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Divergent dietary and defensive adaptations of closely related coexisting seed bugs

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Ecologists use multiple approaches to decipher factors mediating the origin and maintenance of biodiversity. Using closely related species, we retrospectively investigated divergence in diet and defense among seed bugs that co-occur at small spatial scales. The small milkweed bug (*Lygaeus kalmii*) primarily feeds on milkweeds (Apocynaceae) and sequesters toxic cardenolides as an antipredator defense, both traits representing the ancestral condition of Lygaeinae. Little was previously known about the false milkweed bug (*L. turcicus*); we report that the primary host of *L. turcicus* is false sunflower, *Heliopsis helianthoides* (Asteraceae), and that its seeds produce cardenolides, the second report of these toxins in Asteraceae. The dominant cardenolide was novel, which we resolved and named heliopside. We found divergent trends in performance and defense of the two bugs. *L. kalmii* had >40% higher survival and growth on milkweeds compared to *H. helianthoides*, an effect that was primarily explained by *H. helianthoides*' thick seed shell. Nonetheless, *L. kalmii* sequestered toxins equivalently from the two seed species. Conversely, *L. turcicus* had equally high survival and growth on the two seeds, but sequestered toxins more effectively from *H. helianthoides* than milkweed, potentially due to specialized detoxification of *H. helianthoides*' cardenolides. Thus, adaptation to host-plant traits in these two seed bugs is asymmetric for diet and defense, and distinct constraints are likely to limit the bugs' host ranges and contribute to coexistence. Shared seed chemistry apparently facilitated host shifts in the Lygaeinae from Apocynaceae to Asteraceae, with divergent adaptation to their food niches likely facilitating coexistence.

chemical ecology | host range evolution | plant–insect interactions | trade-offs | seed predator

Significance

Insect herbivores are highly diverse and often evolve by shifting onto novel host plants bearing similar chemical defenses to ancestral hosts, a phenomenon known as "chemical bridging." Yet, plants also maintain physical defenses that can constrain insect diets, and the relative role of adaptation to physical and chemical traits is largely unexplored. We document a likely case of chemical bridging for seed bugs from milkweeds, well-known to contain cardenolide toxins, to *Heliopsis helianthoides*, which we unexpectedly discovered contains cardenolides. We show that a physical trait (tough seed shells), in addition to specific cardenolide toxins, constrains host use of the two bug species. This work highlights the importance of diverse plant defense barriers in insect evolution.

Ever since Darwin, it has been predicted that interspecific competition will be strong between close compared to distant relatives and may lead to competitive exclusion when such species co-occur (1, 2). However, several classic examples document the stable coexistence of a striking diversity of congeners within the same local community (e.g., warblers, North American oaks, Caribbean anoles, milkweed aphids) (3–7). Ecologists have theorized that such coexistence must be enabled by niche differentiation, which reduces interspecific competition between species (3, 8). Competition itself may be a driver of such differentiation between species, or niche differentiation may occur due to unrelated forces, especially in cases of allopatric speciation, where niche axes may diverge due to drift or selection imposed by environmental conditions (9).

For the megadiverse insect herbivores, host plant use can be a labile niche axis over evolutionary time (10). Lineages appear to commonly diversify onto novel hosts which are phylogenetically, chemically, ecologically, or nutritionally similar to their ancestral host (11, 12). In herbivorous lineages that specialize on plants containing toxic secondary metabolites, shifts commonly occur onto novel hosts that contain similar toxins as their ancestral host, a phenomenon known as "chemical bridging" (11, 13). Critically, under chemical bridging, herbivore species that use chemically similar hosts are expected to share toxin resistance traits inherited from a common ancestor, in contrast to convergence of toxin resistance, in which lineages independently evolve resistance to similar plant defenses. Chemical niche conservatism is thought to arise from the costly and multifaceted adaptations associated with specialization on toxic hosts. Although resistance to specific plant toxins clearly facilitates chemical bridging, the extent to which host shifts followed by speciation are associated with further adaptation, specialization, and constraints is not well-explored (13–17).

Here we focus on two closely related species of specialized and sequestering seed bugs that commonly co-occur: the small milkweed bug (*Lygaeus kalmii*) and the false milkweed bug (*Lygaeus turcicus*) (Fig. 1). *L. kalmii* is distributed throughout North America and typically feeds on seeds of milkweeds (Apocynaceae: *Asclepias* spp.) which contain toxic cardenolides (18–20). *L. turcicus* is widely distributed in Eastern North America, but prior to this study, less was known about its dietary ecology: A single study indicated that seeds

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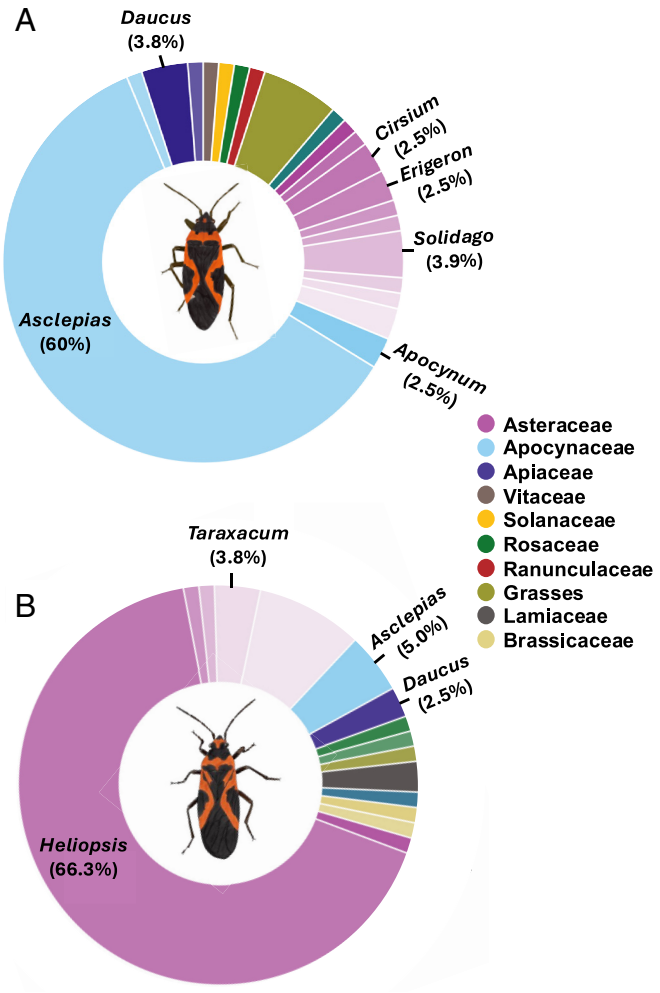

Fig. 1. Pie charts showing plant associations of (*A*) *L. kalmii* and (*B*) *L. turcicus* (n = 80 observations per species) based on iNaturalist records. Pie slices represent unique plant genera within each family. Genera with multiple observations are labeled, along with their percent of total observations. Slices corresponding to genera with a single observation, or observations not identifiable to genus, are left unlabeled (for a fully labeled version, *SI Appendix*, Fig. S1).

of the false sunflower (*Heliopsis helianthoides*, Asteraceae) are likely *L. turcicus's* primary food source (21). The genus *Lygaeus* ancestrally sequesters cardenolides from Apocynaceae and stores them in a specialized dorsolateral compartment (14, 22). Prior to our study, *H. helianthoides* was not known to produce cardenolides; our initial interest in *L. turcicus* was due to observing it on *H. helianthoides*, suggesting an apparent "loss" of sequestration. However, here we report that *H. helianthoides* does indeed produce cardenolides (the second report from the Asteraceae) and the shift of *L. turcicus* onto *H. helianthoides* is a likely case of chemical bridging (13, 14).

We investigate divergent host range, adaptation to host toxicity, and sequestration in these species using a reciprocal transplant approach. Although such studies typically perform reciprocal transplants using populations within a species, this framework also provides insight into the extent of adaptive divergence between closely related species (23–25). Accordingly, we first assessed patterns of plant association by analyzing observations of both bug species using the community science platform iNaturalist (inaturalist.org). Second, to address patterns of performance and sequestration across hosts, we reciprocally reared the two bugs on natural hosts (*A. syriaca* and *H. helianthoides*), as well as along a gradient of cardenolide concentrations among three additional milkweed species. Third, in addition to investigating adaptations to plant chemistry, we tested the impact of physical characteristics, in particular the hard pericarp or "shell" surrounding *H. helianthoides* seeds. Finally, we purified the dominant cardenolide found in *H. helianthoides,* resolved its novel structure using NMR spectroscopy (NMR) and mass spectrometry (MS), and tested its toxicity on the target animal enzyme (Na^+/K^+-ATPase). In sum, this study reveals how host shifts can lead to coexistence of closely related insect species via adaptive specialization to plant toxicity and seed morphology.

Results

Plant Associations of the Two Species. Both bug species were observed on a diverse array of plants (eight botanical families for each species), yet the majority of observations (~65%) were true to the previously reported hosts (*Asclepias* for *L. kalmii* and *Heliopsis* for *L. turcicus*) (Fig. 1 and *SI Appendix*, Fig. S1). All *Heliopsis* observations for *L. turcicus* were identified to the species level as *H. helianthoides*, and accordingly we conclude that it is the main host plant species. Although observations of *L. kalmii* on milkweed were split among several species, the common milkweed (*A. syriaca*) is the dominant milkweed in the eastern United States, and lab colonies used in our experiment were collected from a site where *A. syriaca* was the main host. As such, *H. helianthoides* and *A. syriaca* were used in focal comparisons of bug performance. To better establish feeding associations, we repeated our analysis, restricting sampled observations to photos where a bug was shown with a plant's seeds, the main tissue they feed on (21 total observations for *L. kalmii* and 13 for *L. turcicus*), as these observations are more likely to be feeding visits. This did not affect conclusions about dominant plant associations, with *Asclepias* spp. constituting 90% of these observations for *L. kalmii* and *H. helianthoides* constituting 92% for *L. turcicus*. It is worth noting that each bug can be found on the other's dominant plant in the field. Although we did not find *L. kalmii* on *H. helianthoides* in our iNaturalist survey, we have observed this ourselves in natural populations around Ithaca, NY.

Survival and Growth Performance. Although we did not find a significant difference between bug species for survival based on seed type (likelihood ratio test for bug × seed species interaction: $\chi^2 1 = 0.90$, $P = 0.34$; Fig. 2*A*), *L. kalmii* tended to have higher survivorship on *A. syriaca* than *H. helianthoides* (Estimated Marginal Mean (EMM) ± SE = 65.6 ± 13.4% on *A. syriaca* vs. 30.2 ± 11.4% on *H. helianthoides*), although this difference was not statistically conclusive (odds ratio = 0.23, 95% CI: 0.05 to 1.07, $P = 0.06$). In contrast, survival was comparably high across seed types for *L. turcicus* (66.7 ± 11.7% vs. 58.3 ± 11.7%; odds ratio = 0.61, 95% CI: 0.15 to 2.44, $P = 0.49$). The effect of seed type on growth rate did differ across bug species (ANOVA bug × seed species interaction: $F_{1, 9.33} = 6.51$, $\eta^2_p = 0.41$, 95% CI: 0.03 to 1, $P = 0.03$, Fig. 2*C*). *L. kalmii* grew 43% faster on *A. syriaca* than *H. helianthoides* (0.80 ± 0.071 mg/d vs. 0.52 ± 0.09 mg/d; mean difference = 0.29 mg/d, contrast 95% CI: 0.039 to 0.53, Tukey's Honestly Significant Difference (HSD) $P = 0.027$), while *L. turcicus* grew comparably poorly across seed types (0.49 ± 0.06 mg/d vs. 0.56 ± 0.062 mg/d; mean difference = −0.07 mg/d, contrast 95% CI: −0.257 to 0.122, Tukey's HSD $P = 0.45$). We saw parallel effects on adult mass and development time (*SI Appendix*, Fig. S2). Accordingly, *L. kalmii* seems better adapted to *A. syriaca* seeds as food relative to the alternative host, *H. helianthoides*; this home performance advantage was asymmetric, as we did not find it for *L. turcicus*.

