mutually exclusive, and most sequestering herbivores likely do both. Thus, we propose the 'Selective Sequestration Hypothesis' suggesting that specialists preferentially sequester compounds that are the least toxic to themselves but maintain toxicity to their enemies. For example, we recently found two cardenolide toxins in

milkweed plants that are modified before sequestration and that appear to impose a burden (reduced growth rate) for specialist feeders (Agrawal et al., 2021; Brower & Moffitt, 1974). Because monarch butterflies (*Danaus plexippus*) feed on and sequester cardenolides across all *Asclepias* spp. (Malcolm & Brower, 1989), here we test the generality of their sensitivity to plant and sequestered cardenolides when feeding on four *Asclepias* spp. that span cardenolide concentrations and compositions across the genus.

Milkweed's cardenolides achieve their toxicity by binding to the universal animal enzyme Na^+/K^+-ATPase (Malcolm, 1991; Vaughan & Jungreis, 1977). Indeed, most milkweed species produce greater than 20 distinct cardenolides of varying polarities and potencies (Agrawal et al., 2012, 2021; Agrawal & Hastings, 2023). The highly reduced sensitivity of monarch butterflies to milkweed cardenolides has been functionally attributed to a handful of genetic changes that result in three amino acid substitutions in the alpha subunit of their Na^+/K^+-ATPase (ATPα1); QAN at positions 111, 119 and 122 changed to VSH, respectively; i.e. target-site insensitivity, Holzinger & Wink, 1996; Petschenka et al., 2013; Karageorgi et al., 2019). Thus, plant defence—herbivore offence interactions appear to be modulated by specific toxins and their binding affinity to the animal's sodium pump.

Accordingly, here we test the Selective Sequestration Hypothesis by comparing inhibition of the monarch's enzyme using extracts from leaves and butterfly wings. Leaves represent the raw material for sequestration pre-ingestion; wings represent the end-product of sequestration following any chemical modification, selective uptake, and storage. We then compare the inhibitory effects of the leaf and wing extracts on a sensitive vertebrate enzyme as a proxy for effects on unadapted predators. To address whether cardenolides per se were responsible for differential effects, we next purified the dominant cardenolide toxins from leaves and wings and tested the inhibitory effects of representative mixtures. Finally, because most herbivores, including monarchs, employ multiple mechanisms to cope with plant toxins (Dobler et al., 2011), we also directly test the genetic basis of their adaptation. Here we assessed the impacts of plant and wing extracts not only on the monarch and a generalist vertebrate Na^+/K^+-ATPase, but also on wild-type and CRISPR-engineered Na^+/K^+-ATPase extracted from living *Drosophila melanogaster*. In particular, the Na^+/K^+-ATPase of engineered *Drosophila* were edited to have the three resistance-providing amino acid substitutions found in the monarch's enzyme (Karageorgi et al., 2019). If results with CRISPR-engineered *Drosophila* mirror that of monarchs, relative to their sensitive comparisons, we can conclude that differences in enzyme inhibition between adapted and unadapted species are due to the three amino acid substitutions found in the monarch's enzyme.

Although we specifically predicted that sequestered toxins would be less potent than foliar compounds on the monarch's adapted Na^+/K^+-ATPase, we were less sure about their relative toxicity to a sensitive Na^+/K^+-ATPase, which is typical of predators and highly inhibited by all cardenolides (Agrawal et al., 2021; Petschenka et al., 2018). Nonetheless, our Selective Sequestration Hypothesis posits that specialist herbivores should minimize the ratio of self-harm to toxicity to predators.

MATERIALS AND METHODS

Plant and insect material

We reared monarchs on four North American *Asclepias* spp. that represent diversity in cardenolide concentration, composition and are used as host plants by the monarch butterfly: *A. syriaca* (low cardenolides, a main host), *A. curassavica* (higher cardenolides, a main host), *A. incarnata* (lowest cardenolides, a subsidiary host), and *A. asperula* (highest cardenolides, a subsidiary host). *A. syriaca and A. curassavica* also produce unusual and highly toxic nitrogen-containing cardenolides that appear to be absent in the other two species (Agrawal et al., 2021, 2022). By testing these distinct host species, our study specifically addresses the generality of results as well as other differences between host plants (e.g. trichomes, leaf toughness, latex and nutritional quality) that may predictably alter the plant-herbivore interactions.

Milkweed seeds were obtained from the following sources: *A. asperula*, collected in Boerne, Kendall County, Texas (USA), *A. curassavica*, commercially purchased from Everwilde Farms, Fallbrook, California (USA), *A. incarnata* collected from McLean, Tompkins County, New York (USA), and *A. syriaca* collected from Dryden, Tompkins County, New York (USA). Plant growth and feeding trials were conducted separately for each plant species.

Seeds were surface sterilized with 10% bleach, rinsed, and then nicked to encourage germination. Scarified seeds were sandwiched between layers of moist paper towels and housed in sealed Petri dishes at 30C until germination (3–5 days). Seedlings were planted into 500 mL pots filled with soil and kept in a growth chamber with 400 microeinsteins of photosynthetically active radiation at 27C day /24°C night with a 14 h day length. After plants had grown for at least 1 month, freshly hatched monarch caterpillars from a laboratory colony (reared from wild-caught butterflies in NY, USA, and in the lab for less than 5 generations reared on the main host, *A. syriaca*), were added to plants (typically at low density, up to a few individuals per plant). Most plants had extensive damage; we sampled leaves for chemistry from at least five plants of each species (pooled), from young fully expanded undamaged leaves. Caterpillars were allowed to pupate

and emerging adults were collected (both males and females; at least 10 butterflies were sampled per species and pooled). We focus our analysis of sequestered cardenolides on wing tissue for three reasons: (1) wings have the most concentrated cardenolides among the parts of the adult monarch butterfly (Brower and Glazier 1975; Fink & Brower, 1981), (2) pieces of wings are dispensable and typically provide the first taste of prey to predators (Fink & Brower, 1981), and (3) the cardenolide composition of wings is typically mirrored (although in lower concentrations) in the rest of the monarch body (Fink & Brower, 1981, unpublished manuscript).

