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Costs of toxin ingestion versus costs of sequestration in a community of specialized herbivores

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When multiple insect herbivores specialize on well-defended plants, they often show convergent adaptations to a class of phytochemical defences. However, herbivore communities are typically composed of taxonomically diverse species with different feeding modes and life histories, and thus not all benefits and costs of adaptation are predicted to be the same. Using common milkweed (*Asclepias syriaca*), which produces toxic cardenolides, we investigated convergence in insect performance and cardenolide sequestration in the native community of specialist beetles, caterpillars and bugs, feeding on roots, leaves and seeds, respectively. We found minimal correlations between defence of different plant parts, or in the performance of the insect species, suggesting organ-specific plant defence and distinct insect offence strategies. None of the three species was affected by ingesting cardenolides, despite known differences in counter-adaptations. Nonetheless, all species showed a cost of sequestration, which probably emerged from physiological limits. Although variable among species, our findings provide evidence that the evolutionary benefits of sequestration are accompanied by costs, revealing a general constraint among diverse species coevolving with a shared host plant.

1. Introduction

In most plant–herbivore interactions, our understanding of the ecology and evolution of plant defences has far outpaced that of insect counter-adaptations. When insect herbivores specialize to feeding on specific toxic plants, they often show convergent adaptations—repeated, independent evolution of traits to cope with the plant's class of toxins. For instance, groups of insects have convergently evolved detoxification mechanisms specific to furanocoumarins, cyanogenic glycosides, pyrrolizidine alkaloids or glucosinolates [1–3]. Convergent evolution of resistance to plant toxins probably arose from a combination of shared selective pressures on the insects (i.e. same class of toxins), and evolutionary constraints that limit the number of possible adaptations [4,5]. For example, when the target of a plant defence is a highly conserved animal enzyme, there may be few mutations that are both beneficial and not highly deleterious [6,7]. However, herbivore communities expressing convergently evolved traits are typically composed of taxonomically diverse species with different feeding modes, life histories and specialization on different plant organs that vary in their concentration and composition of defences [1,8–10]. Not all benefits and costs are predicted to be the same in such heterogeneous communities, even when many species share adaptations.

In the interaction between milkweeds (genus *Asclepias*) and their community of herbivores, plants contain toxic cardiac glycosides (i.e. cardenolides), a class of secondary metabolites with an exclusively defensive function that specifically targets animal Na^+/K^+-ATPases (hereafter 'sodium pump') [8,11]. Cardenolides are highly diverse within individual plants, and both the concentration and composition are variable among plant organs [12–14]. In response, many of the specialized milkweed insects have convergently evolved cardenolide resistance, via target site (sodium pump) substitutions. Adding insult to injury, many of these same milkweed feeders sequester cardenolides and exhibit aposematic coloration. In fact, sodium pump resistance to—and sequestration of—cardenolides evolved as two major adaptations to cope with these milkweed toxins in six taxonomic orders of insects, spanning 350 million years of divergence [8]. For specialized sequestering herbivore species, toxic cardenolides represent both something to resist from the plant and to use as protection against predators, complicating the plant–herbivore interaction [15,16].

Among specialized milkweed feeders, the number of amino acid substitutions in the highly conserved sodium pump is directly proportional to enzyme resistance and typically matches the cardenolide concentrations of the preferred tissues of the different insects [13]. Nonetheless, sodium pump resistance to ingested toxins is not equally effective against different cardenolides [17–20], revealing costs associated with the consumption of particular compounds or mixtures. Indeed, costs of ingestion may emerge as the direct effects of toxin consumption on insect performance through inhibition of the insect's sodium pumps, which are expressed in the membranes of all cells. In other words, the consumption of plant tissues with increasingly toxic compounds should reduce insect growth and performance proportionally. To date, the few tests of costs of cardenolide ingestion on growth of milkweed specialists have been ambiguous and typically were conducted using different methods [17,20–24].

Sequestration of plant toxins, in turn, probably evolved to thwart predation, opening novel perspectives within the tri-trophic niche [25,26]. Sequestration involves an active and regulated process of diverse metabolic machinery in the insect's body, involving toxin conversion to less harmful subproducts [27], transport [28,29] and storage [30]. Such simultaneous metabolic demands may be costly at high concentrations of accumulated toxins [17,31,32], potentially yielding negative effects of storage on herbivore performance. In other words, the metabolic demands of physiological mechanisms to maintain toxic compounds in the insect's body may impose negative impacts on insect growth; these costs are deemed to be distinct from the cost of ingestion as they are proportional to the amount stored, and not the amount consumed. For monarchs (*Danaus plexippus*), although few costs of ingesting cardenolides have been reported, a cost of sequestration was previously found via the negative correlation between cardenolides stored and insect growth [17,20,25].

Thus, selection for insect resistance to toxins may be jointly impacted by bottom-up and top-down forces. Across species in the Danaini, which exhibit variation in their sodium pump resistance and ability to sequester, evidence indicates that sequestration probably evolved in response to predators (top-down selection) [33]. In turn, some milkweed specialists show insensitive sodium pumps but do not sequester, suggesting that sodium pump resistance evolved to tolerate plant toxins (bottom-up selection) [8,11,34]. Together, these associations reveal a pattern that the benefits and costs of insect resistance to ingest toxins versus the ability to sequester them probably differ among species. Here we test for the generality of costs of ingestion versus sequestration across three insect herbivores that share a common host plant, show variation in sequestration ability and have variable sodium pump resistance.