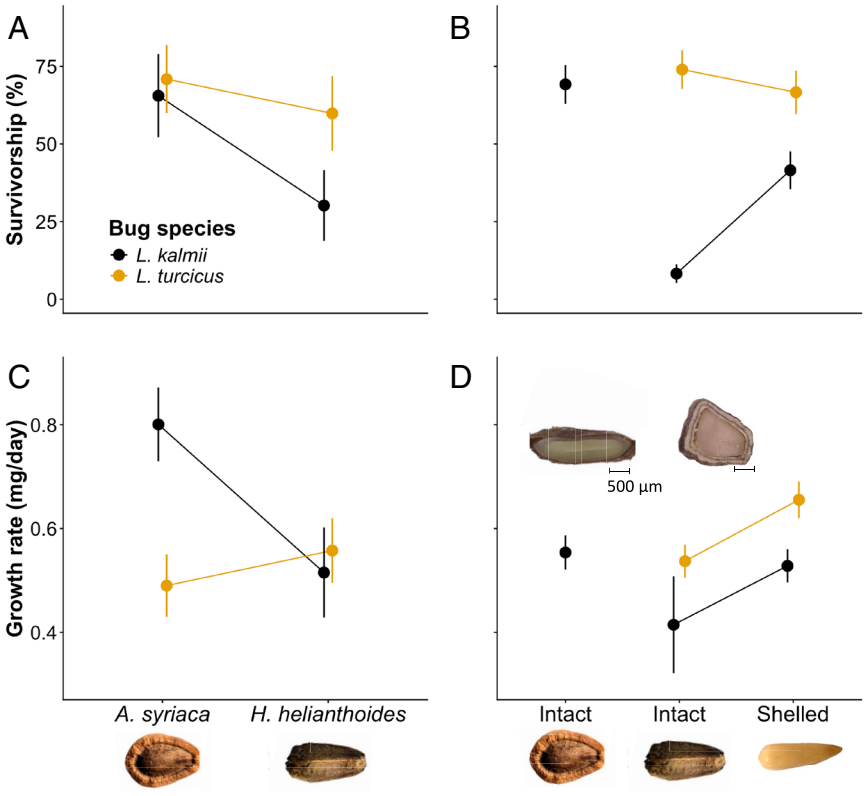

Fig. 2. Reaction norm plots of (*A, B*) per-replicate survivorship and (*C, D*) growth rate for *L. kalmii* and *L. turcicus*. Panels *A* and *C* show performance on each bug's dominant host plant. *B* and *D* show per-replicate survivorship (*B*) and growth rate (*D*) from a separate experiment in which both bugs were reared on intact or shelled *H. helianthoides* seeds, with *L. kalmii* on intact *A. syriaca* included for comparison (*Left*). Shown are least-squares means ± SEs. Cross-sections of *A. syriaca* seeds (panel *B, Top Left*) and *H. helianthoides* seeds (panel *B, Top Right*) illustrate differences in pericarp/seed-coat thickness: *H. helianthoides* ranges from 72 to 160 µm, while *A. syriaca* ranges from 32 to 54 µm.

In our experiment removing the shell of *H. helianthoides* seeds (Fig. 2 *B* and *D*), we found that *L. kalmii*'s poor growth and survivorship on *H. helianthoides* resulted, in part, from the thick shell (more than twice as thick as *A. syriaca*'s seed coat). As in our previous experiment, relatively few *L. kalmii* bugs survived to adulthood on intact *H. helianthoides* seeds (3 out of 85 neonates). In contrast, 80% of *L. turcicus* neonates survived to adulthood on intact *H. helianthoides* seeds (38 out of 50). Removing the *H. helianthoides* seed shell had an asymmetric effect on survival (likelihood ratio test for bug × shelling treatment interaction: $\chi^2 1 = 14.8$, $P < 0.001$; Fig. 2*B*), with *L. kalmii* per-replicate survivorship probability increasing to nearly 50% (8.24 ± 4.96% intact vs. 41.54 ± 5.68% shelled; odds ratio = 0.13, 95% CI: 0.05 to 0.31, $P < 0.0001$), while *L. turcicus* survivorship was unchanged (74 ± 6.47% intact vs. 66.67 ± 6.82% shelled; odds ratio = 1.42, contrast 95% CI: 0.59 to 3.45, $P = 0.43$). Similarly, differences between bug species in development time on *H. helianthoides* disappeared when seeds were shelled (*SI Appendix*, Fig. S3). Shelling seeds had parallel effects on growth rate of the surviving *L. kalmii* and *L. turcicus*, with both species growing approximately 20% faster on shelled seeds (ANOVA bug × shelling treatment interaction: $F_{1, 49.87} = 0.002$, $\eta^2_P < 0.001$, 95% CI: 0 to 1, $P = 0.96$; Fig. 2*D*). Although *L. kalmii's* growth rate was lower on intact *H. helianthoides* seeds relative to *A. syriaca* seeds (0.55 ± 0.032 mg/d on *A. syriaca* vs. 0.35 ± 0.12 mg/d on intact *H. helianthoides*; *t* test $P < 0.001$), this disparity disappeared when *H. helianthoides* seeds were shelled (0.55 ± 0.032 mg/d on *A. syriaca* vs. 0.53 ± 0.039 mg/d on shelled *H. helianthoides*; *t* test $P = 0.6$). *L. kalmii* still had a lower growth rate than *L. turcicus* on shelled seeds (*L. turcicus* on shelled *H. helianthoides*: 0.66 ± 0.035 mg/d; mean difference = −0.127 mg/d, contrast 95% CI: −0.225 to 0.03, Tukey's HSD $P = 0.012$), indicating that the physical barrier of the seed's shell does not fully explain the disparity in growth rate between bug species on *H. helianthoides*.

Characterization of Cardenolide Defenses in *H. helianthoides*. Surprisingly, *A. syriaca* and *H. helianthoides* seeds contained comparable concentrations of total cardenolides on a dry mass basis (*A. syriaca*: 3.08 ± 0.09 mg/g; *H. helianthoides*: 3.06 ± 0.46 mg/g). *H. helianthoides* seeds contained a single compound that constituted 80% of total seed cardenolides, while *A. syriaca* seeds contained a mix of 16 compounds, with the dominant compound glucopyranosyl aspecioside comprising 42% of the total. We further isolated and characterized the dominant compound in *H. helianthoides*, here named heliopside. 1D and 2D NMR data combined with HRMS/MS data indicate the structure of a corotoxigenin to which dideoxy-allopyranosyl and glucose are attached (*SI Appendix*, Tables S1–S2 and Figs. S4–S6). Although the genin was previously known from *Asclepias*, the presence of the two carbohydrate units makes it a novel structure. We verified the inhibitory potential of heliopside and another (unidentified) *Heliopsis* cardenolide against an unadapted (porcine) and adapted (large milkweed bug, *Oncopeltus fasciatus*) Na^+/K^+-ATPase and compared this to an Apocynaceae cardenolide standard, ouabain. The adapted ATPase conferred ≈100× resistance to cardenolides as expected, and heliopside and ouabain were comparably inhibitory of each ATPase (*SI Appendix*, Fig. S7). Note that seeds and leaves of *H. helianthoides* each have >12 cardenolides, and although several of these compounds co-occur in both tissues (including heliopside), seeds are dominated by more polar compounds (*SI Appendix*, Fig. S8).

Sequestration on Alternative Hosts. Differences in cardenolide structure, richness, and profiles between *A. syriaca* and *H. helianthoides* correspond to differential patterns of sequestration between the two bug species. Although we did not detect a significant interaction between bug species and total cardenolide sequestration (ANOVA bug × seed species interaction: $F_{1,9} = 2.42$, $\eta^2_P = 0.21$, 95% CI: 0 to 1, $P = 0.15$; Fig. 3*A*), *L. kalmii* sequestered nearly equivalent

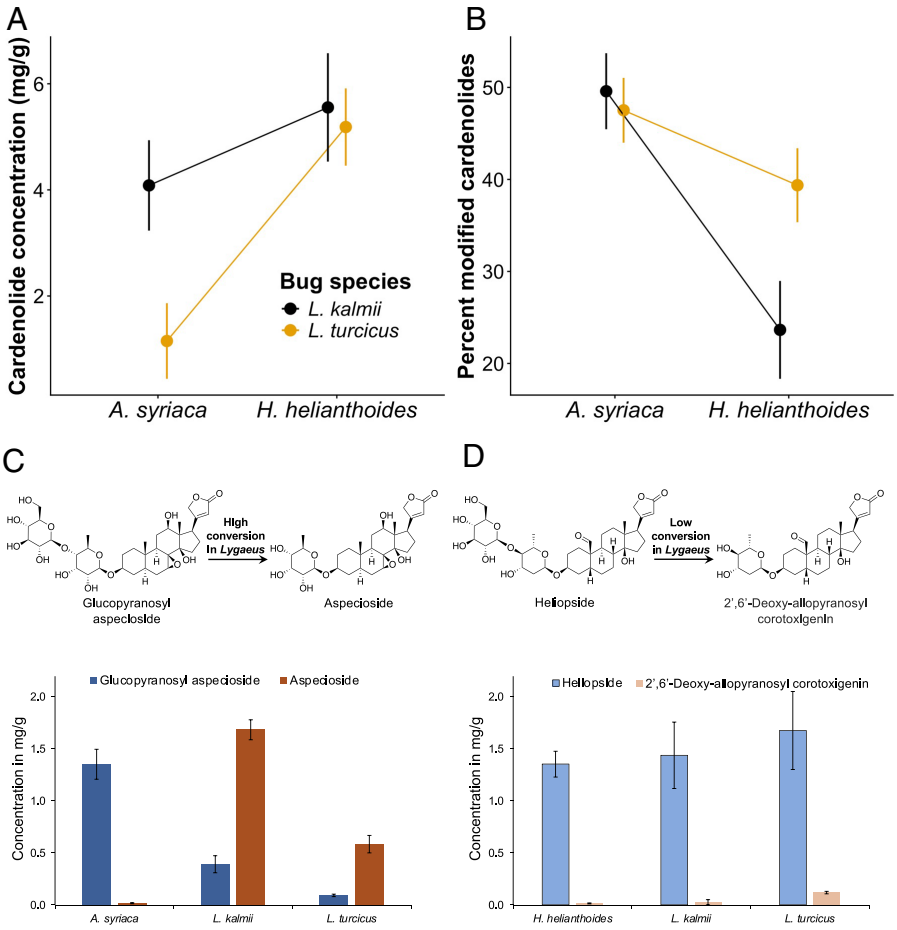

Fig. 3. Cardenolide sequestration and processing by *L. kalmii* and *L. turcicus* when feeding on milkweed (*A. syriaca*) and false sunflower (*H. helianthoides*) seeds. Reaction norm plots of (*A*) total cardenolide concentration in adult bugs and (*B*) percent of total sequestered cardenolides not found in seeds using High-Performance Liquid Chromatography (HPLC) HPLC-UV. (*C*) Structure of the dominant *A.syriaca* seed cardenolide, glucopyranosyl aspecioside, comprising ≈ 40% of total seed cardenolides in *A. syriaca*, and its conversion product, aspecioside, shown in seeds and both bug species based on UPLC-MS quantification. (*D*) the newly described heliopside, comprising ≈ 80% of total seed cardenolides in *H. helianthoides*, and its conversion product (after loss of glucose), shown in seeds and both bug species based on UPLC-MS quantification. Shown are means ± SEs.

concentrations of the toxins from both *H. helianthoides* and *A. syriaca* (5.55 ± 0.85 mg/g vs. 4.08 ± 1.02 mg/g; mean difference = 1.47 mg/g, contrast 95% CI: −1.43 to 4.37, Tukey's HSD $P = 0.29$), while *L. turcicus* sequestered four-fold higher cardenolide concentrations on *H. helianthoides* than *A. syriaca* (5.19 ± 0.73 mg/g vs. 1.15 ± 0.71 mg/g; mean difference = 4.03 mg/g, contrast 95% CI: 1.81 to 6.26, Tukey's HSD $P < 0.01$). Although our ability to detect a significant interaction term was limited by power, these results provide evidence that *L. turcicus* is only an effective sequesterer from its main host, while *L. kalmii* has the capacity to sequester well from both plants.

We found weak evidence that bug species differentially process cardenolides from the two host species. Specifically, we found a marginal interaction in the extent to which bugs modified seed cardenolides, with each sequestering different proportions of modified cardenolides, compounds that were not present in seeds, depending on the host plant (ANOVA bug × seed species interaction: $F_{1,7.3} = 3.36$, $\eta^2_P = 0.31$, 95% CI: 0 to 1, $P = 0.1$; Fig. 3*B*). This result appears to be driven by differential modification of *H. helianthoides* cardenolides. While the two bug species sequestered a similar total concentration of cardenolides from *H. helianthoides* seeds, *L. kalmii* sequestered a larger fraction of cardenolides intact compared to *L. turcicus* (24.4 ± 7.04% modified cardenolides in *L. kalmii* vs. 53.6 ± 5.08 in *L. turcicus*; mean difference = 19.2%, contrast 95% CI: 0.28 to 38.2, Tukey's HSD $P = 0.047$). Potentially divergent cardenolide modification on *H. helianthoides* seeds did not arise from differential conversion of the dominant *H. helianthoides* cardenolide, heliopside (Fig. 3*D*), which comprised approximately 60% of total sequestered cardenolides in both bug species. Instead, the observed pattern is consistent with greater modification of minor *H. helianthoides* cardenolides in *L. turcicus*, which may contribute to the divergent cardenolide profiles observed between the two *Lygaeus* species.