Milkweed leaf tissue and butterflies were frozen at −80°C and then freeze-dried. Wings were clipped from butterfly bodies, and wing and leaf samples were ground to a fine powder, using a coffee grinder (leaf tissue), and 10 mL stainless steel grinding jars with 9 mm stainless steel balls on a Retsch Mixer Mill (MM300; wing tissue). For each leaf and wing sample type, 20–70 mg ground tissue was then extracted with methanol that had been spiked with hydrocortisone (25 μg/mL as an internal standard), and processed in a FastPrep-24 homogenizer (MP Biomedicals, Irvine, CA, USA). Samples were centrifuged at 20,817 *g* for 12 min to remove particulates and the supernatant was taken to dryness in a rotary evaporator (Labconco CentriVap). Wing extracts were defatted twice by dissolving residues in 250 mL methanol, adding 750 mL hexane, vortexing 3 times for 30 s, centrifuging for 10 min at 19,480 *g* and pipetting off the hexane layer. Defatted samples were then taken to dryness and brought back, along with leaf samples, in 300 μL methanol. These samples were filtered (0.2 μm Millipore syringe filter) and 200 μL was set aside for use in sodium pump assays, while the remainder was transferred to a vial for HPLC analysis.

Chemical characterization

Prior to running ATPase inhibition assays, we quantified cardenolides for all leaf and wing samples using HPLC. We followed the methods described in Petschenka et al., 2022. Fifteen microliters of each sample were injected into an Agilent 1100 HPLC for separation on a Gemini C18 reversed-phase Phenomenex column. Separation was achieved using the following gradient of acetonitrile and water: 16% acetonitrile for 2 min, then 16–70% acetonitrile from 2 to 25 mins, then 70–95% acetonitrile from 25 to 30 min, and 95% acetonitrile from 30 to 40 min, followed by a 10 min post-run at 16% acetonitrile. UV absorbance data were recorded for 200–400 nm, and cardenolides were identified by having maximum absorbance between 214 and 222 nm, and a UV spectral peak of characteristic shape. Cardenolide peak areas were integrated, concentrations were calculated using the hydrocortisone internal standard, and total cardenolide concentration for each tissue type was estimated by taking the sum of individual cardenolide concentrations.

We also used high-resolution mass spectroscopy to characterize and quantify the specific chemical composition of leaves and monarch wings focusing on the two primary host plant species (*A. syriaca* and *A. curassavica*; $n=3$ replicates per tissue type per plant species). We follow the protocol described in Agrawal et al. (2022). Briefly, we used reversed-phase chromatography in a 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 × 2.1 mm, particle size 1.8 μm) maintained at 40°C with a flow rate of 0.5 mL/min. Each sample was analysed in positive electrospray ionization mode with *m/z* ranges of 70–1000. MS^2 spectra were obtained via Excalibur software (ThermoFisher Scientific). LC–MS data were analysed using MZmine software (Pluskal et al., 2010). The acquired LC–MS data files were converted to mzXML files using the ProteoWizard MSconvert tool. LC–MS data were then pre-processed with the open-source MZmine 2 software and consisted of peak detection, removal of isotopes, alignment, filtering, and peak filling. We mined the generated feature table to retrieve cardenolide ion adducts known to be present in *A. syriaca* and *A. curassavica* and confirmed their structure by comparing MS2 fragmentation spectra and retention time with pure isolated standards if available in our in-house library. The list of cardenolides can be found in Table S1 and their corresponding chemical structures in Figure S2. The relative concentration (semi-quantification) based on ion counts for all cardenolides was determined using the calibration curve of aspecioside.

In addition to the concentration of cardenolides in milkweed leaves and monarch wings, we characterized the Shannon–Wiener index of cardenolide diversity (Rasmann & Agrawal, 2011; Wetzel & Whitehead, 2020) in each tissue for *A. syriaca* and *A. curassavica*. The index H is calculated as $-\text{sum}(P_i \log[P_i])$, where P_i is the relative concentration of a given cardenolide in divided by the total concentration of cardenolides in that sample. This diversity index combines richness and evenness by weighing each compound by its abundance. As such, the comparison of leaves and wings provides an estimate of whether butterflies change the number and dominance of cardenolides sequestered from leaves.

Physicochemical properties

Physicochemical properties of leaf and sequestered cardenolides were determined using the SwissADME tool http://www.swissadme.ch/ (Daina et al., 2017). Here our goal was to assess properties related to chemical

polarity which govern their movement through an animal body. Cardenolide structures drawn in ChemDraw (PerkinElmer Software, v22.2.0) were transformed into a Simplified Molecular-Input Line-Entry System (SMILES). SMILES line notation was then copied into SwissADME to retrieve the partition coefficient (WLOGP) and topological polar surface values (TPSA) of each cardenolide structure. These values can be found in Table S2.