Despite sharing similar adaptations, we hypothesized that costs of ingestion and sequestration may still differ among milkweed insects, given the phylogenetic, ecological and physiological diversity of milkweed feeders, as well as differences in cardenolide diversity among the plant organs they feed on. Alternatively, should similar costs appear among distinct insect species, it would suggest negative impacts on similar physiological components tied to their shared adaptations, probably limiting plant–herbivore coevolution. Accordingly, here we investigate the impacts of plant toxin ingestion and sequestration for three specialists: the four-eyed red milkweed beetle (*Tetraopes tetrophthalmus*), the monarch butterfly (*Danaus plexippus*), and the large milkweed bug (*Oncopeltus fasciatus*), feeding on roots, leaves and seeds, respectively, of common milkweed (*Asclepias syriaca*). These insects have two, three and five amino acid substitutions in their sodium pumps, respectively, conferring corresponding increases in resistance to cardenolides at the enzymatic level, and all are known to sequester cardenolides [4,18,26,35]. We offered roots, leaves and seeds of common milkweed (*Asclepias syriaca*) to feeding root beetle larvae, monarch caterpillars and seed bugs, respectively, and measured cardenolide concentrations from each plant organ, cardenolides sequestered by insects and insect growth. We first tested whether the cardenolide concentration and composition were different between the three plant organs, whether these differences predicted cardenolides sequestered by each insect, and whether growth was correlated among the three insects. For this last test, as the three insect species were tested on their target tissues of distinct full-sib families of common milkweed, we hypothesized that if different plant families show distinct profiles of defences that are concerted among tissues and costs of ingestion are consistent, then herbivore growth on the different families should be positively correlated between insect pairs. We next performed regressions between plant organ cardenolide concentrations, cardenolides sequestered and insect growth, testing whether insect performance was associated with exposure to the cardenolides (i) present in the plant tissue they fed on (i.e. how cardenolide concentrations in the plant affect insect growth; a cost of ingestion), or (ii) sequestered in their bodies (i.e. how cardenolide concentrations in the insect's body affect insect growth; a cost of sequestration).

2. Material and methods

(a) Plant material

Plant material, germination and plant growth conditions were as described in [12]. Briefly, we collected a single common milkweed fruit from each of 14 randomly selected individual plants separated by at least 5 m from Durland Bird Preserve (42°26'08.0"N, 76°23'46.2"W) in 2018, representing 14 full-sib families. This family structure was used to ensure variation in phytochemical diversity and to provide the three herbivores with a genetically comparable plant diet.

Seeds were sterilized, scarified and stratified for 8 days at 4°C and then germinated at 30°C in the dark. Seedlings were planted in a mix of 25% perlite, 75% soil (LM111; Lamberts, Quebec, Canada) in 10 cm pots and grown for 4 weeks in a growth chamber (14 h daylight, 27°C : 23°C day : night). Slow-release fertilizer (Osmocote Smart-Release®, Marysville, OH) and dilute fertilizer (N : P : K 20 : 20 : 20, 120 ppm N ($\mu g\ g^{-1}$)) were applied once on days 7 and 15, respectively, and plants were watered as needed. Predatory mites (*Amblyseius cucumeris*) were applied to prevent thrips infestation/damage. When plants were eight weeks old, five individuals per family were assigned to each herbivory treatment: control (undamaged), root herbivory or leaf herbivory (see below). We also simultaneously conducted a seed herbivory bioassay with 25 mg of seeds per family in a 9 mm Petri dish, with three replicates per plant family (see below). All plants and dishes followed a completely randomized design.

(b) Herbivory bioassays

All insects used in the experiment were neonates from short-term laboratory-reared insect colonies. We first field-collected insects from the same plant population as above and collected eggs in the laboratory (*Tetraopes*) or started multi-generational colonies. In order to maximize genetic diversity in our laboratory colonies, individuals from each insect species were collected from distant plants within the population and randomly separated into multiple cages. Adult root beetles were reared with fresh milkweed leaves and moistened paper every 2–3 days, and dry grass stems were provided as oviposition sites before the experiment. Monarch butterflies were fed with fresh sugar solution every three days, allowed to mate and common milkweed plants were provided to allow females to oviposit before the experiment. Adult seed bugs were fed with common milkweed seeds from the local area, and cotton-capped microtubes with water were provided for hydration. Seed bugs were allowed to mate, and eggs were put on separate Petri dishes with moistened paper before the experiment. For each insect species, eggs/neonates from different cages were pooled before the start of the experiment.

For the root beetle, two neonate larvae were put on the soil surface next to the stem of each of five plants per family and were allowed to feed for 7 days. For the monarch, one neonate caterpillar was put on the youngest, fully expanded leaf of each of another five plants per family and allowed to feed for 5 days. Another set of five undamaged plants was used as controls. For the seed bug, we initially put five neonate individuals on each Petri dish for each family replicate for 3 days, after which we restricted the experiment to two individuals per replicate. A moistened cotton ball was provided in each replicate and replaced every 2–3 days. Seed bugs were monitored daily until adulthood (approx. 21 days). In summary, we used 14 full-sib families × 3 insect species × 1–5 replicates = 152 herbivore bioassays (110 plant bioassays: 49 for monarch, 61 for root beetle; 42 seed bioassays for seed bug), plus 3–5 undamaged control plants per family = 56 plants.

