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# Tissue-specific plant toxins and adaptation in a specialist root herbivore

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**In coevolution between plants and insects, reciprocal selection often leads to phenotype matching between chemical defense and herbivore offense. Nonetheless, it is not well understood whether distinct plant parts are differentially defended and how herbivores adapted to those parts cope with tissue-specific defense. Milkweed plants produce a diversity of cardenolide toxins and specialist herbivores have substitutions in their target enzyme ($Na^+/K^+$–ATPase), each playing a central role in milkweed–insect coevolution. The four-eyed milkweed beetle (*Tetraopes tetrophthalmus*) is an abundant toxin-sequestering herbivore that feeds exclusively on milkweed roots as larvae and less so on milkweed leaves as adults. Accordingly, we tested the tolerance of this beetle's $Na^+/K^+$–ATPase to cardenolide extracts from roots versus leaves of its main host (*Asclepias syriaca*), along with sequestered cardenolides from beetle tissues. We additionally purified and tested the inhibitory activity of dominant cardenolides from roots (syrioside) and leaves (glycosylated aspecioside). *Tetraopes'* enzyme was threefold more tolerant of root extracts and syrioside than leaf cardenolides. Nonetheless, beetle-sequestered cardenolides were more potent than those in roots, suggesting selective uptake or dependence on compartmentalization of toxins away from the beetle's enzymatic target. Because *Tetraopes* has two functionally validated amino acid substitutions in its $Na^+/K^+$–ATPase compared to the ancestral form in other insects, we compared its cardenolide tolerance to that of wild-type *Drosophila* and CRISPR-edited *Drosophila* with *Tetraopes'* $Na^+/K^+$–ATPase genotype. Those two amino acid substitutions accounted for >50% of *Tetraopes'* enhanced enzymatic tolerance of cardenolides. Thus, milkweed's tissue-specific expression of root toxins is matched by physiological adaptations in its specialist root herbivore.**

cardiac glycoside toxin | chemical ecology | coevolution | sequestration | root herbivory

## Significance

A plant in nature is often attacked by a multitude of insect pests. How does a plant coordinate defending itself, especially if those insects attack different parts of the plant? We show that milkweed plants invest in different chemical toxins in their roots and leaves. A major root pest, the four-eyed milkweed beetle, is physiologically better adapted to toxins in roots than leaves. Furthermore, we attribute >50% of the beetle's enzymatic adaptation to two amino acid substitutions in its sodium–potassium–ATPase. These substitutions, in addition to unidentified adaptations, help the beetle tolerate the root toxin (syrioside). As coevolution proceeds, not only do insects adapt and specialize, but individual plant parts appear to be evolving in response to distinct pests.

Coevolution, reciprocal adaptation in interacting species, appears to be a prominent part of trophic relationships and has been studied using a variety of approaches (1). The matching of phenotypes, for example, defensive traits of hosts and tolerance mechanisms in parasites, is often considered a signature of coevolution (2, 3), although care must certainly be taken to avoid overinterpreting such patterns (4). Advances in our understanding of coevolution over the past 20 y have come from studying phenotype matching among replicate populations of diverse species thought to be under distinct ecological conditions or at different stages of coevolutionary escalation (5–8). This population-level approach has recently been complemented by more mechanistic experiments mixing and matching host and enemy traits in experimental challenges (9–12).

Because most plants have specialized herbivores that feed on distinct plant parts (e.g., roots, leaves, or seeds), it may well be that somewhat independent coevolutionary interactions are possible between a plant and multiple insects (13). In other words, plants may show tissue-specific expression of defenses and these may be countered in a unique way by herbivores. In particular, many classically studied plant–herbivore interactions involve distinct herbivores above and below ground and show tissue-specific expression of chemical defense (e.g., refs. 14 and 15). For example, across tens of species in the Brassicaceae, roots are characterized by a different composition of, higher diversity of, and an average of 4.5-fold higher total concentration of glucosinolates than leaves (16). While much research has delved into the insect counteradaptations to glucosinolates (12) (including isothiocyanate-specific hydrolytic mechanisms used by root flies) (17), in no system to date has the match been made between specific plant defenses expressed in different plant parts and the interactions with different herbivore species adapted to those parts.

We have been studying the diversity of cardenolide toxins produced in milkweed plants (often >20 distinct chemical structures per species) (18) and the impact on their highly specific target, the universal transmembrane animal enzyme, $Na^+/K^+$–ATPase (19). Although leaf and root cardenolides show correlated evolution across milkweed species, plants tend to invest in higher concentrations of a few cardenolides in roots compared to a higher diversity of lower concentration compounds in leaves (20). Because the genetic and physiological