In contrast, although *L. turcicus* sequestered a lower total concentration from *A. syriaca* than *L. kalmii*, the bugs processed cardenolides similarly: both stored comparable fractions of modified milkweed cardenolides (49.6 ± 4.13% modified cardenolides in *L. kalmii* vs. 47.5 ± 3.52 in *L. turcicus*; mean difference = −2.07%, contrast 95% CI: −15.351 to 11.1, Tukey's HSD $P = 0.71$). Moreover, specific *A. syriaca* cardenolides appear to be processed similarly by the two bugs: The dominant cardenolide sequestered from *A. syriaca* seeds in both *L. turcicus* and *L. kalmii* (>30% of the total) was aspecioside, a less toxic breakdown product (loss of a sugar moiety) of the dominant cardenolide in *A. syriaca* seeds (glucopyranosyl aspecioside, previously referred to as glycosylated aspecioside) (Fig. 3*C*) (26, 27).

Performance and Sequestration across a Cardenolide Gradient. We finally reared both bug species from hatch to adulthood on three additional milkweed species' seeds along a greater-than threefold cardenolide concentration gradient to address *L. turcicus*'s relatively poor sequestration observed on *A. syriaca*. There was no clear pattern of bug performance as a function of dietary cardenolides: *L. kalmii* had a higher growth rate than *L. turcicus* on the low-cardenolide *A. incarnata* and the high cardenolide *A. perennis*, but growth was equivalent on the intermediate *A. curassavica* (ANOVA bug × seed species interaction: $F_{2,13.6} = 4.37$, $\eta^2_P = 0.32$, 95% CI: 0 to 1, $P = 0.034$; Fig. 4*A*). While performance was independent of milkweed cardenolide concentration for both species, sequestration followed seed cardenolide concentration (ANOVA bug × seed species interaction: $F_{2,15.67} = 6.2$, $\eta^2_P = 0.42$,

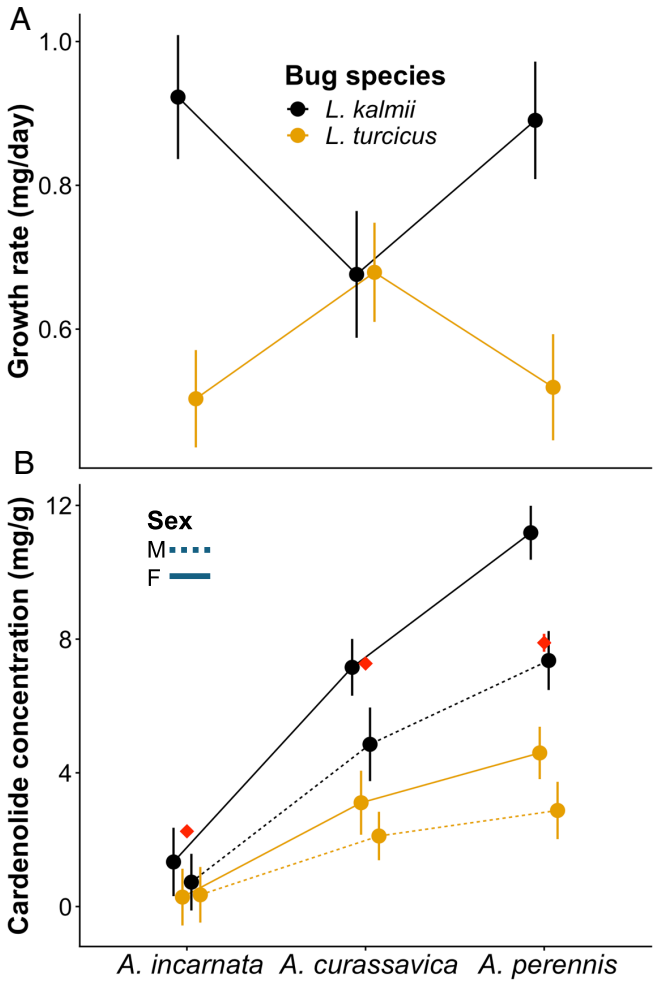

Fig. 4. Growth rate (*A*) and sequestration (*B*) of *L. kalmii* and *L. turcicus* on three different milkweed species' seeds, representing a gradient from low to high cardenolide concentrations. Red points in *B* show the average cardenolide concentration for each seed species. Shown are least squares means ± SEs.

95% CI: 0.08 to 1, $P = 0.01$; Fig. 4*B* and *SI Appendix*, Fig. S9): Both bug species sequestered equivalently little on low cardenolide *A. incarnata* (*L. kalmii*: 1.05 ± 0.81 mg/g vs. *L. turcicus*: 0.31 ± 0.63 mg/g; mean difference = 0.74 mg/g, contrast 95% CI: −1.41 to 2.89, Tukey's HSD $P > 0.1$), but on higher cardenolide milkweed species (*A. curassavica* and *A. perennis*), *L. kalmii* sequestered cardenolide concentrations above the level found in seeds, while *L. turcicus* sequestered approximately 50% of seed concentrations (Fig. 4*B*). Although the patterns were similar between males and females, we also found that females sequestered 50% more cardenolides compared to males for both bug species (ANOVA sex effect: $F_{1,45.5} = 13.6$, $\eta^2_P = 0.23$, 95% CI: 0.07 to 1, $P < 0.001$). Overall, this experiment confirms that *L. kalmii* is a more effective sequesterer of milkweed cardenolides than *L. turcicus*, and its sequestration is accordingly more responsive to higher dietary cardenolide concentrations.

Discussion

Recent work supports the notion that host use in specialized insect herbivores may be reflective of a "host defense chase" dynamic, where herbivore lineages shift via chemical bridges to novel hosts that are similar to their ancestral host (13, 28, 29). This hypothesis is supported by the fact that herbivore phylogenies often show little congruence to host plant phylogenies (i.e., little cospeciation) (29–31). Additionally, even in cases where plant–herbivore cospeciation is found, it is often impossible to determine whether this association simply arises from a shared evolutionary history. Instead, herbivore diversity and host use are often predicted by plant chemical defense traits (29, 31). Under the host defense chase model, chemical specialization represents a preadaptation that allows for subsequent adaptation to different phenotypic traits of novel hosts. Furthermore, for sequestering herbivores, top–down pressure from predators and parasitoids (rather than only conservatism in resistance traits) may reinforce host defense chase dynamics. For example, a diversity of lygaeine bugs perform well when raised on several seed species in the lab (with and without toxins for sequestration) and are known to associate with multiple hosts in the wild (14). Despite their physiological ability to adopt a generalist dietary niche, many such lygaeines remain chemically specialized sequesterers—presumably to gain protection from predators (13, 32).

We investigated a potential case of chemical bridging within a clade engaged in a host defense chase with cardenolide-containing plant species (13, 14). We show that preadaptations to milkweed cardenolides are sufficient for a milkweed specialist to effectively sequester from a novel host (*H. helianthoides*). Accordingly, if defensive chemistry were the only factor determining host use, *L. kalmii* would be equally able to exploit both milkweed and *H. helianthodies*. However, competition between these two species may be reduced reciprocally by *L. kalmii*'s difficulty in coping with *H. helianthoides*' thick seed shell and *L. turcicus's* limited ability to sequester milkweed cardenolides. Where prior research has shown that preadaptations to host chemistry may have facilitated shifts in this group onto novel plants, we find that other host traits (*i.e.*, physical characteristics of seeds) may constrain the use of chemically similar hosts (13, 14).

We have studied both within- and between-species differences in performance and sequestration of cardenolide toxins on the seeds of two host plants. We find that *L. turcicus* shows comparable performance across plants, as measured by survivorship or growth rate, but it sequesters toxins best on its main host, *H. helianthoides*. Conversely, *L. kalmii* performs best on *A. syriaca*, but it sequesters equally well on its primary host plant and *H. helianthoides.* In comparisons between species we find asymmetric divergence along different metrics of performance: *L. kalmii* outperforms *L. turcicus* in sequestration from milkweed, while *L. turcicus* outperforms *L. kalmii* in growth on *H. helianthoides*. Even though cardenolides from *A. syriaca* and *H. helianthoides* are metabolized in similar ways by both *L. kalmii* and *L. turcicus*, the two species show divergent performance on these hosts (even after shelling), likely because of other seed-specific traits. Although we acknowledge that we have not examined competition itself, such between-species differences in fitness-related metrics (in this case, survival, growth, development, and defensive sequestration) on each host plant reflect the potential for competitive asymmetries.

We find that a host shift in this system has given rise to divergent specialization on plant toxins and seed morphology, likely enabling the coexistence of closely related species. Plants in the Apocynaceae (including milkweeds) are the ancestral hosts of the Lygaeinae and *L. kalmii*, which represents this ancestral state, sequesters equally well from *A. syriaca* and *H. helianthoides*. We propose that *L. turcicus*' apparent chemical specialization on *H. helianthoides* (e.g., Fig. 3*A*) has been accompanied by a loss of the ability to effectively sequester milkweed cardenolides. In support of this notion, we find evidence for divergent processing of *H. helianthoides* cardenolides by *L. turcicus*, as it extensively modifies *H. helianthoides* cardenolides when sequestering from this host (Fig. 3*B*). Conversely, *L. kalmii* appears better adapted to milkweed seeds in

terms of survivorship, growth, and development, due to an inability to easily access the endosperm with the thick shell surrounding *H. helianthoides* seeds. Thus, although preadaptations to cardenolides may have enabled *L. turcicus* to exploit a novel host, specific adaptations to nonchemical host traits (in this case, *H. helianthoides* seeds' shell) seem to facilitate niche differentiation between this bug and its coexisting congener (*L. kalmii*).

When raised on milkweed seeds representing a threefold toxicity gradient, we found that growth performance and survivorship in both bug species were unrelated to cardenolide concentration. This is a typical result for the lygaeines, which are the most cardenolide-resistant group of milkweed feeders (27). In contrast, sequestration depended on seed cardenolide concentration for both bugs, but *L. turcicus* body concentration saturated at ≈4 mg/g, 20% less than its maximum concentration on *H. helianthoides*, despite the higher cardenolide concentration in *A. curassavica* and *A. perennis*. Although such saturation is seen in other milkweed-associated species (33, 34), prior work with the lygaeines has shown that sequestration remains strongly correlated with seed cardenolide concentration even on high cardenolide milkweed seeds (35). We speculate that cardenolide saturation in *L. turcicus* may occur for two reasons. First, the bug may be unable to uptake or transport milkweed cardenolides to storage tissues or may lack the physiological machinery necessary to chemically modify specific cardenolides into forms that can be sequestered (36). Second, avoidance of specific cardenolides may be adaptive, as some compounds may be especially toxic to the bug or may be ineffective as defenses against its predators (22, 27, 37). We found that *L. turcicus* and *L. kalmii* similarly broke down the dominant cardenolide in both seeds (27). Thus, it appears that *L. turcicus* retains at least some ability to convert milkweed cardenolides into forms that can be sequestered. Consequently, we suggest that, mechanistically, poor milkweed sequestration in *L. turcicus* may arise from deficiencies in the transport or uptake of compounds, rather than an inability to perform specific chemical modifications.

Conclusion

We have documented cardenolide toxins in Asteraceae (the second report from the family) (38), and we document a likely case of chemical bridging of a bug species (*L. turcicus*) from an ancestrally milkweed-specialized clade onto this novel host. Among the greater than 500 species in the subfamily Lygaeinae, this process appears to have repeated itself contributing to the generation of biodiversity (12). In the current case, host use in these two specialist herbivores was found to be driven asymmetrically by host chemistry and physical defense traits. Our study thus implicates chemical specialization as a key preadaptation that facilitates evolutionary transitions onto novel hosts among herbivores (11, 14, 29, 32, 39), but we find that this preadaptation alone is unlikely sufficient to facilitate coexistence in sympatry. We also show that the *H. helianthoides* specialist *L. turcicus* has lost the ability to effectively sequester cardenolides from milkweed seeds. Accordingly, our study highlights the importance of multivariate trait axes in facilitating divergent host use, even when plants are chemically similar. More generally, herbivorous insects remain an important model for understanding how species diverge and adapt, providing insight into the origin and maintenance of biodiversity.