At the end of the experiment, all insects were frozen at −80°C, freeze-dried and weighed (mg dry weight (d.w.)) prior to analysis of cardenolide sequestration, and plants were harvested for cardenolide analyses. For root beetles specifically, plants were removed from the pots after collecting leaves, and by gently removing soil around the roots, we collected the surviving larvae per plant. Roots were washed clean with water afterwards. For root beetles and monarchs, individuals were pooled together by plant family before chemical analysis to improve accuracy and compound detection given their small size. Adult seed bugs were sexed and analysed individually.

(c) Plant sampling and extraction of plant and insect cardenolides

At the end of the experiment and after insects were removed, roots and the two youngest, fully expanded leaves from all treatments were collected from each plant, frozen at −80°C and freeze-dried. Then, roots and leaves were ground on a mixer mill (Retsch, Haan, Germany) using 5 mm steel beads. Freeze-dried, ground root and leaf samples from each individual plant (166 plants in total) were analysed for cardenolide content using high-performance liquid chromatography (HPLC). Seed cardenolide data were retrieved from [12], with 3–5 biological replicates of randomly selected groups of seeds from the same families. In summary, we used 14 full-sib families × 3 herbivory treatments × 3–5 replicates × 2 tissues = 332 chemical analyses, plus 3–5 replicates per family = 67 records of a previously published database of seed cardenolides from the same plant material.

Plant cardenolides were extracted as described in [12]. Briefly, cardenolides were extracted from 50 mg of ground material of each sample by adding 1 ml of 100% methanol (spiked with 20 µg of hydrocortisone as internal standard) and 20 FastPrep beads, agitated on a FastPrep-24 homogenizer twice for 45 s at 6.5 m s^{-1}, and then centrifuged at 14 000 r.p.m. for 12 min. Supernatants were dried down in a vacuum concentrator at 35°C, resuspended in 250 µl of 16 : 16 : 68 (%) methanol : acetonitrile : water (vol : vol : vol), and filtered using 0.45 µm hydrophilic membranes. For seeds, we followed an identical cardenolide extraction procedure as above [12].

Freeze-dried root beetle larvae, monarch caterpillars and seed bugs (individual) were weighed on a microbalance and then ground with a 3 mm stainless steel bead using a tissue grinder. Each sample was then extracted for sequestered cardenolides in

the same way as plant cardenolides (20 µg of digitoxin was used as internal standard instead of hydrocortisone). Insects were defatted during extraction to avoid shifts in retention times and damage to the analytical column. For monarchs, a volume of 0.7 ml of supernatant was transferred to a fresh tube after centrifugation for defatting. An equal volume of hexanes was added to each monarch sample, vortexed for 10 s each, centrifuged for 15 min at 14 000 r.p.m., and the top hexane layer discarded. This process was repeated twice. An identical procedure was done for seed bugs, but using 0.25 ml of supernatant and 0.75 ml of hexanes instead. No defatting was done for the root beetles given their small size. Defatted samples were taken to dryness at 35°C in a vacuum concentrator (LabConco, Kansas City, MO). Then, all samples were resuspended in 0.2 ml of methanol by agitation at 1000 r.p.m. on a BioShake Iq microplate shaker (Quantifoil Instruments) and filtered using 0.45 µm hydrophobic membranes.

(d) Cardenolide analysis by high performance liquid chromatography–diode array detector/mass spectrometry in plants and insects

Cardenolides from plant organ, monarch and seed bug samples were detected using an Agilent 1100 HPLC with diode array detector and a Gemini C18 reversed-phase column (3 µm, 150 mm × 4.6 mm column, Phenomenex, Torrance, CA) following standard protocols [12,20]. Individual cardenolide concentrations in each sample were quantified by using the peak area and known concentration of the internal standard on a dry mass basis of plant or insect material (µg mg^{-1} d.w.). We summed up the individual compounds to calculate the total cardenolide concentration in plant samples and cardenolides sequestered in insect samples.

Extracts from root beetle larvae samples were injected into a Thermo Fisher Scientific Vanquish UHPLC system coupled with a Thermo Q-Exactive high-field (HF) hybrid quadrupole-orbitrap high-resolution mass spectrometer (MS) equipped with a heated electrospray ionization (HESI) ion source. Metabolites were separated using 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^{-1}, using a gradient of water (A) and acetonitrile (B) (both containing 0.1% formic acid) as follows: 0–2 min at 5% B; 2–11 min from 5 to 98% B; 11–14 min at 98% B; followed by 0.1 min from 98% to 5% B and held at 5% B for 2.9 min to reconditioning. The mass spectrometer parameters were as follows: spray voltage of 3.5 kV, capillary temperature of 380°C, probe heater temperature of 400°C, 60 sheath flow rate, 20 auxiliary flow rate and 1 spare gas; and S-lens radio frequency (RF) level of 50, resolution of 240 000, automatic gain control target of 3×10^6. The instrument was calibrated weekly with positive- and negative-ion calibration solutions (Thermo Fisher). Each sample was analysed in negative and positive ionization modes using a m/z range of 100–800.

