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Convergence and Divergence among Herbivorous Insects Specialized on Toxic Plants: Revealing Syndromes among the Cardenolide Feeders across the Insect Tree of Life

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abstract: Repeatable macroevolutionary patterns provide hope for rules in biology, especially when we can decipher the underlying mechanisms. Here we synthesize natural history, genetic adaptations, and toxin sequestration in herbivorous insects that specialize on plants with cardiac glycoside defenses. Work on the monarch butterfly provided a model for evolution of the "sequestering specialist syndrome," where specific amino acid substitutions in the insect's Na^+/K^+-ATPase are associated with (1) high toxin resistance (target site insensitivity [TSI]), (2) sequestration of toxins, and (3) aposematic coloration. We evaluate convergence for these traits within and between Lepidoptera, Coleoptera, Diptera, Hemiptera, Hymenoptera, and Orthoptera, encompassing hundreds of toxin-adapted species. Using new and existing data on ~28 origins of specialization, we show that the monarch model evolved independently in five taxonomic orders (but not Diptera). An additional syndrome occurs in five orders (all but Hymenoptera): aposematic sequesterers with modest to medium TSI. Indeed, all sequestering species were aposematic, and all but one had at least modest TSI. Additionally, several species were aposematic nonsequesterers (potential Batesian mimics), and this combination evolved in species with a range of TSI levels. Finally, we identified some biases among these strategies within taxonomic orders. Biodiversity in this microcosm of life evolved repeatedly with a high degree of similarity across six taxonomic orders, yet we identified alternative trait combinations as well as lineage-specific outcomes.

Keywords: aposematism, convergent evolution, *Danaus plexippus*, sequestration, sodium-potassium ATPase, specialist-generalist.

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Introduction and Conceptual Background

Convergence—the repeated and independent evolution of phenotypic traits associated with particular environments—is widespread in organismal diversity and has long been interpreted with fascination by biologists. In *On the Origin of Species*, Darwin (1859) considered similarities among organisms and concluded that some were the result of common ancestry (and were thus useful for classification). Others were the result of independent evolution and thus not useful for classification; on this issue he quipped, "thus Linnaeus, misled by external appearances, actually classed an homopterous insect as a moth" (Darwin 1859, p. 427). This duality in the causes of shared phenotypic traits is of long-standing importance in our understanding of biodiversity and its origins.

Convergence highlights that there is predictability in the outcome of the evolutionary process, although the causes of this predictability are often obscure (Stayton 2015*b*; Blount et al. 2018). A conundrum in evolutionary ecology has been interpreting convergence in terms of adaptation versus constraint (Losos 2011; Agrawal 2017*b*). For example, repeated associations with environmental conditions (e.g., plant succulence in hot, arid environments), especially when coupled with functional analyses, is indicative of adaptation. Nonetheless, when phenotypes—and even their molecular mechanisms—converge, one interpretation is that the outcome was constrained: there were few other options. Given that all adaptation is constrained at some level, the question

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becomes: when and why do we see convergent evolution, and what is the relative importance of constraint versus shared selective pressures in driving convergence?

Two aspects of scale bear on the interpretation and the generalizability of convergence. The first is the extent to which phenotypes or their underlying mechanisms are convergent. For example, flight has independently evolved in vertebrates and insects at a coarse-grained level, since they have distinct mechanistic bases. The evolution of wings may thus represent what has been termed "many-to-one mapping," as disparate morphologies have led to the flying phenotype (Wainwright et al. 2005). Conversely, the physiological basis of tolerance to particular toxins has been shown to convergently evolve at the molecular level in some vertebrates and insects (Ujvari et al. 2015; Yoshida et al. 2020; Groen and Whiteman 2021; Mohammadi et al. 2022; Tarvin et al. 2023). Such fine-grained convergence may be due to more general constraints—the existence of very few specific evolutionary solutions to an environmental challenge.

The second aspect of scale is the extent to which taxa with convergent traits have diverged since a common ancestor. Are they recently diverged (e.g., congeners) or in distantly related lineages? Here phylogenetic scale intersects with mechanisms of convergence: a general prediction is that mechanisms of convergent traits are more likely to repeat at shallower phylogenetic scales because the physiological underpinnings are more likely to be shared among close relatives (Storz 2016; Agrawal 2017*b*). Accordingly, recent work has suggested a phylogenetic conservatism of convergence itself—that is, the mechanisms of convergence tend to repeat themselves within lineages while varying across lineages (Natarajan et al. 2016; Edwards 2019; Mohammadi et al. 2022). Although fine-grained convergent evolution at a shallow phylogenetic scale certainly represents repeatability, it is unclear how often such convergence occurs among more distantly distributed clades. Crossing phylogenetic scales, within and between clades, can be important in interpreting the extent and type of convergence.

In the current study, we consider coarse-grained phenotypes (sequestration and aposematism) as well as an underlying fine-grained mechanism of toxin resistance (target site insensitivity [TSI]), within and between clades, to address the causes of convergent evolution in specialist herbivores.