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basis of cardenolide tolerance in the insects' $Na^+/K^+$–ATPases is well studied (21–23), challenging different insects' $Na^+/K^+$–ATPases with distinct cardenolides can provide insight into coevolutionary interactions (10, 19, 24, 25). Among the herbivores of cardenolide-containing plants, insect specialists feed on roots, leaves, seeds, phloem sap, and even pith tissue (26). Genetic substitutions at amino acid positions 111, 119, and especially 122 of the alpha subunit of the $Na^+/K^+$–ATPase have been shown to play the most important role in physiological tolerance (i.e., target-site insensitivity) and survival of these insects (22, 27–29). Here, we focus on the root-feeding four-eyed milkweed beetle *Tetraopes tetrophthalmus* (Cerambycidae) (Fig. 1), which is a sequestering specialist with a modestly tolerant $Na^+/K^+$–ATPase (substitutions only at positions 111 and 119), in comparison to insects with the additional substitution at 122 (e.g., the monarch butterfly) (24, 30–32). We refer to *Tetraopes* as "LSN," meaning that it possesses a single copy of the $Na^+/K^+$–ATPase with a leucine (L) at position 111 compared to the ancestral glutamine (Q), serine (S) at position 119 compared to the ancestral alanine (A), and the ancestral asparagine (N) at position 122 (29). CRISPR-engineered fruit flies with this "LSN" genotype are 4.6× more tolerant to the standard cardenolide ouabain compared to wild type, while the increase in tolerance for the monarch genotype ("VSH" at the same amino acid positions, respectively) is over 350× (17).

*Tetraopes* feeds primarily on roots of the common milkweed *Asclepias syriaca* as larvae and, to a lesser extent, on leaves and flower buds as adults (33, 34). Common milkweed hosts at least 12 species of specialized herbivorous insects and has been well-studied for its interactions with several species of aphids and beetles, as well as the monarch butterfly *Danaus plexippus*, most of which sequester cardenolides from the host plant (26, 34, 35). Little is known about the mechanisms of sequestration or the location of stored cardenolides in *Tetraopes*, but beetles typically upconcentrate cardenolides at the level of whole insects (18, 30). Although other milkweed specialist herbivores detoxify highly potent cardenolides and sequester less potent compounds (10, 25), it is unclear whether *Tetraopes* does the same. We hypothesized that elytra (the hardened protective wing coverings) may be a safer place to store potent toxins than body tissue, while also providing a first line of defense against predators. In particular, *Tetraopes*' elytra are distant and compartmentalized from organs that may be sensitive to cardenolides and are positioned away from the rest of the body during flight when aerial predators may attack. Accordingly, here we asked the following specific questions:

1) How well is Tetraopes' $Na^+/K^+$–ATPase adapted to the composition of cardenolides in A. syriaca roots versus leaves and those sequestered in Tetraopes' adult bodies and elytra, compared to a sensitive insect's $Na^+/K^+$–ATPase (Drosophila melanogaster)? We expected that root cardenolides would either be of high or low potency to the beetle $Na^+/K^+$–ATPase (i.e., well defended or highly susceptible), because of the reciprocal and escalating nature of coevolution. We also predicted that Tetraopes'-adapted $Na^+/K^+$–ATPase would be least inhibited by sequestered body cardenolides, as these are the closest to internal organs and potentially stored only after detoxification. Finally, we expected less variation in the inhibition of the highly sensitive D. melanogaster $Na^+/K^+$–ATPase, which is typically inhibited by any cardenolide.
2) Can the effectiveness of root and leaf defenses against Tetraopes $Na^+/K^+$–ATPase be attributed to specific cardenolides? To address this, we isolated and tested two dominant cardenolides expressed in roots (syrioside A) or leaves (glycosylated aspecioside) as well as an unusual nitrogen-containing minor