Na^+/K^+-ATPase preparations

Preparations of neural Na^+/K^+-ATPases were obtained by homogenization of dissected brains (monarchs) or heads (fruit flies, wild-type QAN and transgenic VSH) in Millipore water, as described in Petschenka et al., 2022. Transgenic flies were generated by genome editing using CRISPR–Cas9 coupled homology-directed repair to introduce the point mutations into the native alpha subunit of *D. melanogaster* region coding for the H1–H2 extracellular loop (details in Karageorgi et al., 2019). Homogenates were freeze-dried and then resuspended in cold Millipore water just prior to running the enzyme inhibition assays. Concentrations used were 0.375 monarch brains per mL, and 27.8 fruit fly heads per mL. For our analysis of a sensitive vertebrate enzyme, we employ the commercially available porcine Na^+/K^+-ATPase (Sigma) at 0.05 Units/mL Millipore water.

Tests for extract potency

All extracts for the enzyme assays were standardized by total cardenolide concentration, as estimated by HPLC. Stock extracts were brought into solution at a concentration of 0.15 μg/μL in 20% DMSO and centrifuged for 10 min at 19,480 *g* to remove insoluble components. We then prepared six 6x serial dilutions of each stock solution. For each milkweed species, leaf and butterfly wing extracts were tested, on the same plate, for inhibition of both resistant (monarch) and sensitive (porcine) Na^+/K^+-ATPase preparations. We tested extracts of only *A. asperula* (leaves and wings of *A. asperula*-reared monarchs) on the wild-type and genetically engineered Drosophila Na^+/K^+-ATPase, as our key motivation was to assess whether the effects on the fly enzymes were the same as the monarch-porcine comparison. Due to the increased resistance of the adapted Na^+/K^+-ATPase to cardenolides, we included the most concentrated stock solution in the extract dilution series for the monarch and CRISPR fly preparations, while the dilution curve for the sensitive enzymes started with 1/6 stock. Assays were replicated at least three times, following the methods in Petschenka et al., 2022. We used a 3-parameter nlme model to fit activity curves for each replicate and estimated the concentration of wing or leaf cardenolides required to inhibit each enzyme type by 50% as the IC50 for each technical replicate. IC50s were reported in units of μg cardenolide per reaction.

Mixtures of purified cardenolides

As a means to isolate the effect of cardenolides on the Na^+/K^+-ATPases in the absence of other extracted products from plant and animal tissues, we used purified cardenolides from the monarch's dominant host, *A. syriaca*. Our mass spectrometric analysis revealed eight cardenolides dominating leaf tissue (accounting for 98.1% of the total cardenolides) and three cardenolides dominating monarch wings (accounting for 90.4% of the total cardenolides; Table S1). Using methods described elsewhere (Agrawal et al., 2021, 2022; Agrawal & Hastings, 2023) we isolated these eight compounds (all three wing compounds were present in leaves) to >90% purity.

Next, we created a synthetic cardenolide mixture representative of the *A. syriaca* leaf extract, as well as one representative of the *A. syriaca*-reared monarch wing extract, by combining purified cardenolides in proportion to their relative abundance in these tissues (see Table S1). The stock concentration of each synthetic mixture matched that of the tissue extracts tested above (0.15 μg/μL in 20% DMSO). We tested each of these mixtures, using a 6-point 6x-dilution series, as above, for inhibition against the monarch and porcine Na^+/K^+-ATPase.

Statistical analyses

All analyses were conducted in JMP Pro (Ver. 16) using linear analysis of variance models. Assumptions of normality and homogeneity of variances were checked in the residuals. Most models consisted of two main fixed effects (extract type, enzyme type) and their interaction.

RESULTS

Chemistry

The four milkweed species' leaves showed a 22-fold variation in cardenolide concentrations from the lowest (*A. incarnata*) to the highest (*A. asperula*), with the two dominant monarch hosts in the middle (*A. syriaca* and *A. curassavica*; Figure 1). Monarch sequestration mirrored the pattern in leaves, with increasing leaf cardenolides across the four milkweed species resulting in increased wing cardenolides (Figure 1, $R^2=0.93$, $F_{1,2}=26.57$, $p=0.036$), although, on average, wings were greater than two-fold more concentrated than leaves (Figure 1).

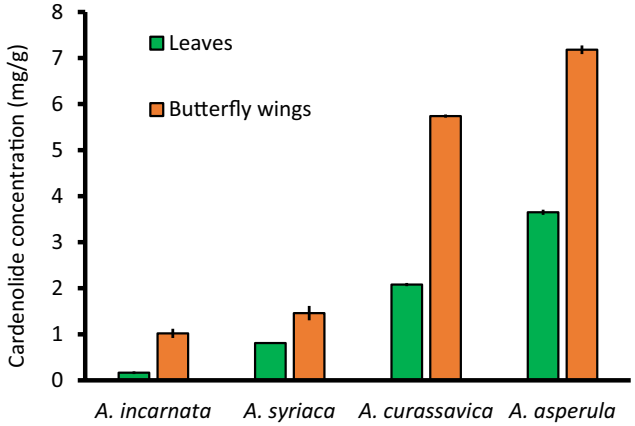

FIGURE 1 Total cardenolide concentration in the extracts of four species of milkweed leaves and adult butterflies reared on those plants. Shown are means±SE based on two replicates of pooled samples run on UV-HPLC. Differences in chemical diversity are given in Figure S1.

For mass spectrometric characterization of cardenolide profiles, we focused on *A. syriaca* and *A. curassavica*. We retrieved 12 cardenolides in *A. syriaca* and 21 in *A. curassavica* (Table S1 and Figure S2). The diversity of cardenolides in *A. curassavica* and *A. syriaca* leaves was 50–70% higher than that sequestered in monarch wings (Figure S1). However, these sequestered compounds were not identical to the cardenolides in leaves due to three significant biotransformations by monarchs (deglycosylation and deacetylation of many cardenolides, as well as the hydrolysis of the thiazoline and thiazolidine motif in labriformin and voruscharin, respectively, Figure 2a,b). Notably, the quantities of sequestered products were substantially higher than the concentrations of the same toxins in the plants (Figure 2c,d).