Materials and Methods

Plant Associations of the Two Species. To determine plant associations each bug species, we downloaded all research-grade iNaturalist observations of each species with geographic coordinates (iNaturalist.com). The geographic range of *L. turcicus* observations is entirely within the range of *L. kalmii*. As such, we calculated the minimum convex polygon (mcp(); adehabitatHR package R) surrounding all *L. turcicus* observations as the range in which the two species co-occur (*SI Appendix*, Fig. S10). We sampled along a grid of 10 evenly spaced latitudes and 20 evenly spaced longitudes between the maximum and minimum latitude/longitude of the polygon encompassing all observations of *L. turcicus*. We next extracted the nearest observation of each species (within the range in which they co-occur) to each grid coordinate and recorded whether the observation contained a correctly identified adult bug on a plant identifiable to the family level. For each observation, the plant was identified to the lowest taxonomic classification possible. This process was repeated until 80 grid points had plant observations for each bug species. The number of observations of bugs on a given taxonomic group was used to determine plant associations for each bug species. The plant part the bug was on in each observation (leaves, seeds, flower, stem) was also noted.

Plant and Insect Material. Bugs used in this experiment came from colonies of wild collected *L. kalmii* and *L. turcicus* from Ithaca, NY, and reared separately on *A. syriaca* (for *L. kalmii*) and *H. helianthoides* (for *L. turcicus*) for less than five generations. *A. incarnata* and *A. syriaca* seeds were collected from field sites near Ithaca, NY, while *A. curassavica*, *A. perennis*, and *H. helianthoides* seeds were purchased from commercial vendors. The experiment was performed in two separate blocks: one in the fall of 2021 and one in the summer of 2022. For each experimental replicate, newly hatched (<24 h old) nymphs were placed in a petri dish with a moistened cotton ball and 0.5 g of seed (either *A. incarnata*, *A. curassavica*, *A. syriaca*, *A. perennis*, or *H. helianthoides*), depending on experimental treatment. Moist cotton balls were replaced as-needed (approximately once every two-to-three days), and 0.5 g of seed was added to each petri dish once weekly. This is far in excess of the dietary requirements for the number of bugs used in each microcosm. Each experimental replicate consisted of one petri dish for each seed-type for each species.

The first experimental block consisted of four replicates (two where each petri dish initially received four nymphs and two where each dish received only two nymphs due to low egg production in colonies). Dishes in this block were placed in a growth chamber (23 °C: 26 °C night:day, 14 h day), and bugs were allowed to feed until reaching adulthood. The second experimental block consisted of three replicates (all of which received four nymphs) and was conducted at a higher temperature (24 °C: 27 °C night:day, 14 h day) due to low survivorship in the first block. Anecdotal experience suggested that these species have greater survivorship to adulthood at slightly warmer temperatures. All bugs were collected within 24 h of making their final molt to adulthood, frozen at −80 °C, and freeze-dried. We recorded sex, development time (number of days from being placed in the experiment to being collected as an adult), and dry mass for each adult bug.

***H. helianthoides* Shelling Experiment.** To understand the effect of seed physical traits on survival and performance of bugs, we performed an experiment in which both species were raised separately on shelled and intact *H. helianthoides* seeds. Each experimental replicate consisted of a deli cup with a section of the lid replaced with a fine mesh containing five newly hatched nymphs of one bug species with access to either *A. syriaca* seeds, intact *H. helianthoides* seeds, or shelled *H. helianthoides* seeds. The experiment was conducted in a growth chamber (24 °C: 27 °C night:day, 14 h day), and the position of each replicate was randomized. For shelled treatments *H. helianthoides* seed shells were cracked gently using a rubber mallet and removed. Bugs were initially presented with 100 mg seed, which was replenished with 250 mg of seed once every week. This amount was well above the feeding requirements of five bugs. Upon reaching adulthood, bugs were removed, freeze-dried, and sexed using reproductive morphology. Development time and adult dry mass were recorded for all bugs.

Cardenolide Quantification. Freeze-dried bugs and seeds (whole insects for bugs and 50 mg for seeds) were ground to a powder and cardenolides were extracted using 1.6 mL methanol (MeOH) with 20 μg digitoxin as an internal standard. We analyzed samples using HPLC following the method used by Züst et al. (2019) (40). Cardenolide concentrations were calculated based on peak areas at 218 nm and were standardized by the concentration of digitoxin in the sample and the sample's dry mass. Peak areas were calculated from HPLC chromatograms using the chromatographR package (41). Detailed cardenolide extraction and analysis methods are in *SI Appendix* text. To assess the inhibition of sodium

pump by *Heliopsis* cardenolides, we tested heliopside, an unidentified *Heliopsis* compound (*SI Appendix*, Fig. S7), and the standard ouabain on a sensitive and resistant Na^+/K^+-ATPase following the methods of Petschenka et al. (42).

Cardenolide Structural Characterization. Seeds (100 g) were freeze-dried, ground, and then extracted with MeOH (1 L) for a week. The extract was washed with hexane (100 mL) a few times followed by drying. The remaining residue was then suspended in 16% acetonitrile and water (9 mL), sonicated for 30 s, vortexed, centrifuged at 20,800 × g for 12 min. After centrifugation, the clear supernatant was immediately injected for preparative HPLC fractionation. The isolated fractions were pooled, dried, resuspended in 0.5 mL of 100% methanol, and then analyzed on the Dionex 3000 LC reversed-phase chromatography system coupled to an Orbitrap Q-Exactive mass spectrometer UPLC–HRMS system in positive and negative ionization modes for quality check following the method used by Agrawal et al. (2022) (27). MS2 spectra were deposited on GNPS link. NMR (NMR) spectra were recorded on a Bruker 600 Avance III HD console and $BBFO^+$ cryoprobe and all 1D data (^1H, ^{13}C) and 2D data (DQCOSY, gCOSY, HSQCAD, gHMBCAD, HSQCTOCSY, ROESYAD) were deposited on Dryad Research Data Repository (https://doi.org/10.5061/dryad.c2fqz61jz). Detailed methods are in *SI Appendix* text.

Statistical Analyses. To assess the performance and sequestration of both species of bugs as a function of seed diet, we performed a series of two-way ANOVAs using the lmerTest package in R version 4.1.2, with petri dish as a random effect [response variable ~ seed diet × bug species × bug sex + block + (1 | petri dish)] (43). Analyses of mass, development time, growth rate, and sequestration, were performed at the individual bug level, with petri dish included as a random effect to account for shared variation among individuals raised in the same dish. Two (out of 22) petri dishes in block 1 were started with four nymphs, while the rest received two starting nymphs. To determine whether this affected our analyses, we conducted all ANOVAs with the initial number of bugs in a replicate as an additional fixed effect (summary of results for all comparisons in *SI Appendix*, Tables S3 and S4). A dish's number of initial bugs was a significant effect for only a single comparison (total sequestered cardenolides for our milkweed cardenolide gradient), and its inclusion did not substantially affect the significance or magnitude of other effects. For the shelling experiment, the model: Response variable ~ seed diet × bug species + bug sex + (1 | petri dish) was used, as the experiment was performed in a single block and no male bugs survived to adulthood on intact *H. helianthoides*, so a sex interaction could not be calculated. Per-petri dish survivorship was analyzed using a binomial Generalized Linear Model (GLM), with seed diet × bug species + block as predictors (block structure was only included for relevant experiments).The significance of GLM effects was assessed using ANOVA with type II likelihood ratio tests. Growth rate was calculated by dividing dry mass at adulthood by the number of days it took for the bug to develop from neonate to adult. Estimated marginal means and pairwise comparisons were performed using the emmeans package in R, and p-values for all pairwise comparisons were adjusted using Tukey's method for multiple comparisons (44).

Data, Materials, and Software Availability. all data have been deposited in Cornell e-commons https://doi.org/10.7298/06h5-cx23 (45).