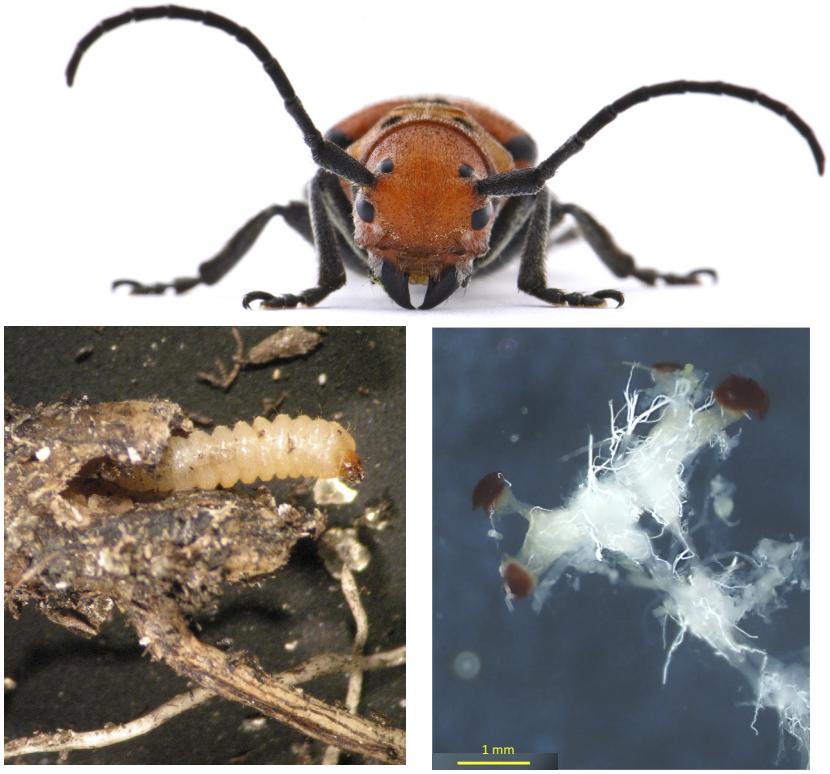


**Fig. 1.** The four-eyed milkweed beetle, *T. tetrophthalmus*. Shown are adult (*Top*), larva in an *A. syriaca* root (*Bottom Left*), and brain (*Bottom Right*; 10× ZeissAXIOZoom. V20). In the brain image, the reddish lobes are retinal tissue (four because there are four functioning eyes) and the whitish tissue includes the entire brain (optic lobes, mushroom bodies, esophageal ganglia) and the thoracic ganglia in the lower right. Some bright white tracheal tissue is present. Photo credits: Ellen Woods (adult), Sergio Rasmann (larva), and Richard Fandino (brain).

cardenolide (labriformin) that is present in both A. syriaca roots and leaves.

3) Can the results for the above questions be explained by two amino acid substitutions (LS at positions 111 and 119) in Tetraopes' $Na^+/K^+$–ATPase? To address this question, we compared the $Na^+/K^+$–ATPase inhibition by cardenolides in a species with a sensitive $Na^+/K^+$–ATPase (unengineered D. melanogaster) to one with those two amino acid changes introduced experimentally (CRISPR-edited D. melanogaster).

## Results

On a dry mass basis, extracts of *A. syriaca* leaves and roots had similar concentrations of cardenolides, and *Tetraopes* bodies and elytra had about twice that concentration (Fig. 2). Of the 28 high-performance liquid chromatography (HPLC) cardenolide peaks found across the four tissues, here, we focus on eight compounds that were each at least 5% of the total for any of the tissues. These eight compounds capture 89 to 98% of the total cardenolides in each tissue (see raw data files in *SI Appendix*). As a means to focus attention on the matching between specific cardenolide composition in different plant tissues and beetle adaptations, our next analyses focus on the inhibition of $Na^+/K^+$–ATPase while controlling for differences in cardenolide concentration between tissues.

Among the four extracts tested, *Tetraopes*' $Na^+/K^+$–ATPase was least affected by root cardenolides and required more than three times the concentration of leaf cardenolides to inhibit its $Na^+/K^+$–ATPase (all statistical comparisons provided in Fig. 3). Sequestered beetle cardenolides were similarly potent to leaves, with elytra cardenolides being slightly less potent than body extracts. As expected, we found lower variation in the potency of cardenolide extracts to the sensitive *Drosophila* enzyme (less than twofold variation) (Fig. 3).

We next isolated the major cardenolides in roots (syrioside A, 46% of total root cardenolides) and leaves (glycosylated aspecioside, 87% of total leaf cardenolides) and a shared minor component that is known to be highly potent and is unusual in containing nitrogen (labriformin, 5.4% of roots and 0.3% of leaves). *Tetraopes*' tolerance of root and leaf cardenolides mirrored that of plant tissue extracts: The beetle enzyme was three times more tolerant of syrioside A than glycosylated aspecioside (Fig. 3). Labriformin, which is known to be among the most potent cardenolides against the monarch butterfly enzyme, was instead only slightly more potent than glycosylated aspecioside against *Tetraopes*. There was again less variation in tolerance of the sensitive enzyme to the three compounds tested.

Overall, across all tissue extracts, *Tetraopes*' LSN enzyme was sixfold more tolerant to cardenolides than the wild-type *Drosophila's* QAN enzyme (Fig. 4). Nonetheless, LSN *Drosophila* was only fourfold more tolerant than wild types. This pattern was similar for *Tetraopes*' tolerance to purified cardenolides, where it was nearly 10-fold more tolerant than wild type, whereas LSN flies were fivefold more tolerant than wild-type flies. Across all enzymes, purified compounds were fivefold less potent than tissue extracts.

## Discussion

Studies of the mechanistic basis of interaction traits and their phenotypic (often physiological) expression have recently yielded insight into ongoing coevolution. For example, we recently showed that some milkweeds produce unusual cardenolides in leaves and seeds that are highly potent against the most adapted specialist sequestering herbivores, although they have mediocre