To gain a better understanding of the physicochemical properties of foliar and sequestered cardenolide structures and hypothesize their ability to cross organismal barriers, we employed the partition coefficient and topological polar surface area (Figure 2e,f). In *A. syriaca*, the cardenolides were categorized into three clusters: a genin group, and a genin group with one or two carbohydrates, respectively. Inspired by the Brain Or IntestinaL EstimateD permeation method (BOILED-Egg; Daina & Zoete, 2016), we propose two physicochemical spaces that correspond to the properties required for crossing the intestinal-lumen barrier and the hemolymph-brain barrier. Interestingly, no non-glycosylated genin capable of crossing the hemolymph-brain barrier was detected in the leaves or butterflies and all sequestered cardenolides were mono-glycosylated. By employing the same method with the cardenolides of *A. curassavica*, we found only a single compound containing two carbohydrates, glycosylated frugoside. All cardenolides sequestered by monarchs and detected in the plant were grouped together in a common space consisting of mono-carbohydrate genins. Once again, no non-glycosylated genin was sequestered.

Toxicity assays

As expected for a highly specialized herbivore, across the four milkweed species tested, the monarch Na^+/K^+-ATPase was 35 times more resistant to inhibition by cardenolides than the sensitive vertebrate Na^+/K^+-ATPase (Figure 3). Among the cardenolide extracts tested, leaf tissue extracts were 48 times more potent on the monarch Na^+/K^+-ATPase (i.e. had greater inhibition of the sodium pump after controlling for cardenolide concentration) than sequestered products from adult wings (Figure 3), while the sensitive enzyme was essentially equally inhibited by wing and leaf extracts (see tissue*enzyme interaction in Table 1, Figure 3). These results were consistent across the four *Asclepias* species tested. We next tested synthetic mixtures of leaf and wing cardenolides from the dominant host, *A. syriaca*, on the monarch and sensitive Na^+/K^+-ATPase. Again, the monarch enzyme was more resistant and wing compounds showed nearly three-fold weaker inhibition than leaf compounds (Figure 4: for extract type, Enzyme, and their interaction all d.f. = 1,8, Fs > 61.0, Ps < 0.001).

Our findings were mirrored when we tested the wild-type Drosophila enzyme (QAN at ATPα1, Na^+/K^+-ATPase residues 111, 119 and 122) against a genetically modified Drosophila enzyme with the monarch's phenotype (VSH; Figure 5). Thus, across all three datasets, and diverse milkweed hosts, leaf extracts were highly potent against sensitive and resistant Na^+/K^+-ATPases, while sequestered cardenolides in wing extracts maintained potency only against a sensitive enzyme. Genetic substitutions in the monarch's Na^+/K^+-ATPase appear to render this specialist especially resistant to the specific cardenolides it sequesters in its body.

DISCUSSION

For specialist herbivores, the ability to cope with plant defences and sequestration of these same compounds go hand in hand, but are typically distinct adaptations (Erb & Robert, 2016). Even herbivores that are highly adapted to chemical defences are typically not completely tolerant, resulting in the need for detoxification and storage of compounds away from their target sites (Heckel, 2014). Many specialists detoxify compounds even though keeping the parent compounds intact may be protective against the third trophic level. As a case in point, *Manduca sexta* detoxifies diterpene glycosides from tobacco, reducing their toxicity to larvae, despite the fact that these compounds can provide protection from spider predation if sequestered (Poreddy et al., 2015). Although the benefits of sequestration are often well-known, the costs that may limit sequestration have been a remarkably difficult nut to crack. Buckeye butterflies feeding on *Plantago* (Camara, 1997) and pipevine swallowtails feeding

on *Aristolochia* (Fordyce & Nice, 2008) each showed some evidence for costs of sequestration; nonetheless, most evidence for costs of sequestration is circumstantial (as is the case for work on monarchs, Brower & Moffitt, 1974; Seiber et al., 1980; Zalucki et al., 2001; Agrawal et al., 2021). Here we have shown the benefits of selective sequestration in terms of reduced toxicity to monarchs, with apparently little loss in terms

