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Evidence for tissue-specific defence-offence interactions between milkweed and its community of specialized herbivores

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Abstract

Coevolution between plants and herbivores often involves escalation of defence-offence strategies, but attack by multiple herbivores may obscure the match of plant defence to any one attacker. As herbivores often specialize on distinct plant parts, we hypothesized that defence-offence interactions in coevolved systems may become physiologically and evolutionarily compartmentalized between plant tissues. We report that roots, leaves, flower buds and seeds of the tropical milkweed (*Asclepias curassavica*) show increasing concentrations of cardenolide toxins acropetally, with latex showing the highest concentration. In vitro assays of the physiological target of cardenolides, the Na^+/K^+–ATPase (hereafter "sodium pump"), of three specialized milkweed herbivores (root-feeding *Tetraopes tetrophthalmus*, leaf-feeding *Danaus plexippus*, and seed-feeding *Oncopeltus fasciatus*) show that they are proportionally tolerant to the cardenolide concentrations of the tissues they eat. Indeed, molecular substitutions in the insects' sodium pumps predicted their tolerance to toxins from their target tissues. Nonetheless, the relative inhibition of the sodium pumps of these specialists by the concentration versus composition (inhibition controlled for concentration, what we term "potency") of cardenolides from their target versus nontarget plant tissues revealed different degrees of insect adaptation to tissue-specific toxins. In addition, a trade-off between toxin concentration and potency emerged across plant tissues, potentially reflecting coevolutionary history or plant physiological constraints. Our findings suggest that tissue-specific coevolutionary dynamics may be proceeding between the plant and its specialized community of herbivores. This novel finding may be common in nature, contributing to ways in which coevolution proceeds in multispecies communities.

KEYWORDS
adaptation, cardiac glycosides, defence trade-offs, insect herbivore community, phenotype-matching, plant-insect interactions

1 | INTRODUCTION

Coevolution is often defined as reciprocal evolutionary change in interacting species (Janzen, 1980), and specialized antagonistic interactions are expected to show a signature of matching defence–offence phenotypes as a result of long and intimate coevolutionary histories (Berenbaum, 1983; Brodie et al., 2002). In natural systems, however, community complexity often disrupts such pairwise coevolution, causing deviations from the expectation of phenotype matching (Thompson et al., 2017; Zangerl & Berenbaum, 2003). If plants are attacked by a community of herbivores, the total selective impact of these attackers on defence may be diffuse, making it challenging to decipher coevolutionary dynamics between any one pair of species (Johnson & Agrawal, 2007; Ohgushi, 2016;

Stinchcombe & Rausher, 2002). Nonetheless, not all herbivores attack the same tissues of a plant, and different plant organs serve as distinct niches exploited by enemies (Herrera, 2009; Joy & Crespi, 2007). Accordingly, a complex community consisting of herbivores adapted to distinct plant organs could favour specialized allocation of defence among these different tissues. This hypothesis is especially relevant to specialized interactions, where coevolutionary history between the plant and its community of attackers that feed on distinct tissues may result in heterogeneous defence-offence interactions across plant compartments.

According to the classic optimal defence framework (McKey, 1974; Rhoades & Cates, 1976), plants should defend their organs proportional to their relative contribution to fitness and the relative attack rate on those tissues. In one of the most prominent empirical examples supporting these predictions, Zangerl and Rutledge (1996) found that defence levels increased from roots to fruits, and that this variation was associated with the probability of attack of those tissues in natural populations of wild parsnip. Since then, many studies made a tremendous effort to understand plant physiological responses and defence signalling between tissues to herbivory (Biere & Goverse, 2016; López-Goldar, Lundborg, et al., 2020; Rotter et al., 2018; Soler et al., 2013). However, plant defence expression across organs, and the extent of insect adaptation to those defended tissues, remains virtually unexplored. This is because research to date has paid more attention to the host plant compared to its herbivores, which in most cases were generalist feeders (Rotter et al., 2018; Strauss et al., 2005; Wise & Rausher, 2013). Thus, to uncover potential evolutionary relationships between tissue-specific defence expression and insect adaptations in plant-herbivore interactions, a focus on specialized, coevolved systems is needed.

In the interaction between milkweeds (*Asclepias* spp.) and their community of specialized insect herbivores, cardiac glycosides (i.e., cardenolides) have an exclusively defensive function and specific physiological target, the Na^+/K^+-ATPase (hereafter "sodium pump") (Agrawal, 2005, 2017; Agrawal et al., 2012). Cardenolide diversity ranges up to 30 compounds in single plants, and both compound diversity and concentration are variable among tissues eaten by insects, and also in nonfood, defensive substances such as latex (Agrawal & Konno, 2009; Rasmann & Agrawal, 2011).

Although the sodium pump is highly conserved among animals and has been well-characterized, independent evolution of sodium pump insensitivity to cardenolides has occurred in six taxonomic orders of insects specialized to feed on cardenolide-containing plants (Karageorgi et al., 2019). The genetic substitutions responsible for sodium pump insensitivity repeatedly evolved via a constrained molecular path, probably explained by amelioration of negative pleiotropy through epistasis (Karageorgi et al., 2019; Taverner et al., 2019). The most beneficial substitutions occur in the amino acid residues 111, 119 and 122, and additionally in 786 and 797 in specific clades, conferring enhanced tolerance to cardenolides (Dobler et al., 2012; Karageorgi et al., 2019). In response, defence evolution in plants has resulted in the production of specific cardenolide compounds with particularly high potency against resistant sodium pumps (Agrawal et al., 2021; Petschenka et al., 2018). Nonetheless, since not all milkweed insects show the same sodium pump insensitivity (i.e., different number of genetic substitutions), whether the degree of cardenolide tolerance in insects targeting different plant tissues matches tissue-specific defence chemistry remains untested.