HPLC-MS data were processed using Mzmine 4.0 followed by a filtering process in R Studio to retrieve cardenolide data (see electronic supplementary material, S1). Briefly, using a filtering vector based on the m/z values of cardenolide genins, we identified the retention times corresponding to cardenolides. We then compared these retention times and parental masses with standards of known compounds previously isolated from common milkweed [36], as well as the internal standard. The area (measured by ion counts) of these retention times was used to calculate the total cardenolide concentration sequestered by root beetle samples. This was done by summing the ion counts of the individual compounds and inferring the concentration based on the ion counts of the internal standard, expressed on a dry mass basis of insect material (µg mg^{-1} d.w.).

Thus, at the level of individuals (or pooled samples within each plant family), plant cardenolide concentrations from each organ were tested for their relationship with insect growth as a proxy for costs of toxin ingestion; a negative correlation would indicate reduced growth upon exposure to increasingly concentrated plant toxins. Cardenolides sequestered by individual insects (or pooled samples) were similarly tested for their relationship to insect growth as a proxy for costs of toxin sequestration; a negative correlation would indicate the impact of the metabolic demands of processing or storing toxins in the insect's body. This contrast between ingestion and sequestration was possible because of the lack of correlation between the concentration of cardenolides ingested and sequestered (see results below).

(e) Statistical analysis

To test for differences in concentration between organs, we used a linear mixed model in SAS v. 9.4 with total concentration of cardenolides from undamaged plants as the response variable, with plant organ as a fixed factor, and plant family as a random factor. The plant organ was considered a repeated measure to account for multiple organs sampled from the same plant individual. Normality requirements were met without transformation. To test for differences in cardenolide composition between organs, we used a permutational multivariate analysis of variance (PERMANOVA) of the individual cardenolides with the package 'vegan' [37] in R v. 4.4.1 [38]. Briefly, we first transformed the cardenolide matrix using the 'log' method from the *decostand* function and used Bray–Curtis dissimilarity. Then, we tested for plant organ differences using PERMANOVA with 999 permutations (*adonis2*). We further tested specific differences between organ pairs with *post hoc* multivariate comparisons with the package 'pairwiseAdonis' [39].

We tested whether differences in cardenolide concentration among organs predicted cardenolides sequestered by each insect at the plant family level by using a general linear model in SAS v. 9.4 for each insect species separately. Concentration of cardenolides sequestered was the response variable, and total concentration of cardenolides of the damaged organ by the insect was the predictor variable. Both variables were $\log(x + 1)$ transformed prior to the analysis. Finally, we performed Pearson correlations of insect growth for each pair of species. Insect growth was square root transformed prior to the analysis.

Costs of ingestion and sequestration were tested via regressions at the plant family level between insect growth and plant organ cardenolides, and between growth and cardenolides sequestered by the insect, respectively. For each test, we used

general linear models in SAS v. 9.4 for each insect species separately, with insect growth as the response variable, and total cardenolides of the damaged organ (i.e. costs of ingestion) or cardenolides sequestered (i.e. costs of sequestration) as the predictor variable. In the case of the seed bug, three separate analyses for each test were conducted: one for the average of both sexes, one for males and one for females. For all analyses, insect growth was square root transformed, whereas cardenolides sequestered and organ total cardenolides were $\log(x + 1)$ transformed. To avoid spurious negative relationships between cardenolide sequestration and growth for each insect owing to their non-independence (i.e. cardenolide sequestration derives from insect growth), we performed 10 000 Monte Carlo simulations for each regression analysis using PROC GLM into SAS v. 9.4, adapting the procedure of López-Goldar *et al*. [17]. The significance of the regressions from the original data was estimated from the fifth percentile of the resulting distribution of simulated slopes. We conducted a similar analysis of costs of sequestration at the individual level for the seed bug only owing to data being limited at the plant family level for the other insects. We performed a linear regression with insect growth as the response variable, and sex and its interaction with cardenolides sequestered as predictor variables. A Petri dish was included as a random factor to account for repeated measures of multiple bugs reared on the same replicate of a plant family.

3. Results

We found strong differences in total cardenolide concentrations between plant organs, which increased from roots (0.95 ± 0.15 mg g^{-1} d.w.) to leaves (1.39 ± 0.15 mg g^{-1} d.w.) to seeds (2.59 ± 0.14 mg g^{-1} d.w.) ($F_{2,159} = 90.85$, $p < 0.001$; figure 1a), as well as their multivariate cardenolide composition (figure 1b). Cardenolide concentrations in insects also increased from root beetles (0.11 ± 0.03 mg g^{-1} d.w.) to monarchs (0.84 ± 0.10 mg g^{-1} d.w.) to seed bugs (3.80 ± 0.43 mg g^{-1} d.w.), qualitatively matching the increase in plant organ chemistry. Nonetheless, we did not find evidence for a quantitative relationship between cardenolide concentrations in the plant organs eaten (i.e. ingestion) and in their respective insect bodies (i.e. sequestration) (figure 1c–e), with the exception of female seed bugs ($\beta = 0.52$, $t_{(12)} = 2.53$, $p = 0.027$, $n = 14$, analyses based on means of multiple replicates per plant family). Finally, variation in growth of the three herbivores was uncorrelated across the 14 plant families (figure 1f–h). In sum, despite the qualitative match between plant and insect cardenolides and less than threefold variation in sequestration among individuals of each species (figure 1c–e), there was (i) a weak quantitative relationship between sequestration and plant variation in cardenolides, and (ii) negligible associations in insect performances across the 14 plant families tested.