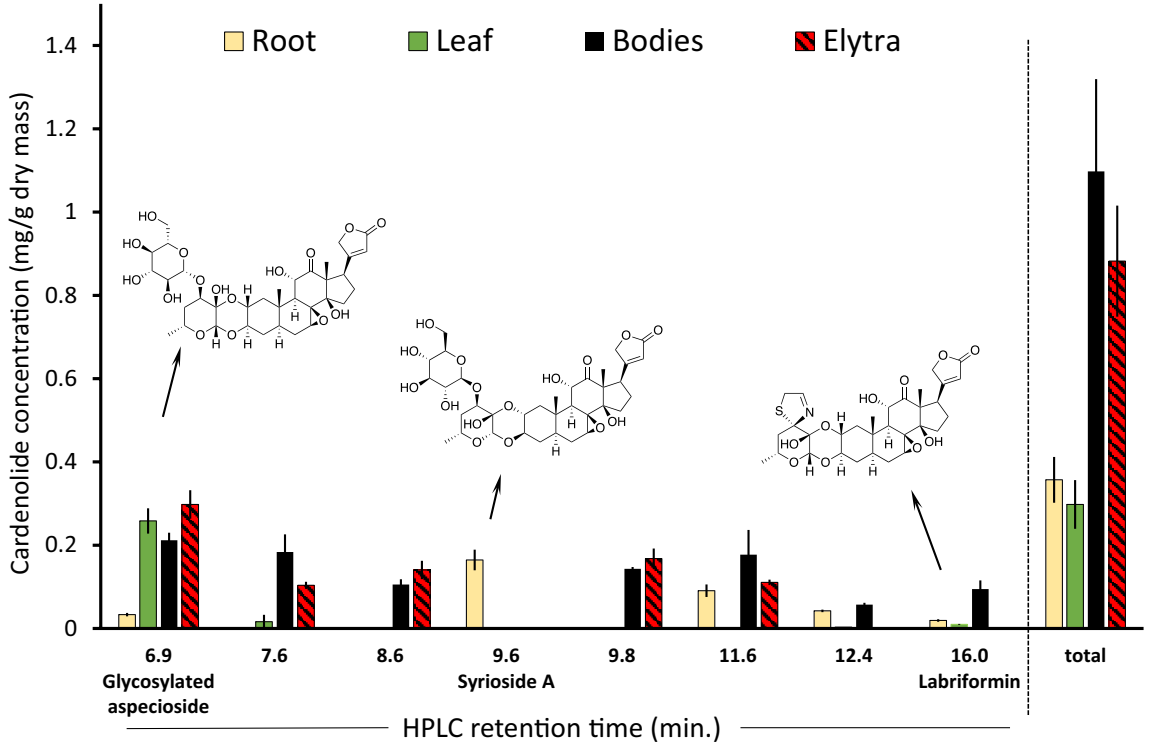


**Fig. 2.** Cardenolide concentrations of the eight major peaks in *A. syriaca* (roots and leaves) and *Tetraopes* (adult bodies and elytra). Numerical labels indicate the HPLC retention time of compounds. Note that glycosylated aspecioside is the dominant compound in leaves and syrioside A occurred exclusively in roots. Labriformin was found in all tissues except elytra. Shown are means ±SEs of three replicates.