Here, we studied the extent of phenotype matching between plant defence and herbivore tolerance of toxins in the community of insect specialists on different plant organs of milkweed. Specifically, we extracted and characterized the cardenolide defences of roots, leaves, flower buds, seeds, and latex of the tropical milkweed (*Asclepias curassavica*) and then tested whether herbivores specialized to distinct plant tissues may be adapted most to extracts from their preferred tissues. In particular, we contrasted herbivore adaptation to cardenolide concentration (sodium pump inhibition per unit tissue dry mass) and cardenolide potency (sodium pump inhibition per unit cardenolides). We studied three specialist herbivores (compared to a nonadapted insect, *Drosophila melanogaster*), in order of increasing molecular substitutions conferring enzymatic tolerance to cardenolides (Figure 1): (1) a cerambycid beetle (*Tetraopes tetrophthalmus*), which feeds on roots as larvae and leaves and flower buds as adults (Agrawal, 2004); (2) the monarch caterpillar (*Danaus plexippus*), which feeds on leaves and flower buds; and (3) the large milkweed bug (*Oncopeltus fasciatus*), which feeds on seeds. The sodium pumps of both *D. plexippus* and *O. fasciatus* have been well-characterized (Dalla et al., 2013, 2017). While the sodium pump of

FIGURE 1 Amino acid substitutions at key residues implicated in cardenolide insensitivity of the Na^+/K^+–ATPase in milkweed insects (red font) compared to the wild-type enzyme (Q111-A119-N122, QAN) (*Drosophila melanogaster*). The root beetle (*Tetraopes tetrophthalmus*) has two substitutions (LSN), the monarch (*Danaus plexippus*) has three (VSH), while the seed bug is TSH, and also has multiple copies of the enzyme with additional substitutions at other residues (786 and 797) (Dalla et al., 2013, 2017; Dobler et al., 2012). The number of genetic substitutions and copies provides increased cardenolide insensitivity (Dalla et al., 2017; Karageorgi et al., 2019; Taverner et al., 2019). Amino acids separated by a slash for a given position in the figure indicate the substituted amino acids in the key sites present in each of the multiple copies of the enzyme (wild-type: QAN-FT; *O. fasciatus*: copy A: TSH-NA; copy B: TSH-NT, copy C: QSH-FS) (Dalla et al., 2017; Zhen et al., 2012) [Colour figure can be viewed at wileyonlinelibrary.com]

T. tetrophthalmus has not been previously studied physiologically, the genetic substitutions of its sodium pump suggest only modest insensitivity to cardenolides (Dobler et al., 2012).

We aimed to answer the following questions in the context of coevolution of plant defence across organs and adaptation of specialized herbivores to cardenolides in the tissues they feed on: (1) Do cardenolide levels differ between plant organs? As postulated by optimal defence theory (ODT), we predicted that cardenolide levels differ between tissues, with highest defence levels in those tissues closely related to plant fitness (i.e., flower buds and seeds); (2) Does cardenolide concentration of the target tissue match the molecular substitutions and relative tolerance of each specialized herbivore species? We predicted that relative insect tolerance to cardenolides would be proportional to the cardenolide concentration present in the tissues they feed on. In order to avoid potential biases from insect adaptations to the toxins present in the tissues they eat, we standardized insect tolerance by using a reference cardenolide, ouabain, which is not present in milkweeds. Also, we tested insect tolerance to latex, a substance with solely defensive function and no nutritional value to herbivores (Agrawal & Konno, 2009); (3) Are each of the three herbivores most adapted to extracts of their target (i.e., food) tissues compared to nontarget tissues which they do not eat? We predicted that insects should be more tolerant of the extracts from tissues they feed on compared to nontarget tissues. Since food tissue extracts may impose joint effects of concentration and potency of toxins on the adaptation of each insect, we also asked (4) To what extent are the insect specialists adapted to the cardenolide concentrations versus potency in their target versus nontarget tissues? Here we predicted that insects should be more tolerant to the specific potency of cardenolides (after controlling for concentration) of their target versus nontarget tissues.

2 | MATERIALS AND METHODS

2.1 | Plant material and tissue sampling

We used *A. curassavica* seeds from a commercial source (Everwilde Farms), surface sterilized with 10% bleach and scarified with a razor blade. Seeds were cold stratified at 4°C on moist paper towels for one week, and placed in the dark at 30°C to synchronize germination. Seedlings were planted in a mix of Lamberts soil (LM111; Lamberts, Quebec, Canada) and 25% perlite in 10 cm plastic pots, and grown in a growth chamber for 3 months (14 h daylight, 27°C:23°C day:night). They were transplanted into 15 cm pots 45 days after planting. Plants were watered as needed and dilute fertilizer (N:P:K 21:5:20, 150 ppm N [μg/g]) was applied every 10–12 days. We also applied slow-release fertilizer (Osmocote Smart-Release) 15 and 45 days after planting. In May, plants were relocated to a field in Ithaca, NY (42.4641159 N, −76.444484 W) and put in mesh cages (3 m length × 1.7 m width × 2 m height) to protect them from natural herbivory. All lateral branches and the main stem (30 cm above soil level) were pruned and allowed to regrow to synchronize flower bud development with tissue sampling. In mid-July, all plants were harvested, and we collected the roots (1–2 mm diameter), the two youngest fully expanded leaves, and the inflorescences from each plant (three tissue types from $n = 19$ plants). All tissues were immediately placed on ice during harvesting and were later freeze-dried. For seeds, we made two separate biological replicates by haphazardly pooling 50 individual seeds each from the same commercial seed source the experimental plants were obtained from (see above). All dried tissues and seeds were ground to a fine powder in a mixer mill (Retsch) using 5 mm steel beads. Then, 40 mg of ground tissue ($n = 18$–19 for each) and seed ($n = 2$) samples were weighed for cardenolide analyses by HPLC. For the sodium pump assays, two replicates of each tissue (as with the seeds) were made by pooling known masses of seven independent ground samples to capture the variation between the different samples, and 30 mg of each replicate were weighed (see below). We used 10 additional *A. curassavica* from the same source material, grown under the same controlled and field conditions, to collect latex from one of the youngest, fully expanded leaves from each plant in preweighed microtubes. Fresh latex was immediately weighed to the nearest 0.1 mg and stored at −20°C until chemical analysis.