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1. C. Darwin, *On the Origin of Species* (D. Appleton and Co., New York, 1871).
2. R. Macarthur, R. Levins, The limiting similarity, convergence, and divergence of coexisting species. *Am. Nat.* **101**, 377–385 (1967).
3. R. H. MacArthur, Population ecology of some warblers of northeastern coniferous forests. *Ecology* **39**, 599–619 (1958).
4. J. B. Losos, D. J. Irschick, T. W. Schoener, Adaptation and constraint in the evolution of specialization of Bahamian *Anolis* lizards. *Evolution* **48**, 1786–1798 (1994).
5. R. A. Smith, K. A. Mooney, A. A. Agrawal, Coexistence of three specialist aphids on common milkweed. *Asclepias syriaca*. *Ecology* **89**, 2187–2196 (2008).
6. J. I. Meier *et al.*, Cycles of fusion and fission enabled rapid parallel adaptive radiations in African cichlids. *Science* **381**, eade2833 (2023).
7. A. L. Hipp *et al.*, Sympatric parallel diversification of major oak clades in the Americas and the origins of Mexican species diversity. *New Phytol.* **217**, 439–452 (2018).
8. J. M. Chase, "5. Ecological Niche Theory" in *The Theory of Ecology*, S. M. Scheiner, M. R. Willig, Eds. (University of Chicago Press, 2011), pp. 93–108.
9. R. J. Rundell, T. D. Price, Adaptive radiation, nonadaptive radiation, ecological speciation and nonecological speciation. *Trends Ecol. Evol.* **24**, 394–399 (2009).
10. M. Ackermann, M. Doebeli, Evolution of niche width and adaptive diversification. *Evolution* **58**, 2599–2612 (2004).
11. E. A. Bernays, Evolution of feeding behavior in insect herbivores. *BioScience* **48**, 35–44 (1998).
12. D. J. Futuyma, A. A. Agrawal, Macroevolution and the biological diversity of plants and herbivores. *Proc. Natl. Acad. Sci.* **106**, 18054–18061 (2009).
13. G. Petschenka *et al.*, Sequestration of defenses against predators drives specialized host plant associations in preadapted milkweed bugs (Heteroptera: Lygaeinae). *Am. Nat.* **199**, E211–E228 (2022).
14. C. Bramer, S. Dobler, J. Deckert, M. Stemmer, G. Petschenka, Na+/K+-ATPase resistance and cardenolide sequestration: basal adaptations to host plant toxins in the milkweed bugs (Hemiptera: Lygaeidae: Lygaeinae). *Proc. R. Soc. Lond. B. Biol. Sci.***282**, 20142346 (2015).
15. A. Mira, E. A. Bernays, Trade-offs in host use by *Manduca sexta*: Plant characters vs natural enemies. *Oikos* **97**, 387–397 (2002).
16. M. S. Singer, J. O. Stireman III, The tri-trophic niche concept and adaptive radiation of phytophagous insects. *Ecol. Lett.* **8**, 1247–1255 (2005).
17. J. B. Joy, B. J. Crespi, Adaptive radiation of gall-inducing insects within a single host-plant species. *Evolution* **61**, 784–795 (2007).
18. L.-M. Hunt, Observations of the habits of lygaeus kalmii angustomarginatus (hemiptera: lygaeidae) in southern michigan. *The Great Lakes Entomologist* **12**, 6 (2017).
19. R. B. Root, The life of a Californian population of the facultative milkweed bug, *Lygaeus kalmii* (Heteroptera: Lygaeidae). *Proceedings of the Entomological Society of Washington* **88**, 201–214 (1986).
20. A. G. Wheeler, The small milkweed bug, *Lygaeus kalmii* (Hemiptera: Lygaeidae): Milkweed specialist or opportunist? *J. N. Y. Entomol. Soc.* **91**, 57–62 (1983).
21. J. A. Slater, On the biology and food plants of *Lygaeus turcicus* (Fabr.) (Hemiptera: Lygaeidae). *J. N. Y. Entomol. Soc.* **91**, 48–56 (1983).
22. P. Rubiano-Buitrago *et al.*, Cardenolides in the defensive fluid of adult large milkweed bugs have differential potency on vertebrate and invertebrate predator Na+/K+-ATPases. *R. Soc. Open Sci.* **11**, 231735 (2024).
23. A. J. Gassmann, A. Levy, T. Tran, D. J. Futuyma, Adaptations of an insect to a novel host plant: A phylogenetic approach. *Funct. Ecol.* **20**, 478–485 (2006).
24. P. Nosil, *Ecological Speciation* (Oxford University Press, 2012).
25. A. M. Roush, L. Zhang, G. R. Hood, J. R. Ott, S. P. Egan, A test of trade-offs in dispersal and reproduction within and between a sister species pair of specialist insect herbivores. *Oecologia* **204**, 529–542 (2024).
26. P. Rubiano-Buitrago *et al.*, Cardenolides in *Asclepias syriaca* seeds: Exploring the legacy of tadeus reichstein. *J. Nat. Prod.* **88**, 49–57 (2025).
27. A. A. Agrawal *et al.*, Functional evidence supports adaptive plant chemical defense along a geographical cline. *Proc. Natl. Acad. Sci.* **119**, e2205073119 (2022).
28. J. X. Becerra, Insects on plants: Macroevolutionary chemical trends in host use. *Science* **276**, 253–256 (1997).
29. M.-J. Endara *et al.*, Coevolutionary arms race versus host defense chase in a tropical herbivore–plant system. *Proc. Natl Acad Sci.* **114**, E7499–E7505 (2017).
30. S. Ibanez, F. Arène, S. Lavergne, How phylogeny shapes the taxonomic and functional structure of plant–insect networks. *Oecologia* **180**, 989–1000 (2016).
31. C. F. H. van der Linden, M. F. WallisDeVries, S. Simon, Great chemistry between us: The link between plant chemical defenses and butterfly evolution. *Ecol. Evol.* **11**, 8595–8613 (2021).
32. F. Beran, G. Petschenka, Sequestration of plant defense compounds by insects: From mechanisms to insect-plant coevolution. *Annu. Rev. Entomol.* **67**, 163–180 (2022).
33. P. L. Jones, G. Petschenka, L. Flacht, A. A. Agrawal, Cardenolide intake, sequestration, and excretion by the monarch butterfly along gradients of plant toxicity and larval ontogeny. *J. Chem. Ecol.* **45**, 264–277 (2019).
34. S. B. Malcolm, B. J. Cockrell, L. P. Brower, Cardenolide fingerprint of monarch butterflies reared on common milkweed. *Asclepias syriaca* L. *J. Chem. Ecol.* **15**, 819–853 (1989).
35. M. B. Isman, Dietary influence of cardenolides on larval growth and development of the milkweed bug *Oncopeltus fasciatus*. *J. Insect Physiol.* **23**, 1183–1187 (1977).
36. S. B. Malcolm, Milkweeds, monarch butterflies and the ecological significance of cardenolides. *Chemoecology* **5**, 101–117 (1994).
37. A. A. Agrawal *et al.*, Cardenolides, toxicity, and the costs of sequestration in the coevolutionary interaction between monarchs and milkweeds. *Proc. Natl. Acad. Sci.***118**, e2024463118 (2021).
38. T.-M. Wang *et al.*, Cardenolides from *Saussurea stella* with cytotoxicity toward cancer cells. *J. Nat. Prod.* **70**, 1429–1433 (2007).
39. A. Termonia, T. H. Hsiao, J. M. Pasteels, M. C. Milinkovitch, Feeding specialization and host-derived chemical defense in chrysomeline leaf beetles did not lead to an evolutionary dead end. *Proc. Natl. Acad. Sci.* **98**, 3909–3914 (2001).
40. T. Züst, G. Petschenka, A. P. Hastings, A. A. Agrawal, Toxicity of milkweed leaves and latex: Chromatographic quantification versus biological activity of cardenolides in 16 *Asclepias* species. *J. Chem. Ecol.* **45**, 50–60 (2019).
41. E. Bass, chromatographR: Chromatographic data analysis toolset (2024). http://doi.org/10.5281/zenodo.14172281.
42. G. Petschenka, T. Züst, A. P. Hastings, A. A. Agrawal, G. Jander, "Quantification of plant cardenolides by HPLC, measurement of Na+/K+-ATPase inhibition activity, and characterization of target enzymes" in *Methods in Enzymology* (Academic Press, 2023), pp. 275–302.
43. A. Kuznetsova, P. B. Brockhoff, R. H. B. Christensen, LmerTest package: Tests in linear mixed effects models. *J. Stat. Softw.* **82**, 1–26 (2017).
44. R. V. Lenth, Emmeans: Estimated marginal means, aka least-squares means. *Health* 15, 3 (2023). https://cran.r-project.org/package=emmeans.
45. N. J. Carlson, A. P. Hastings, C. Duplais, A. A. Agrawal, Data and scripts from: Divergent dietary and defensive adaptations of closely related coexisting seed bugs. Cornell e-commons, https://doi.org/10.7298/06h5-cx23. Accessed 7 February 2026.

PNAS

Supporting Information for

Divergent dietary and defensive adaptations of closely related coexisting seed bugs

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This PDF file includes:

Supporting Information Text

Methods

Cardenolide quantification: Freeze-dried bugs and seeds (whole insects for bugs, and 50 mg for seeds) were ground to a powder (Retsch Mixer Mill 300). 1.6 mL methanol (meOH) with 20 µg digitoxin (Sigma-Aldrich, MO, USA) was added to each sample, along with ~30 FastPrep beads. Samples were agitated twice for 45s at 6.5 m/s on a FastPrep-24 homogenizer and centrifugated for 15 min at 12,000 rpm. Solids were discarded, and samples were dried in a centrifugal concentrator at 35 ^{o}C. Samples were resuspended in 250 µL of meOH, and each resuspended sample was vortexed 3 times with 1.5 mL of hexanes and centrifugated for 15 min at 12,000 rpm. The hexane layer was discarded to de-fat each sample. Samples were once again dried down, resuspended in 250 µL of MeOH, and filtered with a MultiScreen Solvinert 0.45 µm low-binding hydrophobic PTFE 96-well plate (Millipore, Burlington, Mass). We analyzed samples using HPLC following the method used by Züst et al. (2019) (1). Fifteen µL of the samples were injected into an Agilent 1100 series HPLC (Agilent, Waldbronn, Germany) equipped with a diode-array detector. Cardenolides were eluted at a flow rate of 0.7 mL/min across an acetonitrile gradient (0–2 min 16% acetonitrile, 24 min 70% acetonitrile, 30-40 min 95% acetonitrile, and a 10 min post-run with 16% acetonitrile).

Chromatographic quantification: Peak areas were calculated from HPLC chromatograms using the chromatographR package (2). ChemStation *.uv* files were imported into R separately for each bug-species × seed × experimental-block combination because cardenolide extraction and HPLC runs were performed independently for each block. Parametric time warping (using chromatographR's "correct_rt()" function) was performed to correct for between-sample variation in compound retention times at 218 nm, the typical cardenolide absorption maximum (3). Automated filtering was then applied to include only samples with a single absorption maximum between 216 and 240 nm and no other substantial maxima (>1/4 the area of the 216-240 peak area) elsewhere in the spectrum. After automated filtering, candidate absorption spectra were manually assessed to verify a symmetrical absorption maximum between 216 and 222 nm. Peaks meeting this criterion were considered to be cardenolides and their areas at 218 nm were output for downstream analyses (4). Scripts used to identify cardenolide peaks from chromatogram data are available at https://github.com/nathanieljcarlson/cardenolideR and can be applied using the convenience function "ID_cardenolides".

Cardenolide concentrations were calculated from peak areas at 218 nm and standardized by the mass of digitoxin added to each sample and by the sample's dry mass. These individual cardenolide concentrations were summed for each sample to calculate the total cardenolide concentrations presented in Fig. 3a & 4b. Additionally, we identified cardenolides from bugs and their seed diet with shared retention times. These cardenolides were assumed to have been sequestered intact, and the proportion these cardenolides represented of total sequestered cardenolides was calculated for each insect (Fig. 3b).

Cardenolide structural characterization: Seeds (100 g) were freeze-dried, ground, and then extracted with MeOH (1 L) for a week. The extract was washed with hexane (100 mL) a few times followed by drying. The remaining residue was then suspended in 16% acetonitrile and water (9 mL), sonicated for 30 s, vortexed, centrifuged at 20,800×g for 12 min. After centrifugation the clear supernatant then immediately inject for preparative HPLC fractionation. Samples were injected into an Agilent 1260 series preparative LC system with an Agilent 21.2 mm×150 mm, C18, 5 µm column. Each first-pass injection was eluted at a constant flow rate of 14.87 mL/min with a gradient of acetonitrile and water: 0 to 2 min at 16% acetonitrile, 2 to 25 min from 16 to 70%, 25 to 30 min from 70 to 95%, and 30 to 35 min at 95%. Target peaks were detected at 218 nm. In many cases, each first-pass target fraction required drying down, resuspension in 16% acetonitrile, and reinjection for further cleanup. The isolated fractions were pooled, dried, resuspended in 0.5 mL of 100% methanol, and then analyzed on the Dionex 3000 LC reversed-

phase chromatography system coupled to an Orbitrap Q-Exactive mass spectrometer UPLC-HRMS system in positive and negative ionization modes for quality check following the method used by Agrawal et al. (2022) (5).MS2 spectra were deposited on GNPS link.

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 600 Avance III HD console and $BBFO^+$ cryoprobe. All 1D data (^1H, ^{13}C) and 2D data (DQCOSY, gCOSY, HSQCAD, gHMBCAD, HSQCTOCSY, ROESYAD) were deposited on Dryad Research Data Repository (https://doi.org/10.5061/dryad.c2fqz61jz). NMR data were processed with MestReNova version 15.0.0-34764 Mestrelab Research S.L. Sample was measured in CD_3OD (99.5%) and the residual solvent signals were δ_H 3.31/δ_C 49.15.

Figures

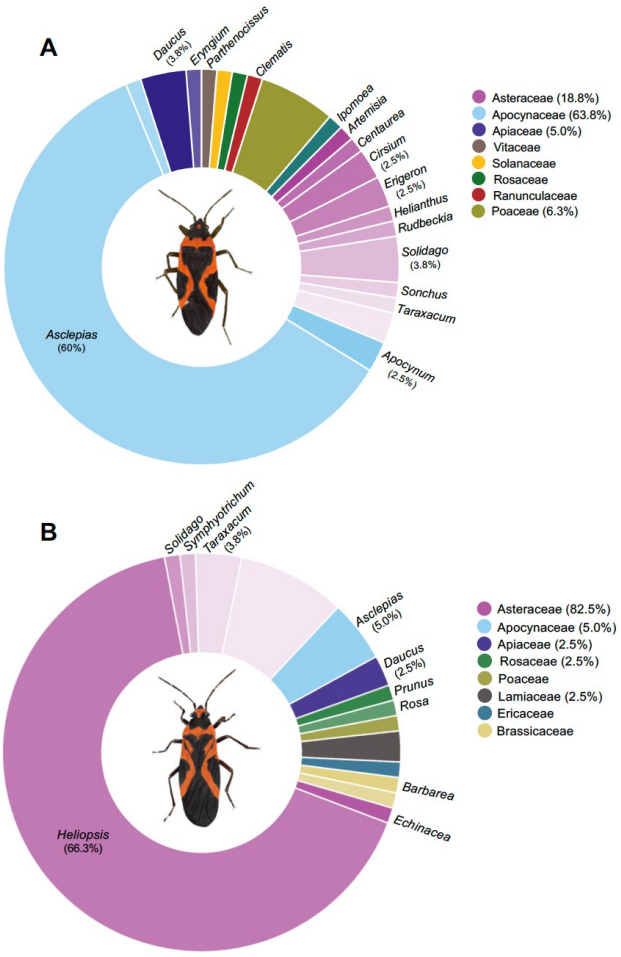


Fig. S1. Pie charts showing plant associations of A) *Lygaeus kalmii* and B) *L. turcicus* (n=80, each species) based on observations reported to iNaturalist. Pie segments are labelled with plant genus and color-coded by family. Plants that could not be identified to genus are grouped in unlabeled slices. The percent of total observations for each plant genus and family are listed next to their respective labels, except for singleton observations.