We next tested for costs of cardenolide ingestion versus sequestration in each species by regressing insect growth against tissue cardenolide levels and cardenolides sequestered (analyses again based on plant family means, figure 2). Despite substantial variation, growth of the three milkweed specialists was not predicted by the cardenolide concentration of the tissues they ate from different milkweed families (figure 2a–c). Nonetheless, when testing the seed bug sexes separately, growth of males was negatively affected by seed cardenolides ($\beta = -0.52$, $t_{(11)} = -2.27$, $p = 0.044$) but not growth of females ($\beta = -0.21$, $t_{(12)} = -0.91$, $p = 0.381$). The concentrations of cardenolides sequestered, however, were negatively correlated with growth in all three insect species (figure 2d,e); this result emerged after correcting for the interdependence of variables using Monte Carlo simulations (see §2). For seed bugs, we found a negative but non-significant trend averaging across milkweed families (figure 2d, females: $\beta = -0.44$, $p = 0.106$; males: $\beta = -0.33$, $p = 0.118$). However, we found sex-dependent costs of sequestration at the level of individual bugs. Specifically, although insect growth did not differ between males and females ($F_{1,25} = 0$, $p = 0.963$), we found a significant interaction between sex and sequestration ($F_{2,25} = 5.49$, $p = 0.01$). Specific contrasts of the slopes showed that the negative relationship between cardenolides sequestered and insect growth was significant, averaged across both sexes (figure 2d, inset panel) and only for males separately ($\beta = -0.49$, $p = 0.015$), supporting the sex by sequestration interaction. Owing to their very small size and lower cardenolide concentrations, larvae of *Tetraopes* and monarchs were not chemically analysed individually but were rather pooled by all replicates on the same plant family (see §2), so individual level assessment of costs was not possible. Nonetheless, even with the smaller sample size of insect cardenolide and growth means for each plant family, significant costs of sequestration were revealed for monarchs and *Tetraopes* (figure 2b,c).

4. Discussion

Convergence of ecological strategies in phylogenetically and ecologically distinct organisms has been typically investigated through the lens of trait evolution as the result of shared selective pressures and limits to alternative adaptive outcomes [5,40]. Nonetheless, whether convergent evolution of ecological strategies also comes with similar costs in different organisms has not been widely tested. Species sharing similar adaptations may not necessarily experience similar costs because different species occupy different niches, have other complementary traits, different life history strategies, and ecological needs that may affect how those costs emerge. However, if similar and strong selective pressures act on key components tied to species' shared physiology, then similar costs (even if the magnitude differs) should be expected between species. Here we have shown that three milkweed insect specialists, representative of three insect guilds, all experienced costs of toxin sequestration in terms of reduced growth, but none experienced costs of ingestion, despite differences in counter-defence adaptations and the plant organ they consume. Such costs, although predicted, have not been well studied and probably arise owing to opposing selection forces acting on toxin metabolism, movement and storage in the insect's body to avoid toxicity (i.e. bottom-up selection) [17,27] but also by predators (i.e. top-down selection) [33,41].

Effects of toxin ingestion have been difficult to detect in specialist herbivores [42,43]. For example, variable levels of glucosinolates and myrosinase activity in *Brassica juncea* had negligible effects on the performance and fitness of the specialist