2.2 | Sample preparation

Cardenolides were extracted from each sample by adding 1 ml of 100% methanol to 40 mg (for HPLC, spiked with 20 μg of hydrocortisone as internal standard) or 30 mg (for sodium pump assays) of ground material (except latex, see below) and 20 FastPrep beads. Samples were extracted by agitation on a FastPrep-24 homogenizer twice for 45 s at 6.5 m/s, and then centrifuged at 20817 *g* for 12 min. Supernatants were dried down in a vacuum concentrator at 35°C. Dried samples for analysis by HPLC were resuspended in 250 μl of 16:16:68 (in %) methanol:acetonitrile:water (vol:vol:vol) and filtered using 0.45 μm hydrophilic membranes. Dried samples for sodium pump assays were resuspended in 250 μl 20% DMSO in deionized water and sonicated twice for 5 min (see below).

Prior to methanol extraction, seed powder was defatted by overnight extraction with 1 ml of hexane, which was then removed and seed material was dried down. For latex, we individually extracted each sample as above and then made two biological replicates by randomly pooling five independent samples for each replicate. Each biological replicate was split in two aliquots before drying down, after of which one aliquot was resuspended for analysis by HPLC as above, and another was resuspended in 20% DMSO for sodium pump inhibition assays (see below).

2.3 | HPLC-UV quantification of cardenolides in plant tissues

We detected and quantified the cardenolides in plant tissues 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) following standard protocols with modifications (Agrawal et al., 2021) (see Methods S1). Cardenolide concentrations for individual and pooled tissue samples were estimated by using the peak area and known concentration of the internal standard (hydrocortisone, RT 13.5 min) on a dry mass basis of plant tissue (μg/mg d.w.). For latex, concentration of cardenolides were converted to a dry mass basis using a wet/dry mass ratio for *A. curassavica* latex (n = 19), collected in a separate experiment. We recorded the number of distinct cardenolide peaks (hereafter "cardenolide richness"), calculated the cardenolide diversity by using the Shannon–Wiener index (hereafter "Shannon diversity"), and constructed a polarity index following Rasmann and Agrawal (2011) for each plant tissue (see also Methods S1). A tentative identification of cardenolides was performed by LC-MS on five randomly selected samples of roots, leaves and flower buds extracted as above (no internal standard was added) following the methods of Agrawal et al. (2021), and compound identity was matched with recently published work from the same seed source (Agrawal et al., 2021).

2.4 | Insect Na^+/K^+-ATPase sensitivity to tissue cardenolides

We quantified the biological activity of diverse cardenolide-containing plant tissues using the Na^+/K^+-ATPase of neural tissue from dissected brains of the fruit fly *Drosophila melanogaster* (wild-type) and those from adults of three milkweed insect specialists (*T. tetrophthalmus*, *D. plexippus*, and *O. fasciatus*) following methods of Petschenka et al. (2018). Briefly, fruit flies, monarchs and seed bugs were reared under controlled conditions (Cornell University, University of Hamburg), and the root beetles were collected in the field in Ithaca, NY (USA). All insects were frozen alive and their brains were dissected and stored at −80°C until use. Insect brains were homogenized in Millipore water as follows: 12.5 heads in 450 μl for fruit flies, 10 brains in 600 μl for root beetles, 0.75 brains in 2 ml for monarchs, and five brains in 600 μl for seed bugs, according to pilot tests to provide comparable enzymatic activity levels for the assays. Na^+/K^+-ATPase activity was then 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). Dried cardenolide extracts from two biological replicates of 30 mg of pooled tissue samples (see Section 2.2 above) were resuspended in 250 μl 20% DMSO in deionized water and sonicated for 5 min. We then prepared four serial dilutions (1:10 of each previous one) to produce a five-point inhibition curve for each tissue type (including latex), and incubated each with all four insect enzyme preparations (5 tissue types × 2 pools × 5 dilutions × 4 enzymes = 200 reactions). Milkweed tissue extracts were analysed alongside the standard cardenolide ouabain (from 2×10^{-3} M to 10^{-8} M) in 20% DMSO to standardize the inherent insensitivity of each enzyme to a nonmilkweed cardenolide. Reactions were performed in 96-well microplates on a BioShake Iq microplate shaker (Quantifoil Instruments) at 200 rpm and 37°C for 20 min and quantified photometrically at 700 nm following Taussky et al. (1953). We performed 1–3 technical replicates per tissue type and enzyme (6–9 technical replicates per pooled tissue type), and from the residual enzymatic activity of each dilution (difference between absorbances of the reaction of each dilution level minus that of completely inhibited reaction) we estimated the sigmoid dose-response curve using a four parameter logistic function using the function nlme with SSfpl from the nlme package v3.1-152 in R v3.6.3 following Züst et al. (2020). From each function, we extracted the relative dilution at the inflection point (i.e., residual enzymatic activity of 50%, or IC50). Raw IC50 values obtained from the logistic model, representing the dilution of the extract needed to inhibit the enzyme by 50%, were multiplied by the amount of tissue extracted for the in vitro assays (30000 μg), *representing* the amount tissue needed to inhibit enzyme by 50% (i.e., IC50 × μg d.w. tissue, hereafter "$IC50_{mass}$"). $IC50_{mass}$ was further multiplied by the total cardenolide concentration present in each tissue as determined by HPLC-UV (μg card/mg tissue), indicating the amount of cardenolides needed to inhibit the enzyme by 50% (i.e., a proxy of cardenolide potency) (IC50 × mg d.w. tissue × μg cardenolide/mg d.w. tissue, hereafter "$IC50_{conc}$").

2.5 | Statistical analyses

We compared plant cardenolide investment between plant tissues (roots, leaves, flower buds, seeds and latex) using a linear mixed effects model in SAS v9.4. Concentrations of individual and total cardenolides, cardenolide richness, Shannon diversity and polarity index (see Methods S1) were analysed independently as response variables and plant tissue was included as a fixed effect. A random term of the plant individual was included to account for nonindependence of cardenolide concentrations in root, leaf and flower tissues from the same plant, whereas seed and latex were considered independent from plant individuals. Variables were log- or square root-transformed when appropriate to meet assumptions of normality. In order to analyse all the variables without inflating type I error due to multiple tests, p-value adjustments were performed using false discovery rate (FDR) (Benjamini & Hochberg, 1995).

To evaluate the differences in overall cardenolide chemistry between plant tissues, we conducted a repeated measures multivariate analysis of variance (MANOVA) in SAS v9.4, including the concentration of all cardenolide compounds as response variables and plant tissue as fixed effect. Plant tissue was also included as a repeated measure of the same plant subject (root, leaf, flower), whereas seed and latex were considered independent from plant individuals as above. We also conducted nonmetric multidimensional scaling (nMDS) in SAS v9.4 to spatially visualize the multivariate differences in chemistry between tissues. Briefly, data were log-transformed and normalized by the standard deviation, and chemical composition of all tissues was analysed based on Euclidean distances.