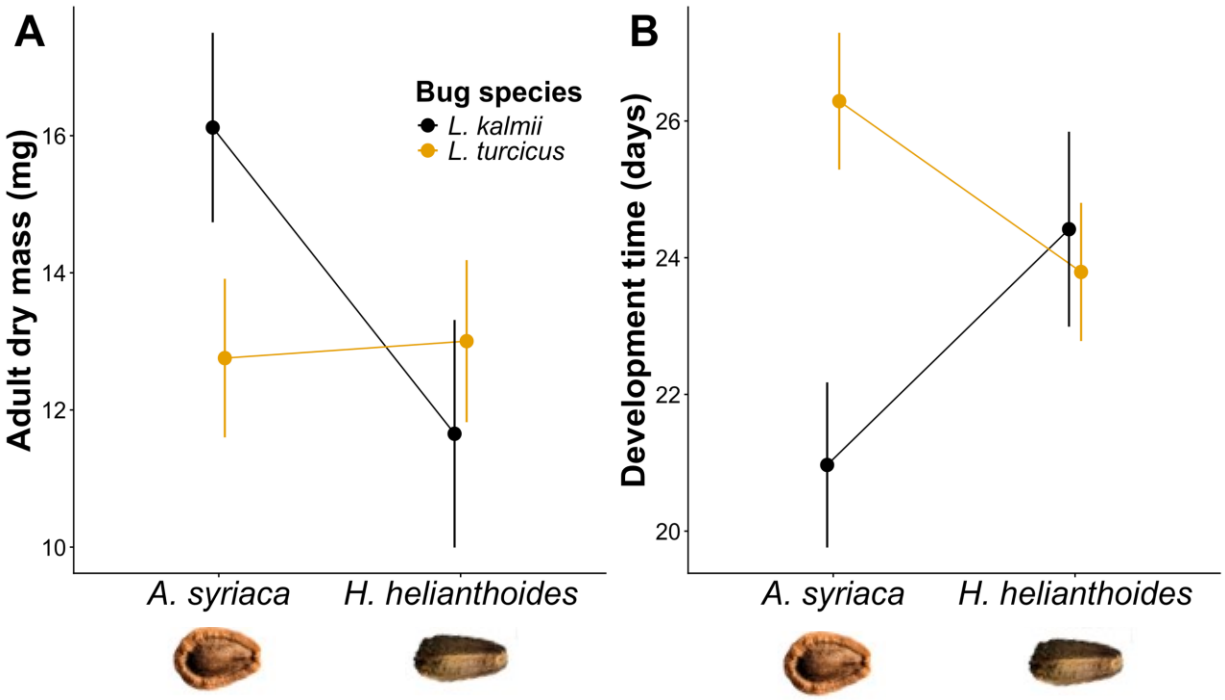


Fig. S2: Development time (A) and adult mass (B) of *Lygaeus kalmii* and *L. turcicus* on each bug's host plant. Both metrics of performance showed a concordant pattern, with *L. kalmii* developing faster and growing to higher masses on milkweed than false sunflower, while *L. turcicus* performed comparably poorly (both in development time and adult mass) on both plants. However, this trend was only significant for development time (adult mass seed type x bug species $F_{1,10.84} = 3.11$, $p = 0.105$; development time seed type x bug species $F_{1,11.19} = 6.59$, $p = 0.025$). Least squares means ± standard errors shown.

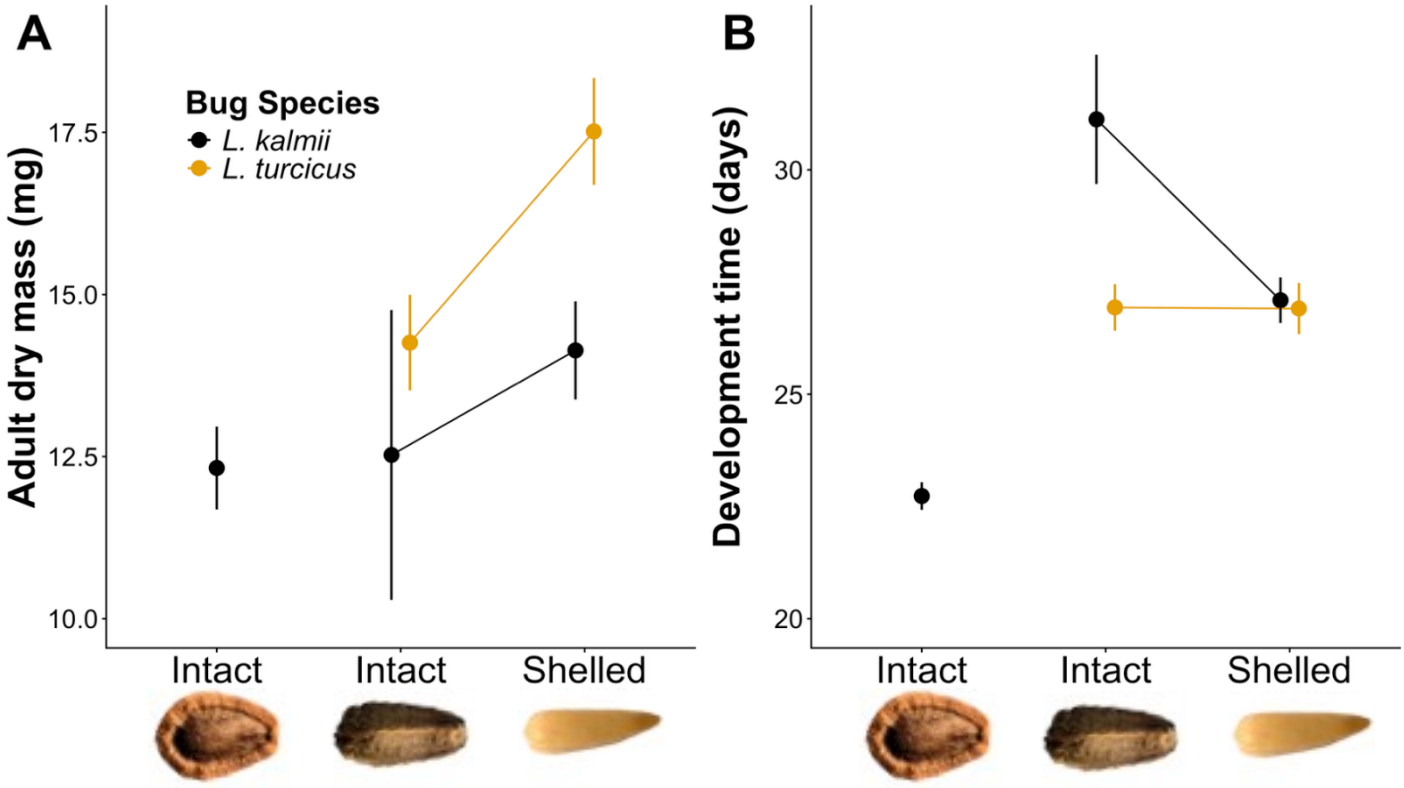


Fig. S3. (A) Adult mass for *L. kalmii* and *L. turcicus* on intact milkweed seed, intact Heliopsis, and shelled Heliopsis. There is only a significant ANOVA effect of sex ($F_{1,91.34} = 21.39$, $p < 0.001$), while there is a nearly-significant effect of bug species ($F_{1,52.54} = 3.79$, $p = 0.057$) and shelling ($p < 0.067$). There is still no shelling x species interaction effect. (B) Development time for *L. kalmii* and *L. turcicus*. There is a significant ANOVA effect of shelling ($F_{1,45.015} = 5.61$, $p = 0.022$) and bug species ($F_{1,45.35} = 6.46$, $p = 0.015$), but no significant sex-level effect. There is a significant shelling x bug species interaction effect ($F_{1,44.45} = 5.53$, $p = 0.023$), with only *L. kalmii* developing faster on shelled than unshelled seeds (Tukey's HSD $p = 0.024$). Least squares means ± SEs shown.

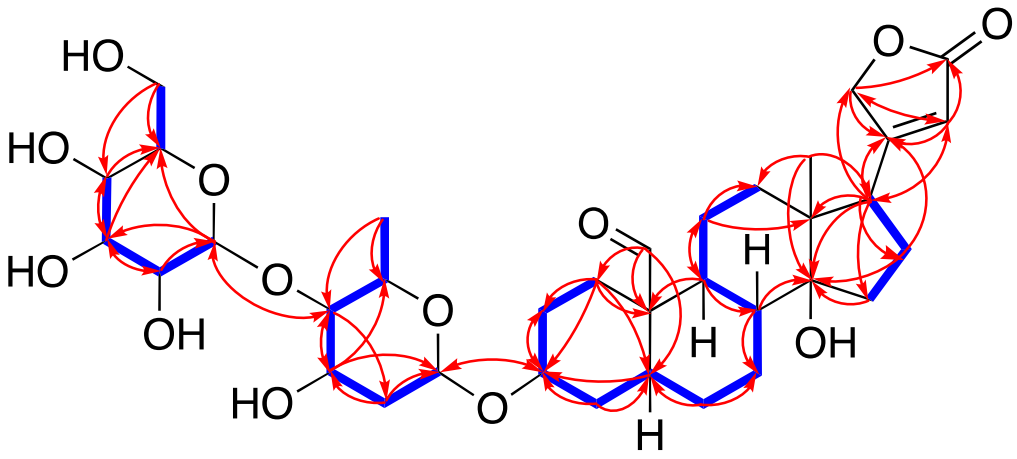


Figure S4. Key COSY (in bold blue lines) and HMBC correlations (in red arrows) from the cardenolide isolated from *Heliopsis helianthoides* (Asteraceae) seeds.

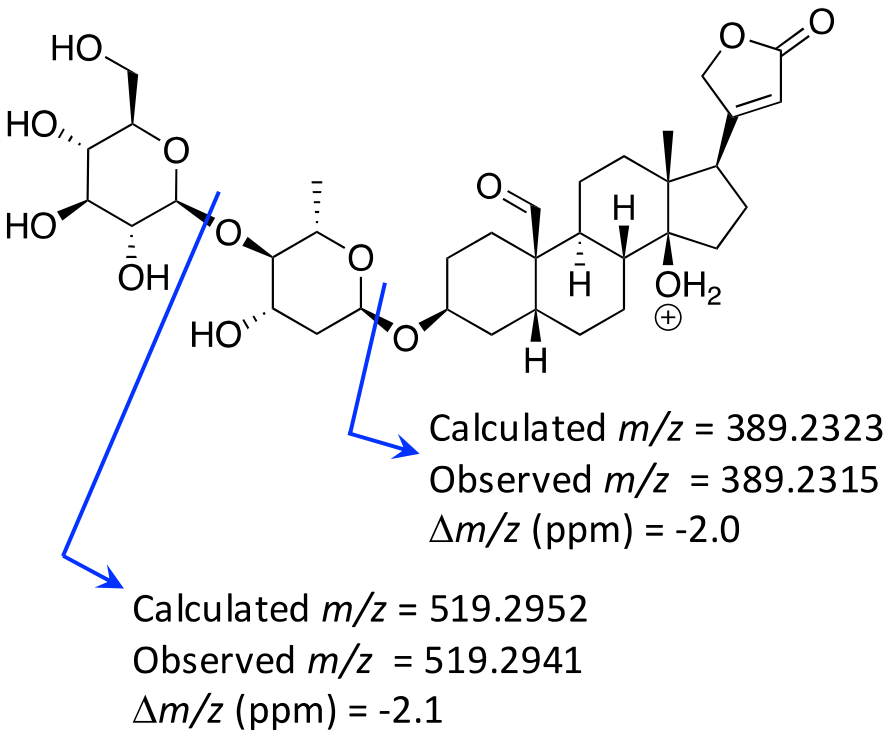
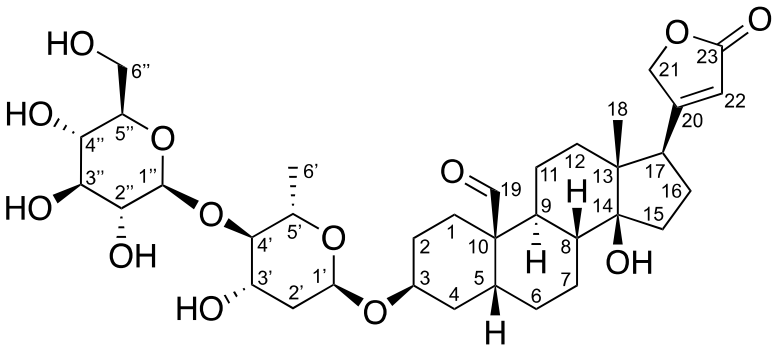


Figure S5. MS/MS fragment detected of the isolated cardenolide from *Heliopsis helianthoides* (Asteraceae) seeds.



Chemical Formula: $C_{35}H_{52}O_{13}$
Exact Mass: 680.3408

4’-O-β-D-glucopyranosyl-3-O-β-D-2’,6’-dideoxy-allopyranosyl corotoxigenin

Figure S6. Structure, formula, exact mass, and name of the main cardenolide isolated from *Heliopsis helianthoides* (Asteraceae) seeds.