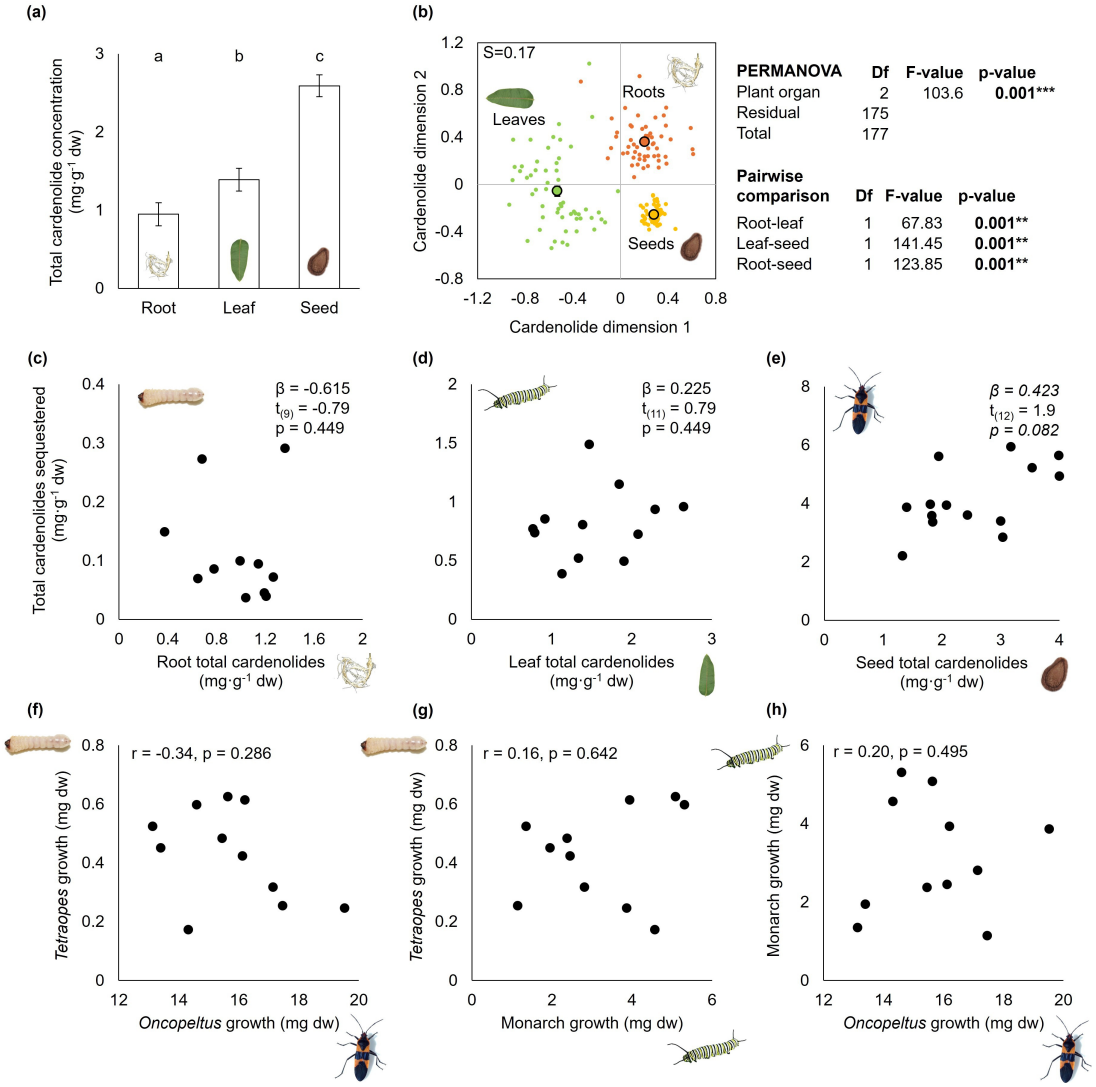

Figure 1. Total (a) and multivariate (b) cardenolide concentrations among plant organs, linear regressions between plant total cardenolides and cardenolides sequestered by insects (c–e), and correlations between performance of the three insects (f–h). Panel (a*)* represents the grand means of plant organs. Panel (b) represents the multivariate plant cardenolide composition from non-metric multidimensional scaling on 33 individual compounds (stress = 0.17) and the companion PERMANOVA results for the effect of plant organ. Panels (c–e) represent linear regressions predicting cardenolide concentrations sequestered in milkweed-specialist herbivores feeding on roots (c), leaves (d) and seeds (e) as a function of the total cardenolide concentrations in the plant organ they feed on. Panels (f–h) represent Pearson correlations of insect growth in pairwise combinations of root (*Tetraopes tetrophthalmus*), leaf (*Danaus plexippus*) and seed (*Oncopeltus fasciatus*) milkweed-specialist herbivores. Dots represent the means of several individuals in a plant family. Different letters represent significant differences between plant organs ($p < 0.05$). Marginally significant regressions are highlighted in italics ($0.05 < p < 0.1$).

turnip sawfly [44]. At a larger scale, variation in plant tropane alkaloid concentrations across *Datura* species weakly affected the performance of the specialist three-lined potato beetle [45]. Indeed, herbivore specialists are typically less affected than generalists when consuming toxic plants [46,47]. For instance, detoxification of glucosinolates occurs via sulfatases and nitrile-specifier proteins [48–50], cyanogenic glycosides are dealt with β-cyanoalanine synthases, rhodaneses and cyanases ([51] and references therein), and pyrrolizidine alkaloids are deactivated by flavin-dependent monooxygenases [52,53]. In our study, cardenolide ingestion was not associated with reduced insect growth despite variation in sodium pump resistance among insects and in plant organ cardenolide concentration and composition. We and others previously hypothesized that phenotype-matching between plant organ toxins and insect resistance may contribute to maintaining multiple 'arms races' [13,54], and that sequestering species probably rely on mechanisms other than target site resistance ([8] and references therein).

Milkweed-specialist herbivores appear adapted to the toxins from the tissues they consume despite their differences in overall sodium pump resistance to cardenolides [13]. Recent *in vitro* assays revealed that insects show greater sodium pump resistance to the dominant cardenolides present in the tissues they consume. Specifically, monarchs and seed bugs showed the strongest relative resistance to glycosylated aspecioside, the most abundant cardenolide in leaves and seeds of common milkweed [55], whereas greater resistance was found in the root beetle's enzyme to syrioside, which is dominant in roots [18]. Although specifically adapted to cope with plant defences that insect species commonly encounter, some cardenolides (e.g. voruscharin) are particularly toxic and can inhibit the insects' sodium pump *in vitro* to similar levels as unadapted generalists [20]. Nonetheless, mechanisms to specifically convert [27], transport [28,29,56,57] and store compounds [17] may be involved in why *in vivo* costs of ingestion may not emerge when explored in whole organisms [17,20]. More importantly, mechanisms to process toxins require simultaneous metabolic demands that are predicted to be costly [20,55,57].