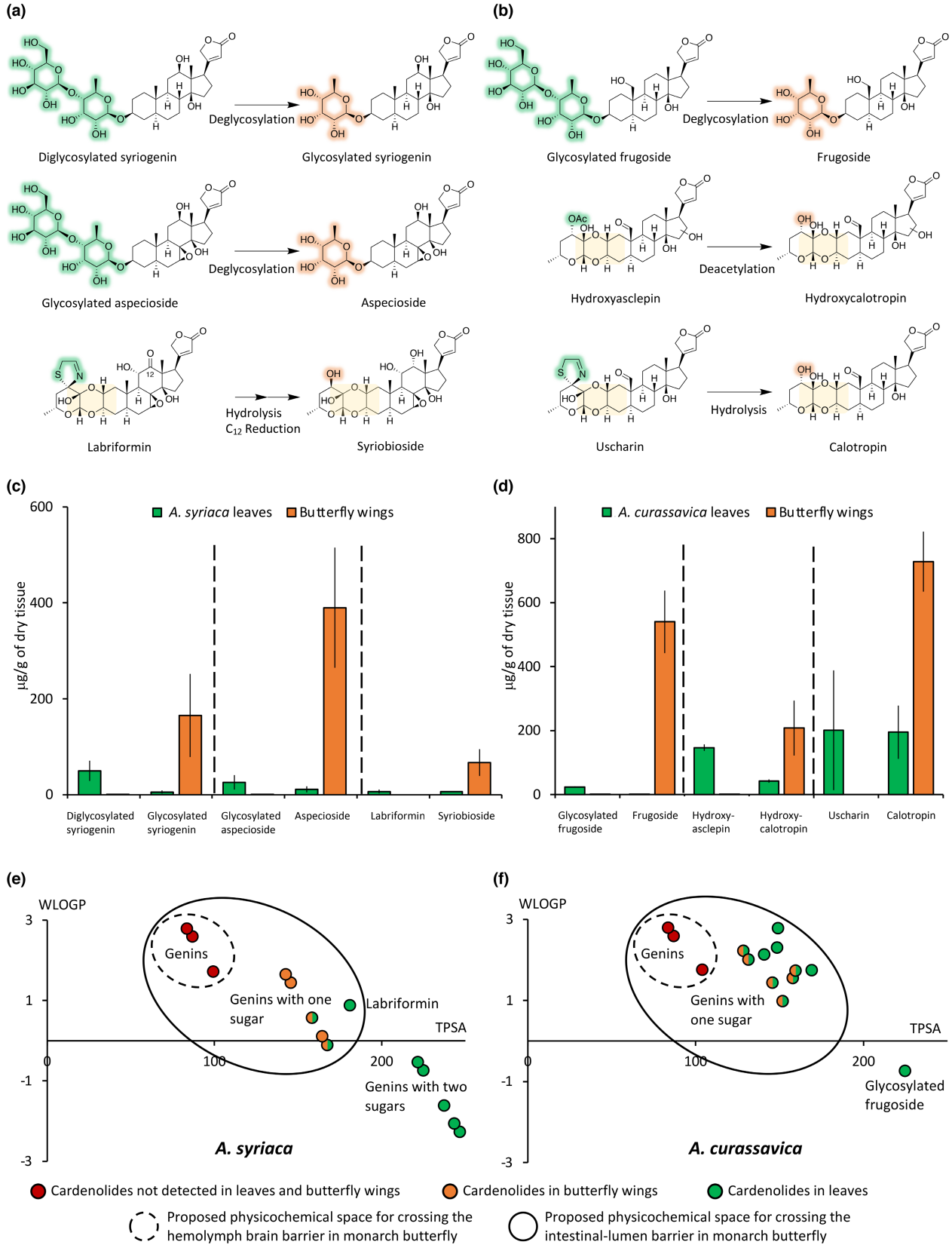

FIGURE 2 Representative biotransformations of leaf cardenolides from *A. syriaca* and *A. curassavica* in the monarch leading to sequestered products in butterfly wings (a, b). The colours emphasize the organic functions present in the chemical structure of the cardenolides present in plants (green) and the wings (orange). Carbohydrate units attached by two O-glycosyl bonds to the cardenolide genin are highlighted in yellow. The comparison of the concentrations of these compounds in the leaves and wings is presented in panels c and d, showing the high concentration of sequestered products (means±SE). Pairs of unmodified and modified cardenolides are separated by dashed lines. Finally, we represent physiochemical properties related to chemical polarity, which govern a compound's movement through an animal body, the partition coefficient (WLOGP) and topological polar surface values (PTSA) for the most representative cardenolides (panels e and f). Although not represented in plants or butterfly wings, the physiochemical properties of genins are shown to illustrate their predicted behaviour relative to glycosides.

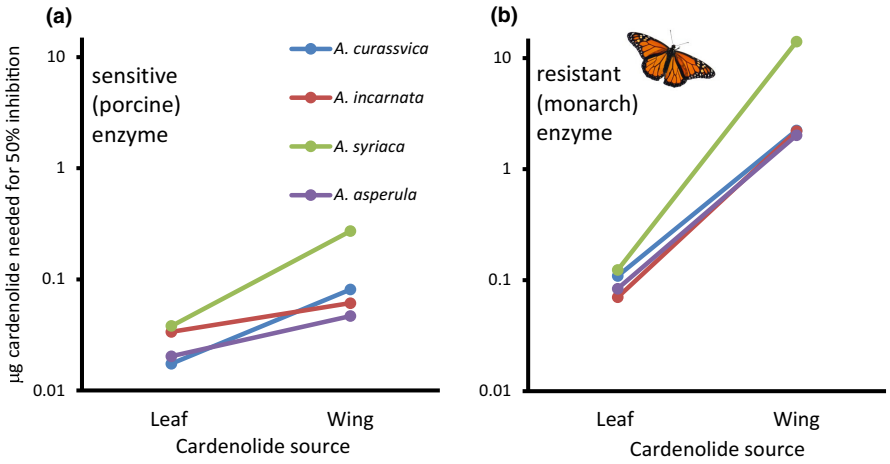

FIGURE 3 Inhibition of the target enzyme by extracts from milkweed leaves and sequestered cardenolides in adult monarch wings. (a) very low concentrations of cardenolides from leaves or wings inhibit the sensitive porcine Na^+/K^+-ATPase. (b) Although leaf extracts of diverse milkweeds contain cardenolides that inhibit the monarch Na^+/K^+-ATPase, sequestered cardenolides from wings are consistently less potent against the monarch enzyme. Shown are the means of at least three replicates; lower values indicate a more potent extract because less is needed to inhibit the Na^+/K^+-ATPase. Note the log *Y*-axis scale.