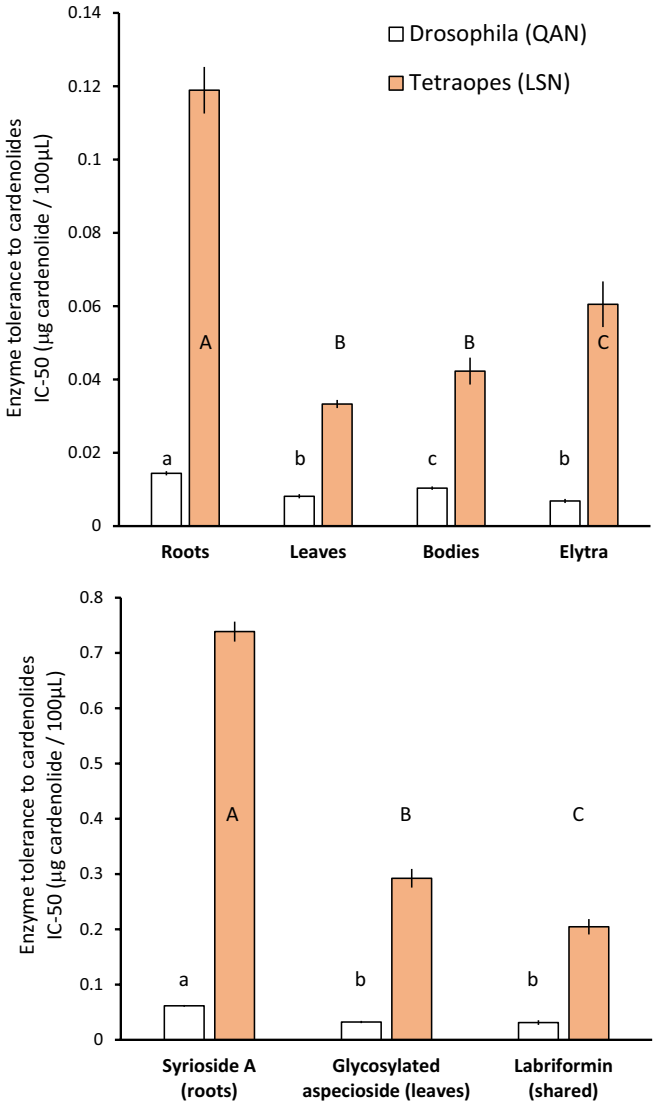


**Fig. 3.** Inhibition of the sensitive *Drosophila* $Na^+/K^+$–ATPase (QAN at amino acid positions 111, 119, and 122, respectively) and that of the *T. tetrophthalmus* (LSN) by cardenolides from crude extracts of *A. syriaca* or adult *Tetraopes* beetles (*Top*) and purified compounds (*Bottom*). Shown on the *Y* axis is the concentration of cardenolides in the tissue extract needed to inhibit the animal enzyme by 50%, or IC-50. Higher values indicate that the enzyme is more tolerant to the cardenolide. Shown are means ±SEs across at least three replicates (each based on a 5-point concentration inhibition curve). Different lowercase letters indicate significant differences (Student's *t* test, $P < 0.05$) between bars for the QAN enzyme; different uppercase letters indicate significant differences between bars for the LSN enzyme.

potency against sensitive insects who have not evolved in response to cardenolides (10, 25). Conversely, leaf- and seed-feeding specialists do not sequester these compounds, but rather chemically alter them to reduce toxicity to themselves. We have interpreted this result as plants producing a range of defenses, some of which are less effective and have been coopted by their herbivores, while others have remained quite toxic, imposing selection for detoxification in those same insect feeders. Here, we have shown that root-specific expression of defense compounds is matched by specific tolerance to those compounds in a major root herbivore. Thus, the complexity of coevolution in this, and likely other, systems may extend to plant parts coevolving with different guilds of herbivores.

In the current study, we used the in vitro tolerance of a beetle's $Na^+/K^+$–ATPase to toxins from leaf versus root extracts and tissue-specific compounds to address the defense–offense match. The concentrations of cardenolides were nearly equal in the roots and leaves of common milkweed, yet the composition was distinct (Fig. 2). *Tetraopes* was found to be three-times more tolerant of root cardenolides than leaf cardenolides; nonetheless, the sensitive control enzyme was only 50% more tolerant of root compared to leaf cardenolides (Fig. 2). Thus, on a proportional basis, *Tetraopes* was eight-times more adapted to roots compared to a sensitive insect enzyme, but only four-times more adapted to leaf cardenolides compared to the sensitive enzyme. Finally, these results were mirrored in the respective enzyme tolerances to the purified dominant cardenolides from roots and leaves (Fig. 3). We interpret these results to indicate that *Tetraopes* is primarily adapted to the toxins in its larval food, milkweed roots.

Among cardenolide-adapted insects, most show genetic substitutions in the $Na^+/K^+$–ATPase corresponding to amino acid changes at positions 111, 119, and 122 (22). On average, substitutions at position 111 provide ≈10-fold tolerance to purified cardenolides, and the later evolving substitution at position 122 provides ≈100-fold tolerance (22, 23, 36); the substitution at position 119 is primarily an epistatic modifier, but is functionally unimportant in cardenolide binding to the $Na^+/K^+$–ATPase (37). Indeed, insects in six taxonomic orders have independently evolved substitutions at these positions, yielding highly tolerant, sequestering, and aposematic species (19). Perhaps more surprisingly, many species (across five taxonomic orders, including *Tetraopes*) only have substitutions at positions 111 and 119, but are nonetheless sequestering and aposematic. In the current study, transgenic LSN flies were less tolerant of cardenolides than *Tetraopes*' native LSN $Na^+/K^+$–ATPase. The comparison of transgenic LSN *Drosophila* to the wild-type amounted to ≈50% of the beetle's tolerance advantage to cardenolides compared to wild-type *Drosophila* (Fig. 4). Accordingly, there are clearly other things going on with the beetle's enzyme itself, beyond LSN, as well as other non-$Na^+/K^+$–ATPase adaptations (e.g., transport, compartmentalization, detoxification, and excretion). In particular, the fact that the genetically engineered enzyme was significantly less tolerant to cardenolides than that of *Tetraopes* indicates that further work is needed to understand 1) the impact of dipteran genetic background effects (38) or 2) other potential sequence changes in *Tetraope*s that may impact cardenolide binding affinity. While known functional $Na^+/K^+$–ATPase substitutions at positions 786, 787, and 797 can been ruled out in *Tetraopes,* this species may possess other subtle changes in the $Na^+/K^+$–ATPase (or epistatic effects), making it more resistant than LSN alone would predict. Amino acid site 315 with an exchange from I to V is one possibility (29). An initial comparison of the *Tetraopes* $Na^+/K^+$–ATPase to that of the cardenolide-sensitive cerambycid *Anoplophora glabripennis,* available on GenBank, also reveals several substitutions that may or may not be functional.