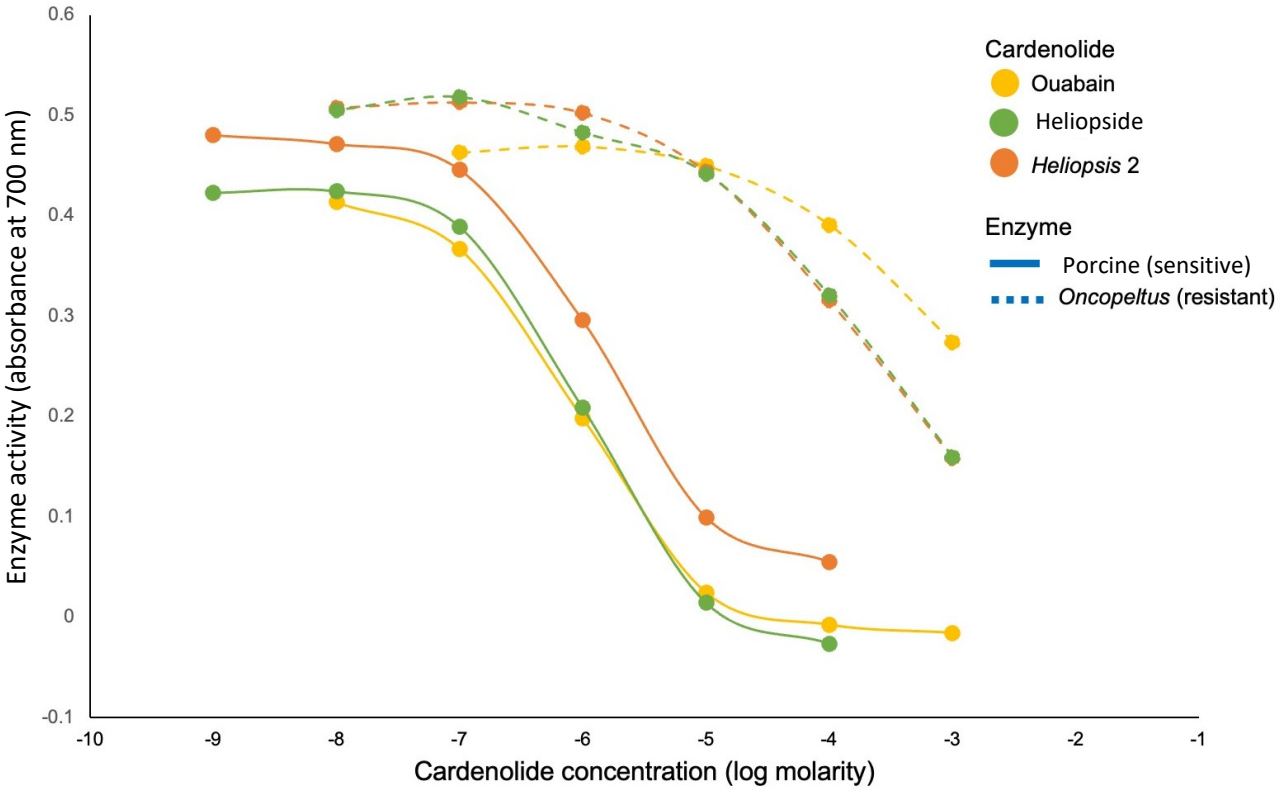


Figure S7. Plot of results from an Na+/K⁺-ATPase inhibition assay of *Heliopsis helianthoides* cardenolides against a sensitive (porcine) enzyme (represented by solid lines) and a resistant (*Oncopeltus fasciatus*—a lygaeine seed bug) enzyme (represented by dashed lines). We tested both enzymes against three cardenolides: ouabain—a cardenolide found in the Apocynaceae, the dominant cardenolide heliopside, isolated from *H. helianthoides* seeds); and the second most abundant compound from *H. helianthoides* seeds (*Heliopsis* 2, unidentified, retention time 13.78 in Fig. S8). The y-axis reflects the level of inhibition of the enzyme at a given cardenolide concentration (x-axis). Both *Heliopsis* cardenolides are comparably toxic to ouabain when on the resistant and nonresistant sodium pumps.

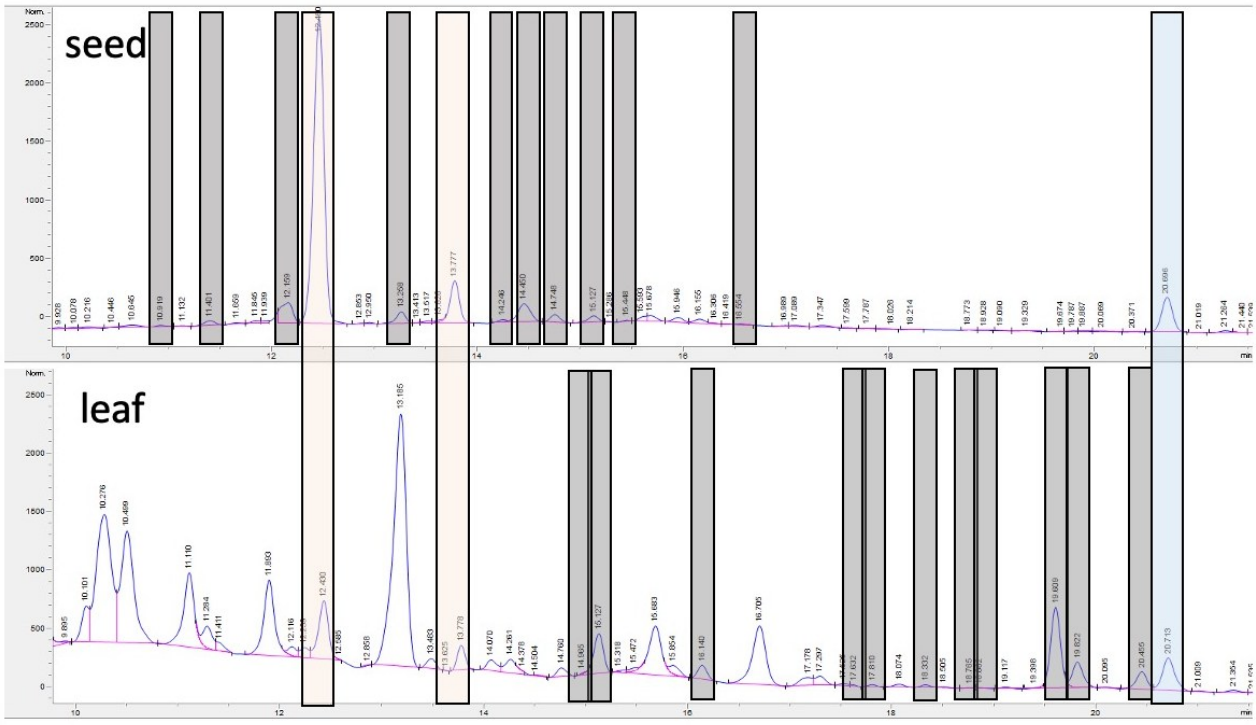


Figure S8. HPLC chromatographic traces of seed and leaf extracts from *Heliopsis helianthoides* (Asteraceae). Digitoxin (internal standard) is highlighted in blue, and the two compounds isolated from *H. helianthoides* seeds are highlighted in tan (retention times 12.4 is heliopside and an unknown cardenolide at 13.78). Then the remaining cardenolide compounds (based on HPLC-UV) are highlighted in grey. The X axis represents minutes of retention on the column, with 10 min corresponding to 34.8% acetonitrile, and 21 min to 60.6% acetonitrile.

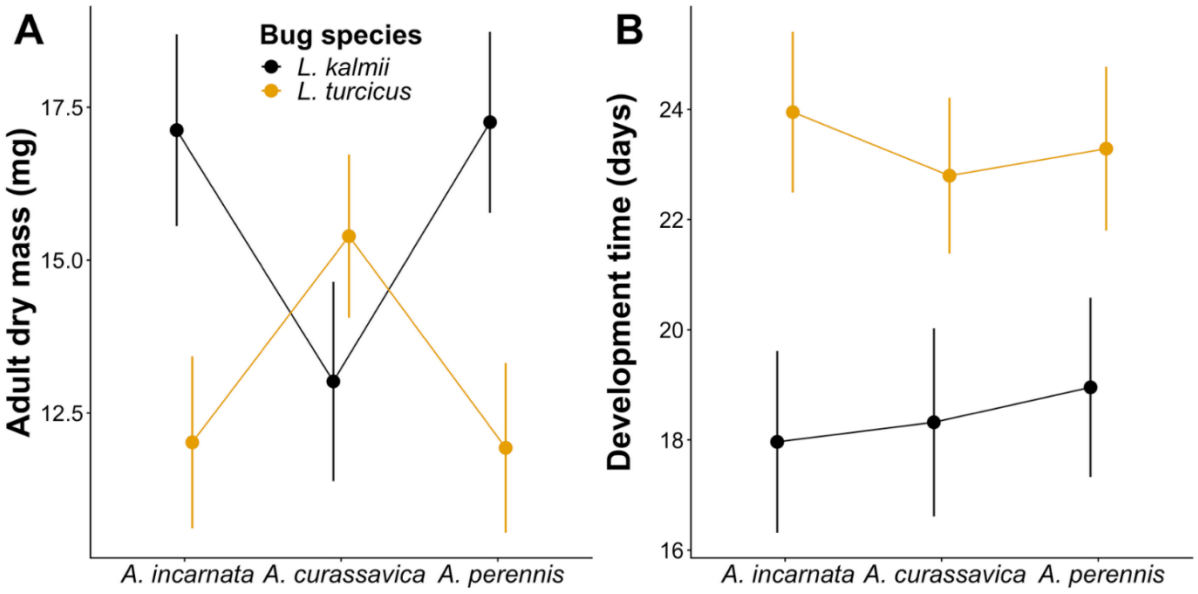


Figure S9. (A) Adult dry mass and (B) development time (days to develop to adulthood) of bugs when reared on milkweed seeds representing a gradient of cardenolide content, from low cardenolides (left) to high cardenolides (right). Least squares means and standard errors are shown. *Lygaeus turcicus* uniformly develops slower than *L. kalmii* across milkweed species (seed type x bug species $F_{2,24} = 0.057$, $p>0.1$), while the two species converge in growth performance (as measured by adult mass) on the intermediate-cardenolide *Asclepias curassavica* (seed type x bug species $F_{2,14.75} = 4.61$, $p = 0.028$; Tukey's HSD $p = 0.26$ on *A. curassavica*).