TABLE 1 Analyses of variance for effects of tissue extract (*Asclepias* spp. leaves vs. monarch wings), enzyme (sensitive vs. monarch) and the tissue-by-enzyme interaction for inhibition of the Na^+/K^+-ATPase.

Host plant	d.f.	Tissue	Enzyme	Tissue × enzyme
A. curassavica	1, 8	57.54***	60.62***	51.05***
A. incarnata	1, 8	50.58***	51.40***	47.95***
A. syriaca	1, 8	9.21*	8.80*	8.57*
A. asperula	1, 20	655.63***	716.08***	623.20***
A. asperula (tested on Drosophila)	1, 22	57.85***	64.35***	57.70***

Note: The response variables analysed were µg cardenolide needed for 50% inhibition of the enzyme (i.e. extracts were adjusted by cardenolide concentrations such that the potency of the extracts was tested). Each row represents a different analysis corresponding to the data shown in Figure 3 (rows 1–4) and Figure 5 (row 5). Shown are *F*-values and significance (***$p<0.001$, *$p<0.05$. In the final row, *A. asperula* leaf and wing extracts were tested on wild-type Drosophila vs. genetically modified Drosophila with the three amino acid substitutions in the monarch's Na^+/K^+- ATPase.

of reduced toxicity to predators who are sensitive to cardenolides.

Biochemistry and physiological relevance of detoxification

The toxicity of cardenolides is influenced by the presence of carbohydrate units attached to the genin. Generally, mono- and diglycosylated genins are more toxic than non-glycosylated genins, while the presence of one versus two sugars does not show a clear tendency to predict the toxicity of cardenolide glycosides (Petschenka et al., 2018). It is surprising then that all the sequestered cardenolides contain at least one sugar, considering that the sequestered products should be the least toxic without any sugars attached. One possible explanation is that the presence of a single sugar allows for their passage across the intestinal wall, either actively or passively while preventing their passive permeation through the hemolymph-brain barrier. In other words, there could be a trade-off between reduced toxicity and increased exposure to non-glycosylated cardenolides. Further investigation is needed to determine where and when cardenolide modifications occur (e.g. Dreisbach et al., 2023), and whether

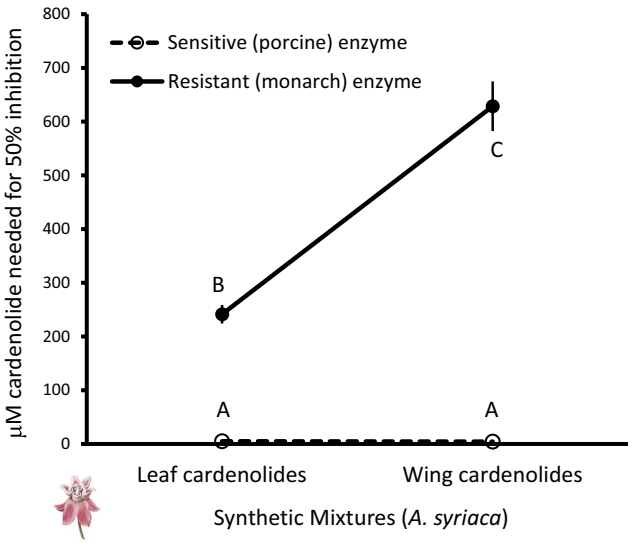

FIGURE 4 Inhibition of the target Na^+/K^+-ATPase enzyme by synthetic mixtures of purified *A. syriaca* leaf cardenolides (eight compounds, in order of dominance: diglycosylated syriogenin, glycosylated aspecioside, aspecioside, labriformin, diglycosylated oxidized syriogenin, syriobioside, glycosylated syriogenin, glycosylated syriobioside) versus sequestered cardenolides in wings from monarchs fed A. syriaca (three compounds, in order of dominance: aspecioside, glycosylated syriogenin, syriobioside; See Table S1). Shown are means±standard error based on $n=3$ for each; lower values indicate a more potent extract because less is needed to inhibit the Na^+/K^+-ATPase. Different letters indicate significant differences ($p<0.05$) using Tukey's HSD.

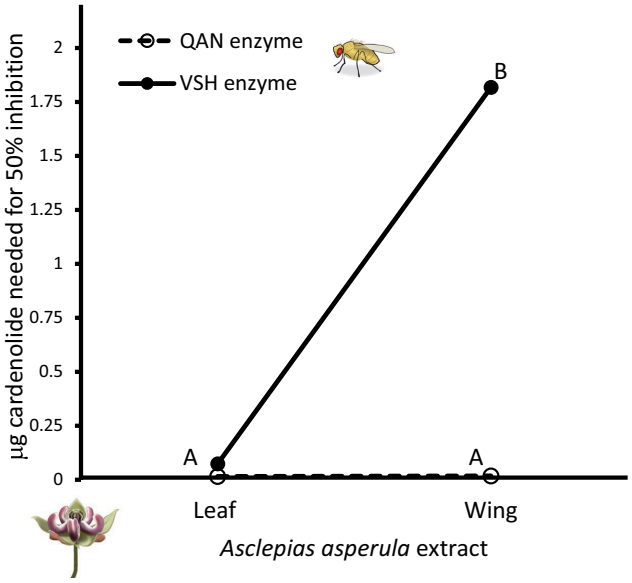

FIGURE 5 Inhibition of the two Drosophila Na^+/K^+-ATPase enzymes by extracts from *A. asperula* leaves and sequestered cardenolides in adult monarch wings. Data are presented for a wild-type (alpha subunit of Na^+/K^+-ATPase at amino acid positions 111, 119 and 122 was QAN) and CRISPR-edited (VSH) Drosophila enzyme. Shown are means±standard error (not visible) based on $n=6$–7 for each; different letters indicate significant differences ($p<0.05$) using Tukey's HSD.