Despite the fact that *Tetraopes*' $Na^+/K^+$–ATPase is only modestly tolerant to cardenolides (about 10-fold less tolerant than monarchs to the standard cardenolide, ouabain) (24), this beetle is a strong sequesterer (equal or greater concentration to monarchs on a dry mass basis when on the same host plant). Additionally, although the monarch is known to detoxify some of the most potent cardenolides before storage (10, 39), the same does not appear to be the case for *Tetraopes*. Although we certainly found sequestered cardenolides in *Tetraopes* that were undetected in leaves and roots (e.g., compounds 8.6 and 9.8 in Fig. 2), suggesting compound modification, sequestered cardenolides were not less toxic to the beetle than plant cardenolides. In fact, cardenolides in *Tetraopes*' bodies (which may be derived from roots and leaves) were equally potent to leaves, while cardenolides in elytra (which are derived exclusively from root

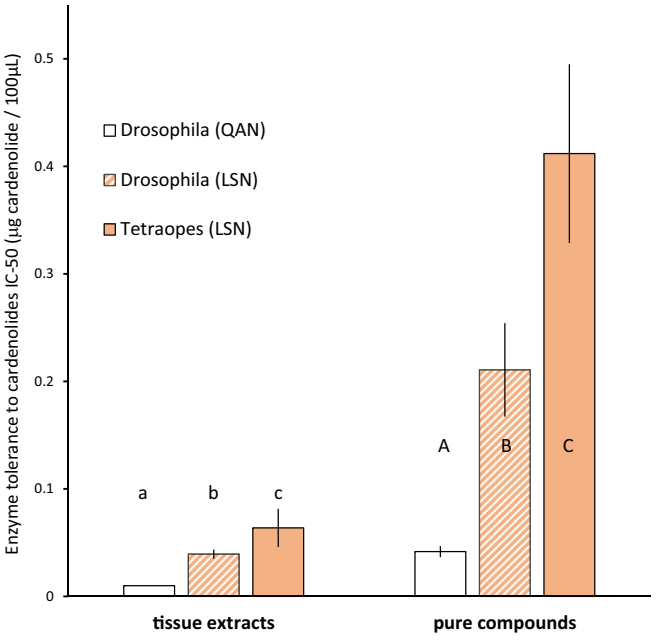


**Fig. 4.** The difference in inhibition of the sensitive *Drosophila* $Na^+/K^+$–ATPase (unengineered, QAN at amino acid positions 111, 119, and 122, respectively), the CRISPR-engineered *Drosophila* enzyme (LSN), and that of the native *Tetraopes* (LSN). Shown on the *y* axis is the concentration of cardenolides necessary to inhibit the animal enzyme by 50%, or IC-50. Higher values indicate that the enzyme is more tolerant to the cardenolide. Shown are means ± SEs across four tissue types and three purified compounds. Different lowercase letters indicate significant differences (Student's *t* test, $P < 0.05$) between bars for tissue extracts; different uppercase letters indicate significant differences between bars for purified cardenolides.

feeding) were 1.8-fold more potent than roots. These relative toxicities were consistent for both *Tetraopes*'-adapted enzyme and that of sensitive *Drosophila,* the latter likely being similarly sensitive to the $Na^+/K^+$–ATPase of many generalist predators of insects. Thus, if anything, *Tetraopes* is selectively storing and modifying plant cardenolides into more potent compounds on the external, most expendable part of its body, the elytra, than is present in its food. This result in concordant with Brower et al.'s hypothesis for monarch butterflies (40), suggesting that more potent cardenolides may be sequestered from plant tissues as means to elicit taste rejection by would-be predators. Finally, we found the highly potent labriformin (a compound typically not sequestered in other adapted milkweed insects) (25) in its body (although not elytra). Taking these results together, we speculate that beetles rely on the use of adaptations apart from the $Na^+/K^+$–ATPase to avoid toxicity. Indeed, the use of transporters and compartmentalization has been shown for other milkweed insects, but not yet studied in *Tetraopes* (41–45).

## Natural Selection and Adaptation

Within and between populations, differences in plant attack by various guilds of herbivores may shape tissue-specific expression of defense traits. Although *Tetraopes* can be highly abundant, and is known to reduce the growth and performance of common milkweed (34), we have not specifically shown selection for plant defense by this species. Nonetheless, root damage is clearly a more important selective agent for this long-lived perennial plant than aboveground herbivory by *Tetraopes* (34, 46). In addition, recent work in our laboratory has shown that even spatially close populations of *A. syriaca* have divergent root cardenolides, more so than would be predicted by neutral genetic differentiation (47). Thus, we speculate that there may be differences in natural selection by root-feeding beetles. The specific drivers of expression of syrioside A in roots versus glycosylated aspecioside in leaves are unknown, and could also have to do with physiological constraints (e.g., there is no latex in the roots). Overall, it appears that cardenolide production in different plant parts can be distinct, relatively uncorrelated, and may be evolving independently (47).