Insect tolerance to plant toxins was compared using linear models in SAS v9.4. Insect tolerance to tissue extracts ($IC50_{mass}$) and to cardenolide potency ($IC50_{conc}$) were the response variables, and insect enzyme, plant tissue and their interaction were included as explanatory variables. Microplate was considered a random factor, and differences were allowed for in microplate variance from each pool of tissue sampled. Insect tolerance to ouabain and to latex were examined independently from plant tissues eaten by the insects, with insect species considered as fixed factor, and microplate as random factor as above. Insect tolerance values for all variables were transformed to the fifth root to meet assumptions of normality. This transformation allows residuals to fit better into a normal distribution compared to more conventional transformations in certain data sets (Kendall et al., 2005).

Pairwise mean differences in univariate analyses between tissues, in tolerance values between enzymes (ouabain and latex), and between tissues within the same enzyme were examined using Fisher's LSD.

We performed simple Pearson correlations to address potential tradeoffs between cardenolide concentration (estimated from HPLC-UV) and potency (estimated as the inverse of the insect tolerance normalized by concentration, $IC50_{conc}$, averaged across insects) across plant tissues eaten by insects and across all tissues (including latex). Potential spurious correlations were accounted for by performing Monte Carlo simulations adapting the procedure of Morris et al. (2006) in SAS v9.4.

Finally, because we recently discovered a few highly potent cardenolides in *A. curassavica* (Agrawal et al., 2021), we explored the potential associations between insect tolerance to tissue extracts and specific chemical compounds in these tissues. Our main goal with this analysis was to generate hypotheses about cardenolides that may show tissue-specific expression and be targeted at particular herbivores. We thus performed simple Pearson's correlations between concentrations of individual cardenolides and insect tolerance to tissue extracts (for each milkweed insect $IC50_{mass}$ and averaged across the three insect specialists), using data from the pooled sample tissues (2 pools × 5 tissues = a maximum of 10 data points). Since significant correlations may arise due to associations with other cardenolides, we also examined pairwise correlations between all cardenolides (only for root, leaf and flower bud individual tissue samples, n = 18–19). p-values from multiple tests were corrected using FDR as above.

3 | RESULTS

We first investigated the cardenolide defences of plant organs using HPLC-UV, and found that 19 out of 20 individual cardenolides (present in at least two tissues) differed in concentration between plant tissues (Table S1). Nine additional compounds showed expression specific to a single tissue: five were unique in seeds, two in latex, one in leaves and one in flower buds (Table S1). Overall, plant tissues strongly differed in cardenolide composition (Figure 2). Total

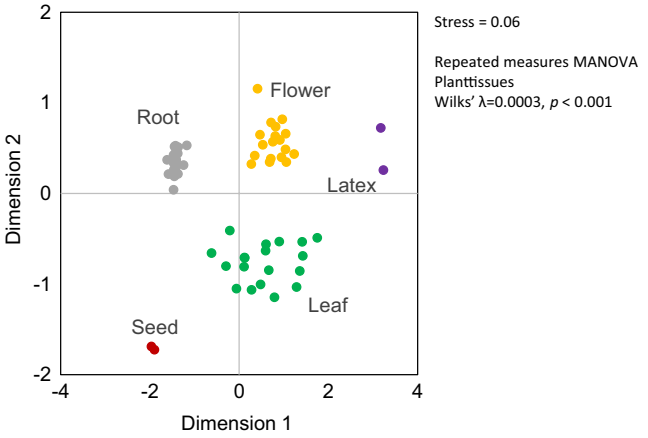

FIGURE 2 Nonmetric multidimensional scaling (nMDS) plot of the cardenolide chemistry of *Asclepias curassavica* tissues. Individual samples from each tissue are depicted by coloured dots (roots, leaves and flowers, N = 18–19 each; seeds and latex, N = 2 each), and the differences in cardenolide chemistry between tissues was supported by a repeated measures multivariate analysis of variance (MANOVA) [Colour figure can be viewed at wileyonlinelibrary.com]

cardenolide concentration between plant parts varied >100-fold ($F_{4,35}$ = 29.9, p < .001, Figure 3a), increasing acropetally across tissues: leaves, buds and seeds showed 5-, 6-, and 12-fold greater cardenolide concentrations than roots, respectively. Latex, a defensive substance not used as food *per se*, showed the highest concentration amongst all plant tissues, with >10-fold more cardenolides than seeds, the tissue with the next highest concentration. Plant tissues also differed in their cardenolide richness, diversity and polarity (Figure S1).

To test how insect species differed in tolerance to cardenolides, we first tested their sodium pump inhibition by the nonmilkweed cardenolide ouabain. Insects differed in tolerance >1000-fold ($F_{3,9}$ = 944.1, p < .001, Figure 3b), with differences closely following from molecular substitutions in their sodium pumps (Figure 1) and also matching the pattern of total cardenolide concentrations in their food tissues (Figure 3a). The root beetle, monarch and seed bug were 6-, 156- and >1600-fold more tolerant than the nonadapted fruit fly, respectively. Similarly, insect tolerance to latex cardenolides differed >150-fold ($F_{3,11}$ = 167.6, p < .001, Figure 3c), closely matching the pattern for ouabain (r > .99, p < .001, n = 4 insect species tested), although latex cardenolides were much more potent, with an average insect tolerance 28-fold lower than ouabain.

We next investigated whether each of the three specialized herbivores are most tolerant to their target versus nontarget, mass-standardized tissue extracts by dosing them on the insect sodium pumps in vitro ($IC50_{mass}$). Insect tolerance varied tremendously ($F_{3,44}$ = 966.7, p < .001, Figure 4) and the root beetle, monarch, and seed bug were on average 7-, 18- and 72-fold more tolerant to cardenolide-containing plant extracts than the fruit fly (i.e., wild-type) sodium pump, respectively. While the seed bug and root beetle were relatively more tolerant to their target compared to nontarget tissues, the monarch was less tolerant to its target foods –leaves and buds– than to other tissues (Figure 4).