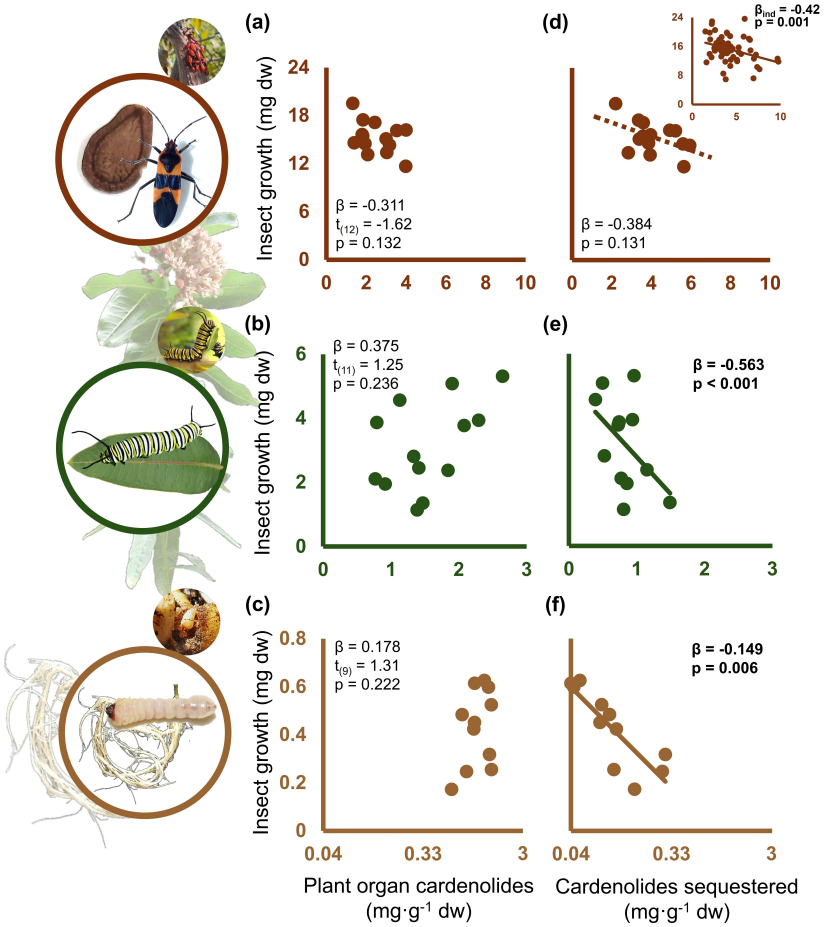

Figure 2. Linear regressions predicting insect growth in specialist herbivores of seeds (a, d), leaves (b, e) and roots (c, f) as a function of cardenolide concentrations in the plant organs they ate (a–c) and cardenolide concentrations sequestered in their bodies (d–f). Each dot represents the mean of several individual plants or insects feeding on a full-sib family of common milkweed. The same plant families were used for all insects. Significant regressions are highlighted in bold with trend lines plotted ($p < 0.05$). A dashed line is included in panel (d) because this regression is highly significant when based on individual adult seed bug sequestration and mass, not averaged across plant families (inset panel). Regressions between cardenolides sequestered and insect growth (panels d–f) were corrected for non-independence of both variables using insect mass with Monte Carlo simulations as described in [17]. Note the log scale on the abscissa of panels (c) and (f) for the root herbivore.

The strong differences in cardenolide profiles between plant organs contrasted with the similar costs of sequestering toxins from the host plant among insects. We note that the strength of the costs did indeed vary in effect sizes (highest in monarchs, intermediate in seed bugs, lowest in root beetles), and these differences may relate to the relative benefits each species enjoys from sequestration. On the one hand, variation in cardenolide concentration and composition in the plant could only weakly explain the costs of ingestion and sequestration in this community of specialists. On the other hand, strong variation in cardenolide profiles among organs [12–14], with concentrations increasing from roots to seeds and toxicity following the opposite direction [12,13], has been argued to play a role in shaping plant organ-specific coevolutionary interactions [13]. Optimization of the cost–benefit ratio of investing in plant defences to cope with herbivores with variable resistance to toxins can explain our findings regarding the costs of sequestration (i.e. bottom-up selection). In seed bugs (high resistance), costs probably emerged owing to hyperaccumulation of less toxic seed cardenolides (1.6-fold more total cardenolides than those from seeds), and in root beetles (modest resistance) owing to lower total accumulation but mostly of more toxic cardenolides (8.5-fold less total cardenolides than those from roots). In the case of monarchs, handling moderate concentrations with typically greater cardenolide diversity in leaves may exacerbate costs via synergistic impacts of toxin mixtures on multiple physiological mechanisms [17]. Regardless of whether variation in cardenolide composition among organs is a cause or an effect of specialized coevolution is still under debate. For instance, high concentrations of less toxic cardenolides may indirectly derive from plant physiological constraints in mobilizing more non-polar defences (i.e. more toxic and non-water-soluble) in seeds [13,58].