In another case of arms race coevolution, that between newts which produce the highly poisonous tetrodotoxin (TTX) and garter snakes which have genetic substitutions in their voltage-gated sodium channels (NaV), population variation has been used to decipher the interaction history (48). Remarkably, four substitutions in NaV provide increasing levels of tolerance to TTX, and populations with higher frequencies of substitutions show greater tolerance to TTX overall (49). Population variation in the number and frequency of these genetic substitutions is hypothesized to be driven by the intensity of exposure to TTX. Additionally, the costs of these substitutions, variation in the specific profiles of toxins in prey, benefits of sequestration, and the presence of alternative food items may all contribute to variation in the level of NaV adaptation (9, 50). In our system, although most *T. tetrophthalmus* populations feed on *A. syriaca*, host-shifting populations have been observed, which could generate similarly variable selection among populations of this herbivore (51). Across *Asclepias* spp., we find that milkweed species with an associated specialized root herbivore (*Tetraopes* sp.) have higher concentrations of root cardenolides than those of their close relatives that do not experience such root herbivory (52). Accordingly, there may be population-level variation in herbivory and defense that scales up to variation among species.

## Conclusion

Many coevolutionary interactions are not strictly pairwise. This is decidedly the case for milkweed–herbivore interactions where a single plant species is attacked by >10 specialist herbivores, with feeding niches spanning essentially all plant tissues. Our evidence that cardenolide toxins have evolved by natural selection against these specialists is manifold. Most recently, the finding that some cardenolides are unusual in their structure (with nitrogen, and likely costly for the plant), not sequestered by insects, and highly potent against adapted $Na^+/K^+$–ATPases (but not against more generalized herbivores) is certainly suggestive. Conversely, the specialist milkweed herbivores have somewhat distinct adaptations to cope with milkweed's defenses. The current work underscores a root herbivore's strong adaptation to the dominant toxin in roots, largely although not completely attributable to two amino acid changes in its $Na^+/K^+$–ATPase. That such dynamics can be compartmentalized within different plant organs suggests that somewhat independent coevolutionary interactions are possible between a plant and its community of insect pests.

## Materials and Methods

**Plant and Insect Material.** Roots, leaves, and beetles were collected from a fallow field in Dryden, NY (42.455208, −76.396388). We collected and pooled leaves and roots of *A. syriaca* from at least five individuals at least 5 m apart from each other. Hundreds of beetles were pooled. All organisms were stored at −80 °C. Plant tissues and beetle bodies and elytra were freeze-dried before extraction (Labconco Corp.), while beetle brains were dissected on ice for use in enzymatic assays (see below). *Drosophila* stocks (wild-type W1118 and CRISPR knock-in LSN strain) were obtained from Noah Whiteman (University of California Berkeley). The flies were maintained at Cornell University in plastic vials on S food (https://cornellfly.wordpress.com/s-food/) and kept at 22 to 25 °C on a benchtop under the natural daylight cycle. The lines are available from the Bloomington Stock Center (86512w[*]; Atpalpha[LSN]).

**Preparation of Tissue Extracts.** One pooled sample each of freeze-dried milkweed roots, leaves, beetle bodies (not including heads), and elytra was prepared for HPLC and $Na^+/K^+$–ATPase inhibition assays by extracting 100 mg tissue with 1 mL methanol and spiked with 30 μg hydrocortisone as a noninhibitive internal standard, using a Fast Prep homogenizer and zirconia/silica beads (MP Biomedicals), as in ref. 53. Cleared supernatant was removed and the extraction was repeated with 1 mL methanol. The supernatants were pooled, taken to dryness in a rotary evaporator (Labconco Corp.), and brought up in 0.25 mL methanol by sonicating for 30 s and shaking at 1,000 rpm for 10 min. The extracts were defatted by adding 0.75 mL hexanes, vortexing three times for 30 s, shaking at 1,500 rpm for 10 min, and removing the upper layer following a 15 min centrifugation at 13,200 rpm. This defatting process was repeated for a total of three times, before extracts were again taken to dryness. Final residues were resuspended in 0.3 mL methanol, sonicated and shaken as above, and filtered through a 0.2-μm nylon membrane syringe filter (Millex, Merck KGaA). 0.2 mL of each extract was removed to a separate tube to dry down for sodium pump assays, while the remainder was analyzed for cardenolide concentration via HPLC. Two additional HPLC replicates were generated using this same protocol except that we started with 50 mg of tissue (except for elytra, 21.5 mg), with final residues brought up in 0.2 mL methanol, and none of the extract was removed for sodium pump assays.

**HPLC and Purified Cardenolides.** Samples were analyzed, as in ref. 53, on an Agilent 1100 HPLC (Agilent) using a Gemini C18 reversed-phase column (3 μm, 150 × 4.6 mm, Phenomenex) and a constant flow of 0.7 mL/min of the following gradient of acetonitrile and Millipore-filtered water: 0 to 2 min at 16% acetonitrile; 2 to 25 min from 16 to 70% acetonitrile; 25 to 30 min from 70 to 95% acetonitrile; 30 to 35 min at 95% acetonitrile; followed by 10 min reconditioning at 16% acetonitrile. Ultraviolet (UV) spectra of peaks from 200 to 400 nm were recorded, and cardenolides were identified as peaks with a single maximum of UV absorption between 214 and 222 nm. These peaks were quantified using the peak area of the internal standard hydrocortisone (converted to the equivalent peak area of the standard cardenolide digitoxin) and standardized by dry mass to give a concentration of each cardenolide (and total cardenolide) in units of μg per mg dry tissue.