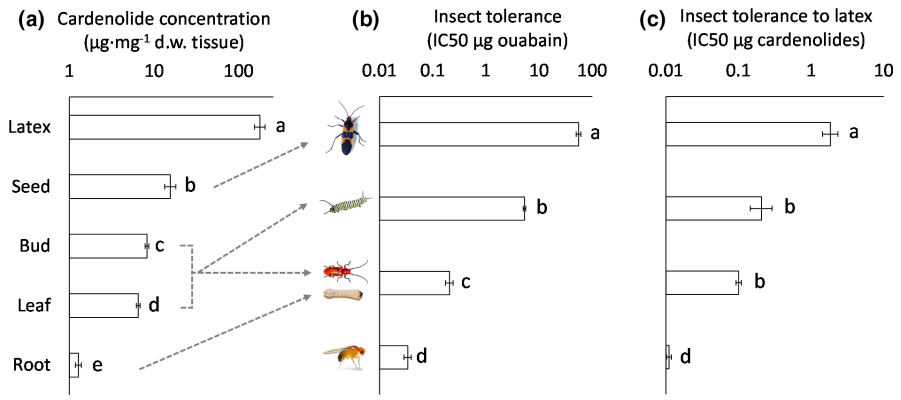

FIGURE 3 Allocation of defences between plant tissues in *Asclepias curassavica* and tolerance of insect specialists to cardenolides. (a) Total cardenolide concentration in *Asclepias curassavica* food (root, leaf, bud and seed) and nonfood (latex) tissues for insects estimated by HPLC. (b) Standardized evaluation of insect tolerance (IC50) estimated as the µg of ouabain necessary to inhibit enzyme activity by 50%. (c) Insect tolerance (IC50) to latex, a cardenolide-rich defensive substance. Bins and error bars represent back-transformed least square means and standard errors. Different letters in each panel indicate significant differences between factors (Fisher's LSD). Grey arrows connect insects with the plant tissue they typically eat [Colour figure can be viewed at wileyonlinelibrary.com]

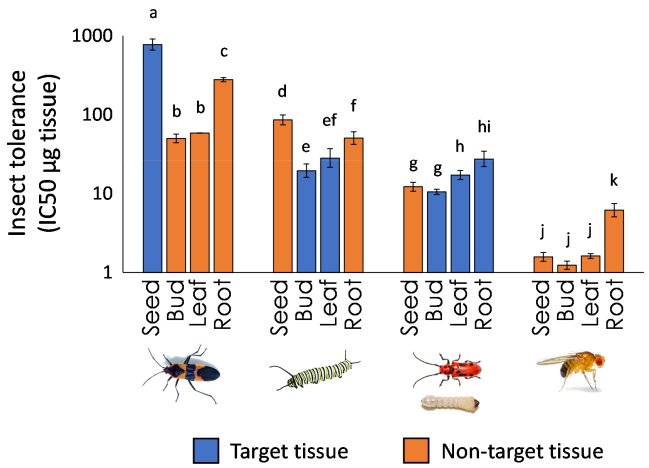

FIGURE 4 Insect tolerance to target versus nontarget plant tissues based on µg of tissue extract needed to inhibit their enzymes by 50% ($IC50_{mass}$). This assessment of net toxicity includes concentration and potency of cardenolides. Bars represent back-transformed least square means and standard errors. Enzymes significantly vary in their average tolerance to plant tissues. Different letters indicate significant differences (Fisher's LSD) in enzyme sensitivity between tissues for each insect. Insects from left to right: Seed bug, monarch caterpillar, root beetle, and fruit fly (wild-type enzyme). The root beetle feeds on roots as a larva and buds and leaves as an adult. Note the *y*-axis is on a log scale [Colour figure can be viewed at wileyonlinelibrary.com]

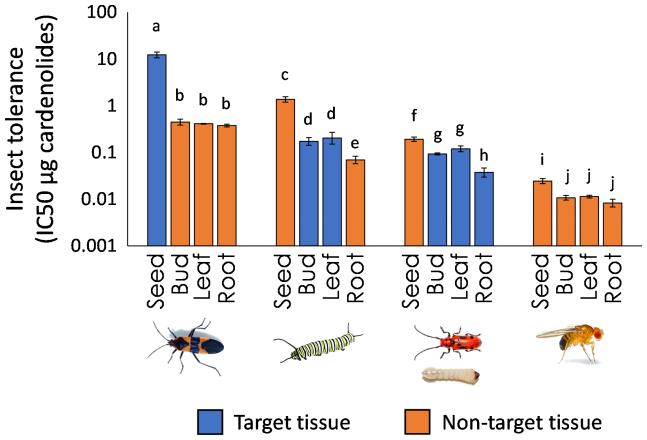

FIGURE 5 Insect tolerance to the cardenolide composition (potency) from target versus nontarget plant tissues. Here, the potency of cardenolides is assessed by presenting the µg of cardenolides from those tissues needed to inhibit enzyme activity by 50% ($IC50_{conc}$). Bars represent back-transformed least square means and standard errors. Enzymes significantly varied in their average tolerance to plant cardenolides. Different letters indicate significant differences (Fisher's LSD) in enzyme sensitivity between tissues for each insect. Insects from left to right: Seed bug, monarch caterpillar, root beetle, and fruit fly (wild-type enzyme). The root beetle feeds on roots as a larva and buds and leaves as an adult. Note the *y*-axis is on a log scale [Colour figure can be viewed at wileyonlinelibrary.com]

To address whether insect tolerance is adapted to the particular toxicity of the cardenolide composition present in their target tissues, we considered the potency of tissue extracts by normalizing the extracts by their total cardenolide concentration (measured independently by HPLC-UV, $IC50_{conc}$). While insect tolerance to the specific cardenolide compositions varied strongly ($F_{3,44}$ = 722.3, p < .001, Figure 5), all insects generally showed the greatest tolerance to seed cardenolides (which were also the most polar), moderate tolerance to that of flower buds and leaves, and the lowest tolerance to that of roots (except for the seed bug and the nonadapted fruit fly) (Figure 5). On average, insects showed 1.7-, 1.9-, and 13.9-fold less tolerance to cardenolides from flower buds, leaves, and roots, respectively, compared to that of seeds ($F_{3,44}$ = 184.7, p < .001, Figure 5).