Cardenolide concentrations present in the plant organs do not always predict cardenolides sequestered by the insects quantitatively, supporting the notion that cardenolide sequestration involves an active and selective process regulated by the insect [17,59]. For example, monarch caterpillars initially sequester more passively, typically reflecting their diet, but concentrations eventually saturate and plateau in their bodies during the later stages of larval development [30,60,61]. The sequestration process generally involves accumulating some less toxic cardenolides intact, whereas more toxic ones are transformed into less harmful subproducts first [27,30].

Despite this process being relatively well understood in monarchs, whether similar mechanisms occur in other milkweed specialists has not been well studied. For the seed bug, cardenolides are accumulated and apparently processed in a similar manner as monarchs, but are eventually stored in specialized dorsolateral spaces at higher concentrations, and their content is

actively regulated by the insect [62–64]. Previous studies found that larger seed bugs, which are typically females, can secrete larger amounts of cardenolide-rich fluid from the dorsolateral space, eventually leading to lower cardenolides in their bodies per dry weight compared with males when depleted of fluid [65]. On the other hand, the root beetles may have physiological limitations in accumulating cardenolides, given the lower cardenolide amounts typically found in their bodies compared with seed bugs [11,66]. However, recent evidence found that root beetles accumulate comparatively more toxic cardenolides in their bodies than monarchs and seed bugs [18], which may indicate compensation for lower cardenolide accumulation with more potent toxins. This compensation may explain why root beetles generally experience greater larval mortality on highly defended plants [22,67]. Thus, although the active process of cardenolide transformation and sequestration is contingent on the diet to some extent, it is largely insect-specific.

We speculate that in coevolutionary interactions, once specialists crack the code of resisting plant defences, costs of sequestration may emerge and be exacerbated through bottom-up and top-down selection from plants and predators. From the perspective of plant defence, sequestration of especially toxic compounds can negatively impact different physiological mechanisms, eventually leading to a reduction in insect performance. For instance, mixtures of structurally diverse toxins may require different and simultaneous metabolic demands that can eventually reduce insect growth [17]. Increasing toxin sequestration may also impose other metabolic costs, including reduced respiration rate, lipid storage and impaired flight energetics [32,68–70]. Lastly, toxin accumulation has been associated with increased oxidative stress in cardenolide-sequestering insects, with potential consequences for their performance and surviving predator attacks [71,72]. Because other factors, like variation in plant nutritive value, may influence how insects cope with consumed plant toxins [73,74], consistency in costs of sequestration suggests a more fundamental limitation on processing and storing toxins.

From the perspective of reducing predation, presumably higher levels of toxins sequestered are favoured by most predators. Nonetheless, sequestering insect species have been reported to accumulate diverse toxins in different proportions, probably to cope with multiple predators they can potentially encounter in nature [27,65]. For monarchs, sequestered cardenolides are more effective against vertebrate compared with invertebrate predators [4]. In addition, not all insect species are exposed to predators equally [75], which can influence selection on sequestration and associated costs. Despite the similar costs of sequestration in our study (figure 2d–f), we speculate that the different slopes provide indirect evidence of different selection intensities by predators. Seed bugs and monarchs are typically unconcealed in their juvenile stages, and especially monarchs tend to be preyed upon by many enemies [76], supporting potential benefits countering the steeper negative slope. Regardless, we suggest interpreting these results with caution since insects fed for short periods of time not representative of their developmental cycle (i.e. monarch caterpillars typically feed for approximately 14 days before pupating, whereas root beetle larvae take months feeding on roots before pupating), they were examined at different life stages (i.e. adult seed bugs versus monarch and root beetle larvae), and fed on chemically distinct plant organs, therefore making comparisons of slopes difficult to interpret.

Lastly, selection by predators favouring toxin sequestration and their toxicity may conflict with selection by plants that favour reduction of toxicity of compounds [27]. In other words, both plants and predators probably impose similar costs of sequestration for insect specialists (e.g. reduction in growth, survival), but the cause of those costs is probably different. Plants probably impose costs of sequestration through selection for multiple mechanisms to reduce toxicity in the insect's body, whereas predators may do it via selection for increased toxicity in the herbivore, imposing costs through accumulation and handling of harmful chemicals for themselves. If this is the case, selection favouring sequestration of less toxic compounds could facilitate the evolution of predator resistance to the herbivores' sequestered compounds over time [41]. Thus, given that specialist sequesterers must tolerate the toxins in their diet, yet probably pay a cost of sequestration, we predict that species will maintain heritable variation in their level of sequestration contingent on the intensity of selection from plants and predators within a population.

Our findings suggest that the evolutionary benefits of sequestration are not free of costs and also provide evidence that similar costs occur among diverse species sharing a host plant. Despite large differences in plant organ toxicity and target site (sodium pump) mutations of the milkweed feeders, species showed negligible costs of toxin ingestion. Costs of sequestration are a commonality shared by this specialized community of insect herbivores which may be coevolving with the host plant and their natural enemies through conflicting selective pressures. In other words, convergence in the costs of sequestration may result from bottom-up selection by plant defences to reduce sequestration, and from top-down selection by natural enemies to increase sequestration to thwart predation.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. Data and code are available in Zenodo [77].

Supplementary material is available online [78].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. X.L.-G.: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing—original draft, writing—review and editing; P.A.R.-B.: data curation, methodology, writing—review and editing; A.P.H.: data curation, methodology, writing—review and editing; A.A.A.: conceptualization, funding acquisition, investigation, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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