selective mono-deglycosylation, potentially catalysed by a β-glucosidase enzyme or by the basic pH environment in the caterpillar gut, is responsible for the presence of a single carbohydrate motif. β-Glucosidases are plant enzymes primarily known for their ability to disrupt insect digestion by activating a pro-toxin through the removal of a carbohydrate motif (Vassão et al., 2018). However, in the case of cockchafer larvae (*Melolontha melolontha*), β-glucosidases have an interesting effect: they actually reduce the toxicity of taraxinic acid β-D-glucopyranosylester in the common dandelion (Huber et al., 2021). Alternatively, the complete deglycosylation of two carbohydrates could be followed by a glycosylation reaction with a glycosyl transferase, resulting in the attachment of one carbohydrate unit.

The second scenario involving a glycosyl transferase enzyme is more complex but certainly possible. Indeed, among the various enzymes involved in detoxification, glycosyl transferases are widely distributed in herbivorous insects and have been reported for the detoxification of capsaicin, gossypol, and benzoxazinoid by *Helicoverpa* species (Ahn et al., 2011; Krempl et al., 2016; Wouters et al., 2014). These enzymes react with plant toxins and add a carbohydrate unit to the chemical structure reducing passive penetration of toxins into the intestinal wall. This process increases the toxin's hydrophilicity and restricts its passive penetration into the intestinal-lumen barrier. To our knowledge, only *Diabrotica* spp. beetles have been found to employ a glycosylation reaction, facilitated by glycosyl transferase, to sequester toxins (cucurbitacins and benzoxazinoids; Andersen et al., 1988; Robert et al., 2017).

Labriformin in *A. syriaca* and voruscharin in *A. curassavica* exhibit a thiazoline and thiazolidine motif, which appears to restore strong binding to the highly adapted sodium pump of monarchs. Hydrolysis of these chemical functions results in the formation of less toxic products that are subsequently sequestered (Agrawal et al., 2021, 2022). Another notable feature of these structures is the attachment of a carbohydrate group to the triterpene motif through two O-glycosyl bonds (highlighted in yellow in Figure 2a,b). Within *A. syriaca*, labriformin and reduced labriformin are the only cardenolides that possess this specificity. In *A. curassavica*, in addition to voruscharin and uscharin, most cardenolides have their carbohydrate motif fused to the genin, except for glycosylated frugoside. The double attachment of the carbohydrate does not seem to alter the physicochemical properties or affect toxicity. Consequently, the exact role and chemical evolution of this rare carbohydrate attachment to the genin remains unknown. Similarly, deacetylation reactions led to products with overall similar physicochemical properties as the acetylated structures as shown in Figure 2 and Table S2. There is currently limited information available regarding the differences in toxicity between these acetylated and non-acetylated

cardenolides, and the precise role of deacetylation is yet to be elucidated.

Detoxification, selective uptake, and the costs of sequestration

Although it is widely known that animals selectively sequester chemical toxins from their foods (Abdalsamee & Müller, 2012; Beran & Petschenka, 2022; Kuhn et al., 2004; Opitz et al., 2010), the combination of this selectivity with detoxification and degradation is less understood. Insects feeding on plants with iridoid glycosides completely degrade some compounds and differentially uptake others, and these strategies are used more so than compound modification or detoxification (Boros et al., 1991; Bowers & Collinge, 1992). Detoxification of pyrrolizidine alkaloids by diverse sequestering herbivores involves modifications for stable storage, only to be reactivated during predation (Hartmann & Ober, 2000; Wang et al., 2012). For monarchs on milkweed, we have long known of their tolerance mechanisms to cardenolides (Holzinger et al., 1992; Karageorgi et al., 2019; Vaughan & Jungreis, 1977), as well as selective storage of these compounds (Malcolm, 1995; Roeske et al., 1976). Nonetheless, our recent demonstration of the breakdown of particular cardenolides and their differential potency (Agrawal et al., 2021, 2022), coupled with the reduced toxicity of sequestered compounds in monarch wings compared to leaves shown here, represent novel insights (Figure 2).

In the current study, we provide strong support for the selective sequestration hypothesis. Indeed, across four host plant species, monarch butterflies broke down and sequestered a subset of cardenolide toxins, and these were less toxic to monarchs than the cardenolides extracted from the leaves they eat. It was known that monarchs concentrate some toxins while not sequestering others, and this often results in a pattern of increased polarity of sequestered cardenolides compared to those in leaves (Brower et al., 1982; Malcolm, 1995; Roeske et al., 1976; Seiber et al., 1980) In particular, the three dominant compounds stored by monarchs feeding on *A. syriaca* are one-sugar glycosides converted from two sugar compounds more abundant in leaves (Figure 2). The mechanistic basis, generality, and implications of removing one sugar before sequestration remain to be discovered. Importantly, we tested the toxicity of the sum total milkweed leaf- vs. monarch wing extracts controlling for their different concentrations of cardenolides. In other words, we specifically show differences in the potency of the different cardenolide compositions rather than differences in cardenolide concentrations.