We found a strong negative relationship between concentration and potency of cardenolides across food tissues (Figure 6), supporting a greater concentration of less potent cardenolides acropetally, from roots to seeds. The relationship, however, disappeared after

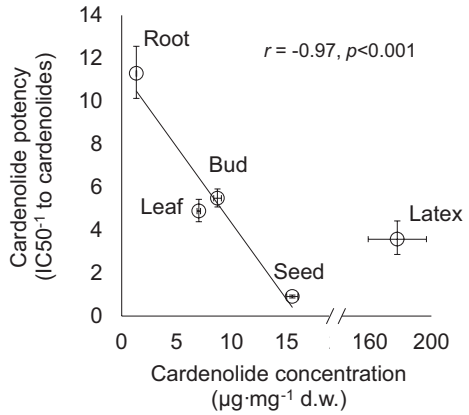

FIGURE 6 Negative association between concentration and potency across tissues eaten by insects ($n = 4$, solid line); not significant after including latex ($n = 5$). Each dot represents the mean and standard error of HPLC cardenolide concentration (*x*-axis) and potency (inverse of insect tolerance to cardenolide concentration, $IC50_{conc}$, averaged across insects; *y*-axis) from two biological replicates (each pooled from several independent samples) of tissue. The *r* and *p*-value are corrected for spurious correlations using the Monte Carlo procedure by Morris et al. (2006). Note that the *x*-axis is broken for ease of visualizing latex

including latex, which is strictly defensive and not utilized as a food *per se* ($r = -.38$, $p = .534$, $n = 5$).

Finally, we provide an initial attempt to link insect tolerance and specific cardenolide chemistry across plant tissues. We found nine out of 23 individual cardenolides were correlated with the average $IC50_{mass}$, taken across the three insect specialists (Table S2). Three of these were positively associated, and corresponded to more polar compounds, whereas six were negatively associated with this average $IC50_{mass}$, and corresponded to more nonpolar cardenolides. We next evaluated whether the strength of inhibition could be specifically predicted by the retention time of the compounds (a proxy of their polarity). We found a significant negative association between retention time of cardenolides and the *r* values of the correlation between the concentration of the compounds and average $IC50_{mass}$ (i.e., more nonpolar compounds have greater negative impacts on the insects) (Table S2). We found similar patterns for individual insects, especially for the seed bug and the monarch, but not for the root beetle, which did not show significant correlations between individual cardenolides and $IC50_{mass}$ (Table S2). Nonetheless, a negative correlation between cardenolide retention time and *r* values of cardenolide concentration-$IC50_{mass}$ correlations was found for all individual insects (Table S2). Expression of many individual cardenolides were themselves correlated (Figure S2). Only voruscharin (RT 20.0 min), present in leaves, flowers and latex, was virtually uncorrelated with other compounds, highlighting its known role in toxicity (Figure S2). Interestingly, seed bug $IC50_{mass}$ was negatively correlated with voruscharin, despite not being present in the seed tissue the insect eats (Table S2).

4 | DISCUSSION

Specialized interactions between plants and their herbivores often show phenotype-matching between key defensive and offensive traits that may have resulted from coevolution (Futuyma & Agrawal, 2009). For example, in the interactions between Pierid butterflies and plants in the Brassicaceae, evolution of novel genes for detoxification in insects were accompanied by duplication and neofunctionalization of defensive glucosinolate genes in host plant species (Edger et al., 2015; Wheat et al., 2007). In another system, insect tolerance to plant defence evolved by cytochrome P450-mediated metabolism of toxic furanocoumarins, and P450 activity matched the defence levels of host populations (Berenbaum & Zangerl, 1998; Zangerl & Berenbaum, 2003). Less is known about the consequences for coevolution in interactions involving a community of insect herbivores that feed on different parts of the same host. Here we found that the community of specialist milkweed herbivores were proportionally matched to tissue specific cardenolide concentrations of their foods. Nonetheless, they differed substantially in their degree of adaptation to toxin composition in the plant tissues they feed on, potentially pointing to organ-specific coevolutionary host-enemy interactions.

4.1 | Cardenolide concentrations strongly vary between plant tissues and increase acropetally

Historically, optimal defence theory (ODT) was used to predict within-plant defence allocation, suggesting it should be proportional to the relative contribution of a tissue to fitness (McKey, 1974; Rhoades & Cates, 1976) and their rate of herbivore damage (Zangerl & Bazzaz, 1992). Previous studies generally found positive associations between tissue value and plant defence (Hunziker et al., 2021; McCall & Fordyce, 2010; van Dam et al., 1996; Zangerl & Rutledge, 1996), with a concomitant reduction in herbivory (Hunziker et al., 2021; van Dam et al., 1996; Zangerl & Rutledge, 1996) under the ODT framework. Our findings support this notion, in part, as cardenolide concentrations increase >10-fold from roots to shoots to seeds, the latter assumed to have higher contribution to fitness (especially since *A. curassavica* is not clonal). However, since we lack a reliable measure of herbivory risk, it is unclear whether these patterns are a consequence of inherent plant investment to protect its most valuable structures, or from evolutionary responses to different herbivore pressures across tissues. Latex was also an outlier under the ODT framework, as it showed the highest cardenolide concentration despite not having a primary function for the plant other than defence. Indeed, the use of tissue value as a proxy of how well defended it should be is subjective (e.g., root damage may be lethal, but floral damage is typically not; McCall & Fordyce, 2010) and, despite the importance of ODT in the origins of plant defence theory, we argue that a more nuanced and coevolutionary view of defence allocation is warranted (see Section 4.5 below).