The cardenolides syrioside A, glycosylated aspecioside, and labriformin were purified from a bulk methanolic extract of *A. syriaca* seeds collected from Dryden NY (42.473120, −76.322413), using vacuum filtration and standard phase separation techniques coupled with separation and fraction collection using an Agilent 1260 Preparatory HPLC (10). Isolated compounds were >90% pure based on NMR spectroscopy. Briefly, extracts were defatted and depigmented, and extracted cardenolides were collected by injection into an Agilent 21.2 mm × 150 mm, C-18, 5 μm column. Compounds were identified by their exact parental mass ($[M+H]^+$), as well as the corresponding sodium adduct ($[M+Na]^+$) on a Thermo Scientific Q-Exactive Hybrid Quadrupole-Orbitrap Liquid chromatography–mass spectrometry (LC-MS) system in positive ionization mode (10). Prior to use in $Na^+/K^+$–ATPase inhibition assays, purified cardenolide solutions were prepared in methanol (except acetonitrile for labriformin) and quantified using HPLC-UV with an external calibration curve of digitoxin.

**Preparation of Neural $Na^+/K^+$–ATPases.** Frozen beetle heads were thawed on ice and the brains were dissected in ice-cold Millipore water using a dissection microscope. The brains were pooled in batches of 10 to 30 brains, homogenized in 0.5 mL Millipore water using an all-glass grinder (Wheaton Industries), and aliquoted into tubes for freeze-drying. For each batch, a small portion of homogenized brain tissue (2 to 3 brains) was freeze-dried and set aside for pilot assays to determine the appropriate concentration to use in assays. Fly heads were removed by snap-freezing flies in liquid nitrogen in a centrifuge tube, shaking frozen flies vigorously three times for 10 s, and then separating fly heads from bodies using a prechilled 710 μm mesh sieve and collection pan. Batches of 100 to 200 fly heads were homogenized as above and then aliquoted into tubes for freeze-drying. Just prior to running assays, freeze-dried homogenates of 10 beetle brains (0.5 to 1 mL) or 12.5 fly heads (0.45 mL) were brought up in ice-cold Millipore water, using a sonicating ice bath.

**Preparation of Whole-Tissue and Single-Compound Dilution Curves.** The dried-down portion of each of the 100 mg tissue extracts (one each for milkweed roots, leaves, *Tetraopes* bodies, *Tetraopes* elytra) was brought up in the appropriate volume of 20% dimethyl sulfoxide (DMSO) to standardize the concentration of total cardenolide at approximately 0.05 μg/μL extract (based on preliminary HPLC quantification), using repeated sonication. Each extract was then diluted serially with 20% DMSO to create the following five dilutions: 1, 1/7, 1/49, 1/343, and 1/2,401. These dilutions were chosen in a pilot assay to maximize resolution of the inhibition curve. Purified cardenolide compounds were also brought up in 20% DMSO, to a concentration of $5 \times 10^{-4M}$, and then subjected to 10-fold serial dilution to create the following five solutions: $5 \times 10^{-4M}$, $5 \times 10^{-5M}$, $5 \times 10^{-6M}$, $5 \times 10^{-7M}$, and $5 \times 10^{-8M}$.

**Sodium Pump Inhibition Analyses.** Assays were run to test the purified compounds (plates 1 to 2) and whole-tissue extracts (plates 3 to 7) above for the extent of inhibition of the unengineered wild-type fly (QAN), CRISPR-edited fly (LSN), and *Tetraopes* (LSN) $Na^+/K^+$–ATPase using the methods outlined in ref. 41. For the pure compound plates, wells with 20% DMSO alone were also included for determination of control (full activity) and background (fully inhibited) activity levels. For tissue extracts, a background well was included for every dilution. A master-mix for the reaction (with potassium) and background (without potassium) solutions was made up on ice, with the ATP and appropriate enzyme preparation added. This solution was added to the inhibitor solution in each well using a multichannel pipette just prior to mixing and incubation. Following 20 min incubation, reactions were stopped by the addition of sodium dodecyl sulfate (SDS) and then reactions were stained with Taussky-Shorr (TS) reagent for phosphate quantification using spectrophotometric reading of absorbance at 700 nm (BioTek, Agilent). This dataset includes three technical replicates for each purified cardenolide or whole-tissue extract and $Na^+/K^+$–ATPase combination.

We fit the reaction minus background absorbance data with a four-parameter nlme logistic model (RBG ~ SSfpl(log.conc, A, B, $x_{mid}$, scal) using R v4.2.2 and extracted $e^{xmid}$ as the concentration required for 50% inhibition of the insect $Na^+/K^+$–ATPase (IC-50, as in ref. 53). We statistically compared IC-50 values using ANOVA and Student's *t* test pair-wise comparisons (JMP Pro ver. 14) with fixed main effects of insect enzyme and tissue extract (or isolated compound).

**Data, Materials, and Software Availability.** All study data are included in the article and/or supporting information.

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