Evidence has accumulated over the past 50 years that coping with cardenolides, or sequestration itself, may impose a burden on monarchs. For example, our recent work on tropical milkweed, *A. curassavica*, has shown that a dominant cardenolide (voruscharin) is not sequestered, is associated with reduced caterpillar growth, and that sequestering higher levels of compounds (selected cardenolides and breakdown products) reduces caterpillar performance. Similarly, reduced growth and increased melanization have been reported for ingestion or storage of other cardenolides (Agrawal et al., 2021; Brower & Moffitt, 1974; Seiber et al., 1980; Zalucki et al., 2001). Thus, it would seem that sequestration involves trade-offs for this highly specialized herbivore. As in any coevolutionary scenario, defensive traits of the plant may be beneficial to (and coopted by) adapted herbivores while also imposing a burden or cost for their performance. Despite being challenging to decipher, we believe this complex scenario is the likely case for many coevolutionary interactions.

Toxicity of unsequestered cardenolides

One consequence of selective sequestration is that the costs of sequestration may be reduced, but sequestered compounds may also be less effective as an anti-predator defence than those in leaves (Poreddy et al., 2015). Although we have not conducted predation bioassays, our enzymatic analysis of the target of cardenolides showed that sequestered cardenolides were less toxic to monarchs but were equally toxic to the sensitive Na^+/K^+-ATPase as cardenolides from leaves. This suggests that selective sequestration may not carry with it a reduced effectiveness against the monarch's predators. Most predators of monarch butterflies are generalist vertebrates (lizards, mice, birds) and invertebrates (spiders, stinkbugs, parasitoids) with sensitive Na^+/K^+-ATPases (Agrawal, 2017). Yet, recent evidence indicates modest molecular changes in the resistance to cardenolides of at least some species that consume monarchs at their overwintering sites (Groen & Whiteman, 2021). The genetic basis of adaptation to cardenolides is very well-known in monarchs, perhaps more so than in any other coevolving plant-herbivore interaction (Karageorgi et al., 2019). By comparing wild-type and transgenic fruit flies for inhibition by milkweed leaf and monarch-sequestered cardenolides from extracts, here we have shown that simple genetic changes affecting three amino acids of the Na^+/K^+-ATPase are sufficient to explain the difference in potency between these extracts. Accordingly, the few predators that have one or two changes in their Na^+/K^+-ATPase (Groen & Whiteman, 2021) would be ripe for study in terms of the impacts of leaf vs. monarch-sequestered cardenolides. One prediction would be that the lower toxicity cardenolides stored by monarchs also show reduced toxicity to the few enemies that also show substitutions in their Na^+/K^+-ATPases (Groen & Whiteman, 2021).

Two unusual nitrogen-containing cardenolides, voruscharin and labriformin, found in *A. curassavica*

and *A. syriaca*, respectively, are not sequestered by monarchs (Agrawal et al., 2021, 2022; Malcolm, 1995; Roeske et al., 1976; Seiber et al., 1980). Both compounds are detoxified by chemical conversion, apparently at some cost to the caterpillars, and when tested on the monarch's sodium pump are among the most toxic cardenolides (Agrawal et al., 2021, 2022). Nonetheless, these compounds are unremarkable, or even less toxic against the sensitive (highly conserved) Na^+/K^+-ATPase (Agrawal et al., 2021, 2022). In prophetic experiments conducted >40 years ago, labriformin was demonstrated to be a rather weak cardenolide when administered to mice (Benson et al., 1978), blue jays (Brower et al., 1982), and equally inhibitive to ouabain (one of the weakest standard cardenolides) when it was dosed on the Na^+/K^+-ATPase of sheep or pig (Benson et al., 1978). Collectively, this work suggests that these unsequestered nitrogen-containing cardenolides are specifically targeted against the highly resistant Na^+/K^+-ATPase of the monarch and other adapted specialist herbivores (Agrawal et al., 2021, 2022). The final conclusion of this work is that there appears to be little trade-off for monarchs in detoxifying these compounds. In other words, the breakdown products, which are safer for monarchs than the parent compounds, likely maintain potency against most predators.

Conclusion and speculation

Across plant-herbivore coevolutionary interactions, different levels of offence and defence have apparently been reached over time and space. For monarchs and milkweed, there is substantial evidence for an arsenal of functional plant defences (latex, trichomes, diverse cardenolides, volatile organic compounds, etc.) that reduce insect performance (Agrawal, 2017; Edwards et al., 2023). At the same time, one could conclude that each of these defences has been cracked via behaviours (e.g. trenching of laticifers), physiological adaptations (detoxification, selective sequestration, etc.), and genetic substitutions (target site insensitivity) of the monarch (Agrawal, 2017; Karageorgi et al., 2019). This has led us to delve into the adaptive nature of specific cardenolide toxins, especially those that are structurally distinct from others or that are modified during sequestration. Here too we find evidence for plant defence and counter-adaptations by the specialist herbivore. Remarkably, the most toxic cardenolides to monarchs are so specialized that they are rather weak agents of defence against unadapted animals (Agrawal et al., 2021, 2022). Nonetheless, milkweed plants present a cocktail of many cardenolide toxins. Accordingly, monarchs detoxify some of these compounds and selectively sequester others. Although monarchs pay a cost to sequester cardenolides, this may not come with the additional trade-off of reduced protection against their enemies.

AUTHOR CONTRIBUTIONS

AAA, APH, and CD jointly conceived the research and experimental design; APH conducted empirical work, curve fitting, and HPLC chemical quantifications. CD identified chemical compounds and conducted all LC-HRMS analyses. AAA conducted statistical analyses and wrote the first draft of the manuscript; APH and CD substantially contributed to revisions.

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DATA AVAILABILITY STATEMENT

The data supporting the results of this study have been archived with Figshare https://doi.org/10.6084/m9.figshare.24204381.v1 and https://doi.org/10.5061/dryad.wpzgmsbtv.

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