4.2 | Proportional insect tolerance to cardenolides in their target tissues may not imply best adaptation when compared to nontarget tissues

On the one hand, inherent tolerances of insects to ouabain and latex were proportional to the cardenolide concentration present in the tissues they eat, which indicates defence-offence phenotype matching between this plant and the three herbivores. In addition, the lower average insect tolerance to latex compared to ouabain (28-fold) supports the role of latex as a defensive substance in milkweeds (Agrawal & Konno, 2009). On the other hand, our experimental design allowed us to investigate the extent of which the insects are adapted to their target versus nontarget tissues, and discuss the potential mechanisms behind the patterns. Overall, the milkweed seed bug and the root beetle were more adapted to their target compared to nontarget tissue extracts, whereas the monarch was less adapted to its target tissues (leaves and buds) compared to the tissues it does not eat (roots and seeds) (Figure 4). We offer two nonmutually exclusive interpretations of this apparent mismatch for the monarch. First, our in vitro assay is highly mechanistic, and may miss other traits insects utilize to overcome plant toxicity. For example, behavioral strategies, toxin transport, and metabolic conversion in vivo all contribute to tolerance of cardenolides in monarchs but were not assessed in the current study (Agrawal et al., 2012; Dobler et al., 2015; Groen et al., 2017; Jones et al., 2019). Second, as in any coevolutionary interaction, given both constraints and the current moment in the interaction history, one partner may appear to be better adapted than the other. In a recent study, we have shown that a specific cardenolide present in the leaves and latex of *A. curassavica* (voruscharin) strongly inhibits the monarch's enzyme activity and impairs caterpillar performance (Agrawal et al., 2021). The current results are in line with these findings, since leaf and flower bud extracts (which also contain residual latex) were the only food tissues containing voruscharin (Table S1), and those were more inhibitive for the monarch than other tissues. Furthermore, voruscharin concentration was negatively associated with average insect tolerance across tissues, and was virtually uncorrelated to that of other cardenolides, supporting our previous study (Agrawal et al., 2021). This result could be extended to individual insects (Table S2), especially for the highly tolerant seed bug which does not encounter voruscharin in seed tissue. Therefore, our results point towards differences in the degree of adaptation of specialist insects to the tissues they eat, probably due to evolution of tissue-specific expression of novel, highly toxic compounds in response to milkweed herbivores, especially the monarch.

4.3 | All insects are consistently more adapted to seed cardenolides and less to that of roots

In addition to variable amounts of total defences, individual compounds within the same class of defence vary in their toxicity (i.e., potency) against herbivores (Agrawal et al., 2021; Jones et al., 2019; Klepzig & Schlyter, 1999). Disentangling concentration and potency effects of phytochemicals on insect tolerance, however, is challenging because different chemical compounds within the same class may present multiple functions other than defence (Kessler & Kalske, 2018; Moore et al., 2014), and may have opposing effects depending on the herbivore identity (Rotter et al., 2018). Nonetheless, in coevolved systems in which phytochemicals have known functions and specific physiological targets, such as cardenolides, normalizing by concentration allows assessment of potency (Jones et al., 2019; Petschenka et al., 2018). In our study, all insects, regardless of the number of genetic substitutions in their sodium pumps, consistently showed the highest tolerance to cardenolide potency of seeds and lowest to that of roots overall (Figure 5). Thus, our findings suggest tissue-specific coevolutionary dynamics between milkweed and its herbivores, probably mediated by differences in insect tolerance to toxins, but also by distinct patterns of cardenolide concentration and potency across plant tissues derived from physiological constraints. In this regard, we found that cardenolide concentrations increased from roots to seeds, but their potency increased from seeds to roots, reflecting a tradeoff between concentration and potency of defences across tissues (Figure 6). Tradeoffs between types of defence (Moles et al., 2013; Thaler et al., 1999), between constitutive and induced levels within a defence (Agrawal & Hastings, 2019; López-Goldar, Zas, et al., 2020), or between defences and growth-related traits (Agrawal, 2020; Sampedro et al., 2011) are pervasive in plants. However, the nature of constraints on the simultaneous investment of greater amounts of more potent defences within the same plant, and their implications in the coevolution with herbivores that feed on distinct tissues are largely unexplored.

4.4 | Implications of plant defence tradeoffs and relative insect tolerance to toxins in milkweed-herbivore coevolution

We speculate that tradeoffs between concentration and potency of defences may be related to costs of phytochemicals differing in toxicity across plant tissues in this system, mediated by their effectiveness against herbivores with specific tolerance mechanisms to plant defence. Different costs of individual constituents within a class of defence have been reported (Gershenzon, 1994), suggesting that biosynthesis of more expensive compounds should take place when they increase plant fitness. In contrast to generalist insects, the increasing tolerance to toxins in milkweed insects allows them to cope not only with greater cardenolide concentrations but also their relative potency (Dobler et al., 2011; Petschenka et al., 2018). This may increase costs for the plant when investing in progressively potent cardenolides, especially if they become ineffective against specialist herbivores. Our results indicated that the root cardenolides were the most potent against all insects except the seed bug, which showed similar levels of tolerance to the cardenolide potency of flower buds and leaves (Figure 5). This suggests, on the one hand, that investing in more potent cardenolides is an effective strategy against less

tolerant insects such as the root beetle and monarch, but may be too costly against the highly tolerant seed bug. This is also in line with the predictions of Feeny's Apparency Hypothesis (Feeny, 1976), in which low concentrations of acutely toxic compounds may be effective against insects in the lower end of the tolerance gradient to plant defences, whereas high concentrations of less potent compounds may be more effective against insects at the upper end of the specialist gradient.

Defence trade-offs may also arise from physiological constraints that prevent defences to be homogeneously distributed between different tissues. Mobilization of plant defences between tissues do not occur in the same way for all classes of compounds, and may probably be restricted to more polar (i.e., water-soluble) substances (Gershenzon & Ullah, 2022). This may generate a gradient in cardenolides across milkweed tissues, in particular restricting nonpolar cardenolides from seeds. Our results may suggest the existence of physiological constraints limiting the synthesis or transport of more potent toxins to seed tissue. Unlike more polar cardenolides that are probably synthesized and mobilized through the vascular system connecting milkweed tissues, less polar (i.e., more toxic) cardenolides seem to be restricted to mobilization through latex canals (Seiber et al., 1983), which can be present in most tissues (including fruits) depending on the milkweed species (Agrawal & Konno, 2009), but not specifically in seeds. This could explain the strong differences in toxin concentration between seeds (mostly polar) and other milkweed tissues (Figure S2), suggesting that local biosynthesis in the seeds or transport of nonpolar cardenolides from other tissues is highly unlikely. Altogether, our findings may explain why plant cardenolide concentrations increase acropetally, with a compensatory overaccumulation of less toxic cardenolides against highly insensitive insects (*Oncopeltus*), indirectly mediated by plant physiological limitations, and lower concentrations of more toxic cardenolides against less tolerant insects (*Tetraopes*), potentially as a cost-saving strategy for the plant. Although speculative, this pattern is robust among the tissues that are consumed by specialist herbivores (Figure 6). Nonetheless, the tradeoff vanishes when considering latex, suggesting that either resource costs of the simultaneous investment of greater concentration of more potent defences or physiological constraints differ between edible and defensive plant compartments. In particular, although latex had >10-fold higher concentration of cardenolides than seeds, latex is mobile in the plant, is largely carbon-based, and does not provide a nutritional benefit for insects (Agrawal & Konno, 2009).