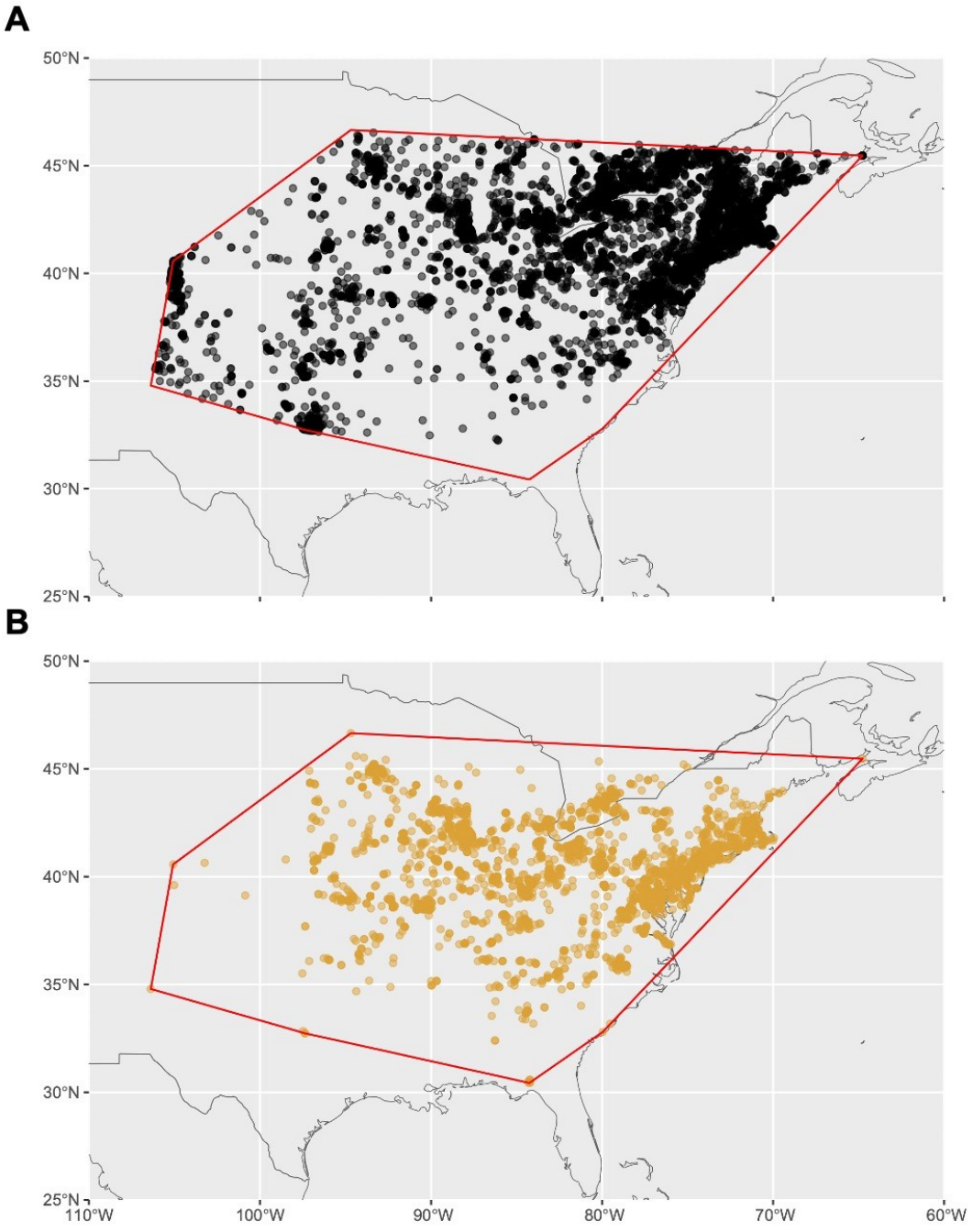


Figure S10. Distribution of iNaturalist observations of (A) *Lygaeus kalmii* and (B) *L. turcicus* to generate Figure 1. Observations were only used from the minimum convex polygon entirely bounding the ranges of both species (red polygon shown). As *L. turcicus*'s range is entirely bounded within the range of *L. kalmii*, this polygon represents the full extent of *L. turcicus's* range. Datapoints for plant identification were sampled from 10 evenly-spaced latitudes and 20 evenly-spaced longitudes from within this polygon.

Tables

Table S1. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) in CD_3OD (*J* in Hz, chemical shifts in ppm) for heliopside, the main cardenolide isolated in *Heliopsis helianthoides.*

Position	$\delta^{1}H$	$\delta^{13}C$
1	1.04 td (14.6, 3.6)	29.9
	2.40 td (13.6, 3.6)	
2	1.26 m	29.5
	1.96 m	
3	3.71 m	75.5
4	1.20 m	39.9
	1.85 m	
5	1.40 m	41.9
6	1.59 m	27.4
	1.98 m	
7	1.26 m	26.4
	2.21 m	
8	1.60 m	41.7
9	1.31 m	47.4
10		50.7
11	1.24 m	20.7
	1.73 m	
12	1.42 m	38.2
	1.50 m	
13		48.6
14		83.6
15	1.68 m	30.6
	2.08 m	
16	1.86 m	25.7
	2.15 m	
17	2.82 dd (9.3, 5.5)	49.7
18	0.82 s	14.0
19	9.99 s	208.1
20		176.0
21	4.90 dd (18.5, 1.6)	73.1
	5.01 dd (19.2, 1.5)	
22	5.89 s	115.7
23		175.0
1'	4.92 dd (8.8, 2.0)	95.5
2'	1.64 m	33.0
	1.86 m	
3'	3.83 q (3.2)	64.3
4'	3.44 m	73.7
5'	4.03 m	67.9
6'	1.24 d (6.5)	15.0
1''	4.36 d (7.7)	100.0
2''	3.19 m	72.7
3''	3.34 m	75.7
4''	3.26 m	69.7
5''	3.23 m	75.9
6''	3.64 dd (11.7, 5.6)	60.8
	3.86 dd (11.7, 1.8)	

Table S2. HRMS data of heliopside, the main cardenolide isolated in *Heliopsis helianthoides*.

Formula	Precursor ion	Observed *m/z*	Calculated *m/z*	Δ*m/z* (ppm)
$C_{35}H_{52}O_{13}$	$[M+H]^+$	681.3474	681.3481	-1.0
	$[M+NH4]^+$	698.3727	698.3746	-2.7
	$[M+Na]^+$	703.3281	703.3300	-2.7
	$[M-H]^-$	679.3320	679.3335	-2.2
	$[M+FA-H]^-$	725.3375	725.3390	-2.0

Table S3: ANOVA summary of complex model (including the initial number of bugs in a replicate) for all *A. syriaca* vs. *H. helianthoides* comparisons. Seed = seed species. Species = bug species.

	Effect	Sum Squares	Mean Square	Num DF	Den DF	F Stat	P Value	
Development Time	Seed	2.301	2.301	1	11.345	0.846	0.377	
	Species	3.475	3.475	1	10.938	1.277	0.283	
	Sex	0.515	0.515	1	23.899	0.189	0.667	
	block	6.128	6.128	1	9.116	2.252	0.167	
	Initial_bugs	3.917	3.917	1	9.192	1.44	0.26	
	Seed:Species	22.385	22.385	1	11.61	8.228	0.015	*
	Seed:Sex	1.235	1.235	1	23.914	0.454	0.507	
	Species:Sex	5.577	5.577	1	23.893	2.05	0.165	
	Seed:Species:Sex	1.636	1.636	1	23.918	0.601	0.446	
Growth Rate	Seed	0.035	0.035	1	10.9	2.024	0.183	
	Species	0.033	0.033	1	10.266	1.935	0.194	
	Sex	0.055	0.055	1	23.924	3.216	0.086	
	block	5.84E-04	5.84E-04	1	7.978	0.034	0.858	
	Initial_bugs	1.16E-03	1.16E-03	1	8.019	0.068	0.801	
	Seed:Species	0.088	0.088	1	11.288	5.122	0.044	*
	Seed:Sex	0.018	0.018	1	23.942	1.042	0.318	
	Species:Sex	0.022	0.022	1	23.915	1.278	0.27	
	Seed:Species:Sex	0.019	0.019	1	23.95	1.095	0.306	
Adult Mass	Seed	8.419	8.419	1	11.865	1.684	0.219	
	Species	2.005	2.005	1	11.371	0.401	0.539	
	Sex	18.193	18.193	1	23.935	3.64	0.068	
	block	2.32	2.32	1	9.305	0.464	0.512	
	Initial_bugs	0.012	0.012	1	9.375	2.39E-03	0.962	
	Seed:Species	10.755	10.755	1	12.178	2.152	0.168	
	Seed:Sex	4.131	4.131	1	23.947	0.826	0.372	
	Species:Sex	3.509	3.509	1	23.929	0.702	0.41	
	Seed:Species:Sex	5.121	5.121	1	23.952	1.024	0.322	

Proportion Cardenolides Modified	Seed	477.471	477.471	1	10.44	3.438	0.092	
	Species	127.347	127.347	1	9.614	0.917	0.362	
	Sex	91.957	91.957	1	23.868	0.662	0.424	
	Block	279.321	279.321	1	7.132	2.011	0.198	
	Initial_bugs	52.837	52.837	1	7.121	0.38	0.557	
	Seed:Species	188.614	188.614	1	10.913	1.358	0.269	
	Seed:Sex	0.584	0.584	1	23.904	4.21E-03	0.949	
	Species:Sex	8.376	8.376	1	23.855	0.06	0.808	
	Seed:Species:Sex	4.058	4.058	1	23.917	0.029	0.866	
Total Cardenolides	Seed	12.159	12.159	1	10.372	6.615	0.027	*
	Species	3.213	3.213	1	9.896	1.748	0.216	
	Sex	3.218	3.218	1	23.915	1.751	0.198	
	block	0.327	0.327	1	7.944	0.178	0.684	
	Initial_bugs	0.571	0.571	1	8.009	0.311	0.592	
	Seed:Species	4.552	4.552	1	10.678	2.476	0.145	
	Seed:Sex	0.197	0.197	1	23.931	0.107	0.746	
	Species:Sex	6.876	6.876	1	23.908	3.741	0.065	
	Seed:Species:Sex	0.33	0.33	1	23.937	0.18	0.675	

Table S4: ANOVA summary of complex model (including the initial number of bugs in a replicate) for all comparisons across a milkweed cardenolide gradient (*A. incarnata*, *A. curassavica*, and *A. perennis*. Seed = seed species. Species = bug species.

	Effect	Sum Squares	Mean Square	Num DF	Den DF	F Stat	P Value	
Development Time	Seed	0.075	0.037	2	16.53	0.022	0.979	
	Species	51.568	51.568	1	15.997	29.93	5.13E-05	*
	Sex	3.437	3.437	1	42.054	1.995	0.165	
	block	7.517	7.517	1	12.535	4.363	0.058	
	Initial_bugs	0.872	0.872	1	13.148	0.506	0.489	
	Seed:Species	4.694	2.347	2	16.322	1.362	0.284	
	Seed:Sex	0.736	0.368	2	40.177	0.213	0.809	
	Species:Sex	2.627	2.627	1	42.169	1.525	0.224	
	Seed:Species:Sex	1.808	0.904	2	40.712	0.525	0.596	
Growth Rate	Seed	3.15E-03	1.57E-03	2	14.404	0.075	0.928	
	Species	0.25	0.25	1	13.897	11.855	4.00E-03	*
	Sex	0.227	0.227	1	41.968	10.77	2.08E-03	*
	block	7.00E-04	7.00E-04	1	10.507	0.033	0.859	
	Initial_bugs	3.76E-03	3.76E-03	1	11.075	0.178	0.681	
	Seed:Species	0.143	0.071	2	14.203	3.387	0.063	
	Seed:Sex	0.015	7.44E-03	2	40.068	0.353	0.705	
	Species:Sex	3.89E-03	3.89E-03	1	42.091	0.185	0.669	
	Seed:Species:Sex	0.014	7.25E-03	2	40.668	0.344	0.711	
Adult Mass	Seed	0.573	0.287	2	13.681	0.04	0.961	
	Species	28.281	28.281	1	13.185	3.918	0.069	
	Sex	108.325	108.325	1	42.546	15.006	3.64E-04	*
	block	4.101	4.101	1	9.723	0.568	0.469	
	Initial_bugs	1.644	1.644	1	10.281	0.228	0.643	
	Seed:Species	58.677	29.338	2	13.477	4.064	0.042	*
	Seed:Sex	5.538	2.769	2	40.793	0.384	0.684	
	Species:Sex	6.628	6.628	1	42.669	0.918	0.343	
	Seed:Species:Sex	6.32	3.16	2	41.409	0.438	0.648	
Total Cardenolides	Seed	142.232	71.116	2	18.31	37.074	3.65E-07	*
	Species	35.941	35.941	1	17.739	18.736	4.17E-04	*
	Sex	30.807	30.807	1	45.884	16.06	2.23E-04	*
	block	8.455	8.455	1	13.036	4.407	0.056	
	Initial_bugs	9.533	9.533	1	13.775	4.969	0.043	*
	Seed:Species	27.703	13.851	2	18.047	7.221	4.97E-03	*
	Seed:Sex	9.332	4.666	2	45.016	2.432	0.099	
	Species:Sex	5.773	5.773	1	45.961	3.009	0.089	
	Seed:Species:Sex	1.557	0.779	2	45.416	0.406	0.669	

SI References

1. T. Züst, G. Petschenka, A. P. Hastings, A. A. Agrawal, Toxicity of Milkweed Leaves and Latex: Chromatographic Quantification Versus Biological Activity of Cardenolides in 16 Asclepias Species. *J Chem Ecol* **45**, 50–60 (2019).

2. E. Bass, chromatographR: Chromatographic Data Analysis Toolset. (2024). https://doi.org/10.5281/zenodo.10934303. Deposited 6 April 2024.

3. "Quantification of plant cardenolides by HPLC, measurement of Na+/K+-ATPase inhibition activity, and characterization of target enzymes" in *Methods in Enzymology*, (Academic Press, 2023), pp. 275–302.

4. S. B. Malcolm, M. P. Zalucki, Milkweed latex and cardenolide induction may resolve the lethal plant defence paradox. *Entomologia Experimentalis et Applicata* **80**, 193–196 (1996).

5. A. A. Agrawal, *et al.*, Functional evidence supports adaptive plant chemical defense along a geographical cline. *Proceedings of the National Academy of Sciences* **119**, e2205073119 (2022).