It is possible that the patterns of plant defence allocation we observed are influenced by other adaptations of insects to circumvent defence that our in vitro assay did not capture. For instance, milkweed insects often convert and sequester more polar, less potent, cardenolides in their bodies (Agrawal et al., 2021; Jones et al., 2019; Rasmann & Agrawal, 2011) compared to the tissues they eat (Agrawal et al., 2012; Ali et al., 2017; Malcom, 1991). We found that more polar cardenolides are dominant in seeds, which contrasts with the more diverse and less polar composition in other tissues (Figure S1), the latter of which consistently showed greater inhibition against all insects. Structure-affinity relationships between cardenolides and the amino acid residues of the sodium pump seem to be driven by polar interactions of hydrogen bonds with the hydroxyl groups in the steroidal skeleton of cardenolides (Bejcek et al., 2021; Laursen et al., 2013). In other words, more polar compounds seem to have greater affinity to a more polar enzyme. It is possible that increased insensitivity to cardenolides may be driven by reduced polarity in the binding site of enzymes with greater number of amino acid substitutions, especially decreasing the affinity for more polar toxins. Although less polar compounds seems to be more toxic to milkweed insects (e.g., root cardenolides), their negative impact seems to be ameliorated with increased number of genetic substitutions, especially in the seed bug (Figure 5).

We found that the seed bug and the root beetle are adapted to their target tissues, but we hypothesize that the underlying mechanisms for each insect are probably different. The seed bug seems to be one step ahead in the coevolutionary arms race with milkweeds due to its highly insensitive enzyme, enhancing tolerance to high concentrations of relatively less potent (i.e., more polar) cardenolides that can be easily sequestered, and reducing the negative impacts of more toxic (i.e., less polar) cardenolides. The seed bug's gene duplications (Dalla & Dobler, 2016) with further heterogeneous expression of the enzyme in the insect's body (Lohr et al., 2017) may have significantly contributed to its evolutionary advantage over milkweed defence. First, these evolutionary innovations most probably occurred to minimize negative pleiotropic effects in the seed bug by duplication and neofunctionalization of the enzyme (copies A, B and C) (Dalla & Dobler, 2016). Copies A and B (four and three substitutions, respectively) confer high insensitivity to cardenolides and significantly reduced enzymatic activity compared to the copy C (two substitutions), which is more sensitive to cardenolides, but functionally more efficient (Dalla & Dobler, 2016; Lohr et al., 2017). Whereas copy A and B are mostly expressed in the Malphigian tubules for cardenolide handling and excretion, copy C is mostly expressed in nervous tissue (i.e., brain), indirectly allowing for cardenolide ingestion and sequestration while maintaining sodium pump efficiency under challenging cardenolide environments (Lohr et al., 2017). The root beetle also seems well-adapted to its target tissues, despite being the most impaired by cardenolides, probably because the highly potent cardenolides in root tissues are in concentrations below toxicity thresholds (Figure 3). In contrast, the monarch seems to be trailing in the arms race with milkweeds, probably because of substantial amounts of moderately potent cardenolides and the specific expression of highly potent toxins such as voruscharin in the tissues it feeds on (Agrawal et al., 2021). Alternatively, because monarchs feed on multiple milkweed species which differ in both concentration and potency of leaf cardenolides, it is plausible that counter-adaptation of the insect may be constrained (Rasmann & Agrawal, 2011; Seiber et al., 1983). Evaluation of complementary adaptive strategies in vivo would certainly add insightful information to the patterns we observed.

4.5 | Synthesis and speculation

In specialized coevolutionary interactions where multiple enemies feed on the same plant, niche specialization originates from divergence in where and how each herbivore feeds (Futuyma & Moreno, 1988). Therefore, the heterogeneous structure of the herbivore community is expected to impact the evolution of host's defence allocation differently between tissues. For example, milkweed leaves and flower buds present large amounts of toxic latex which is absent in seeds and roots; this pattern may be driven by past selection by distinct herbivores or constraints on deployment of latex. Similarly, the evolution of novel toxic compounds with specific expression in leaves, flowers and latex, such as voruscharin, may have arisen in response to monarch feeding on those tissues. Second, specialization should be viewed as a continuum rather than discrete, especially when addressing coevolutionary hypotheses. For example, milkweed herbivores are all highly specialized in comparison to generalist herbivores but, in spite of that, they show high variability in tolerance, transport, conversion and sequestration of cardenolides (Agrawal et al., 2012). In addition to their specialized preference for distinct compartments of the plant, that variability in handling plant defences combined with plant physiological limitations in the expression and transport of certain defences may exacerbate the divergence in defence allocation across plant tissues – not only in concentration, but also in their potency. Third, current patterns of attack may be highly impacted by coevolutionary history and may not reflect the intensity of recent selection. Thus, predicting defence allocation is not only dependent on the current fitness impact of herbivores, but also on the level of herbivore specialization, coevolutionary history, and constraints.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Xosé López-Goldar and Anurag Agrawal conceived and designed the research. Xosé López-Goldar performed the experiment. Xosé López-Goldar performed the chemical analysis, enzyme inhibition assays, and analysed and interpreted the data with the help of Amy Hastings, Tobias Züst and Anurag Agrawal. Xosé López-Goldar wrote the manuscript with input from Anurag Agrawal, Tobias Züst and Amy Hastings. All authors approved the final version for publication.

DATA AVAILABILITY STATEMENT

The data supporting the results have been archived in the Zenodo Digital Repository (https://doi.org/10.5281/zenodo.6323713) (López-Goldar et al., 2022).

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