Milkweeds, Monarchs, and Beyond

Monarchs and milkweeds have provided a model for how coevolution may proceed, leading to specialization and the evolution of offense-defense syndromes in plants and their herbivores. We define syndromes as repeatedly evolved sets of traits associated with particular ecological conditions (Wilson et al. 2004; Agrawal and Fishbein 2006; Adler et al. 2014; Lagomarsino et al. 2017; Healy et al. 2019). In this study, we address the sequestration syndrome in insect herbivores specializing on plants that produce toxic cardiac glycosides (cardenolides and bufadienolides, with a focus on the former; see the history presented in table S1; tables S1–S4 are available online). In particular, we are interested in when, how, and why there is repeatability in the evolution of trait combinations associated with these specialized herbivores.

We have been studying the community of specialized insect herbivores that consume milkweed plants (genus *Asclepias*), which produce cardenolides, a class of steroidal toxins that poison the universal animal transmembrane enzyme, the Na^+/K^+-ATPase (sodium pump, for short). Past studies have suggested that this group of insects typically (1) gain tolerance to cardenolides via shared specific amino acid substitutions at a handful of positions in the Na^+/K^+-ATPase, (2) sequester cardenolides as a defense against predators, and (3) are aposematically colored (figs. 1, 2). These three traits form the basis of what we have termed the "sequestering specialist syndrome," as demonstrated for monarch butterflies (history summarized in Agrawal 2017*a*) and subsequently for beetles (Labeyrie and Dobler 2004), true bugs (Bramer et al. 2015; Moore and Scudder 1986), and grasshoppers (Dobler et al. 2019; Yang et al. 2019). Although this bird's-eye view revealed remarkable convergent evolution at several levels, tremendous variation exists in each of these characteristics within and between lineages. Convergence in the milkweed herbivore community appears incomplete (or imperfect; Kikuchi and Pfennig 2013; Stayton 2015*a*), perhaps due to differences in the genetic backgrounds, habitats, or specific diets of the different insect groups that have independently evolved specialization on cardenolide-producing plants.

Given the diversity of insect species adapted to cardenolide toxins (hundreds of species across six taxonomic orders), here we address the generality of the sequestering specialist syndrome, linking a mechanistic basis for toxin resistance (mainly substitutions at amino acid positions 111 and 122 in the alpha subunit of the sodium pump, ATPα1; Dobler et al. 2012; Zhen et al. 2012), sequestration of cardenolides into the adult stage, and aposematic coloration. We note that although there are likely manifold mechanisms for animals to tolerate dietary cardenolides, here we focus on TSI—that is, sodium pump substitutions—because they provide a repeatable, mechanistic, and functionally validated basis for physiological resistance (first described in monarch butterflies; table S1; fig. 2).

Across animals, non-cardenolide-adapted species typically have a glutamine residue at position 111 and an asparagine residue at position 122 (Q111 and N122) in their

Figure 1: Specialist herbivores of Apocynaceae examined in this study. *A*, Larva and adult of the monarch butterfly (*Danaus plexippus*). *B*, Large and small milkweed bugs (*Oncopeltus fasciatus* and *Lygaeus kalmii*, respectively). *C*, Four-eyed red milkweed beetle (*Tetraopes tetrophthalmus*). *D*, Milkweed tiger moth (*Euchaetes egle*). *E*, Dogbane tiger moth (*Cycnia tenera*). *F*, Milkweed leaf-miner fly larva and adult (*Liriomyza asclepiadis*). *G*, Unexpected cycnia (*Cycnia inopinatus*). *H*, Milkweed leaf beetle (*Labidomera clivicollis*; orange larva morph [*left*] and adult [*right*], respectively). *I*, Dogbane beetle (*Chrysochus auratus*). *J*, Dogbane saucrobotys moth (*Saucrobotys futilalis*). *K*, Black cone-headed grasshopper (*Poekilocerus bufonius*). *L*, Milkweed stem weevil (*Rhyssomatus lineaticollis*). Insects are not shown to scale. Photo credits: Ellen Woods (*A*, *B*, *C*, *D*, *F* [larva], *L*), Anurag Agrawal (*E*, *F* [adult], *G*, *H*, *I*, *J*), and Michael Blecher (*K*).

Na^+/K^+-ATPase. In contrast, monarch butterflies possess nucleotide substitutions in the underlying gene (ATPα1), yielding an enzyme subunit with valine at position 111 and histidine at position 122 (V111 and H122). These two substitutions are associated with the cardenolide-binding pocket of the enzyme and provide ~10-fold and ~25-fold resistance to cardenolides, respectively, but together synergize to provide >100-fold resistance (Dalla et al. 2013; Karageorgi et al. 2019; table 1). Accompanying this resistance in monarchs and some other species is sequestration of cardenolides in the wings and aposematic coloration, thus forming the model tested in the current study. Here we use the following working definition of aposematism: highly contrasting bright coloration that is typically not cryptic to humans when in a natural habitat.

The model provided by monarchs during the evolution of the Danaini (Petschenka et al. 2013*a*) has been suggested to hold across five taxonomic orders of milkweed

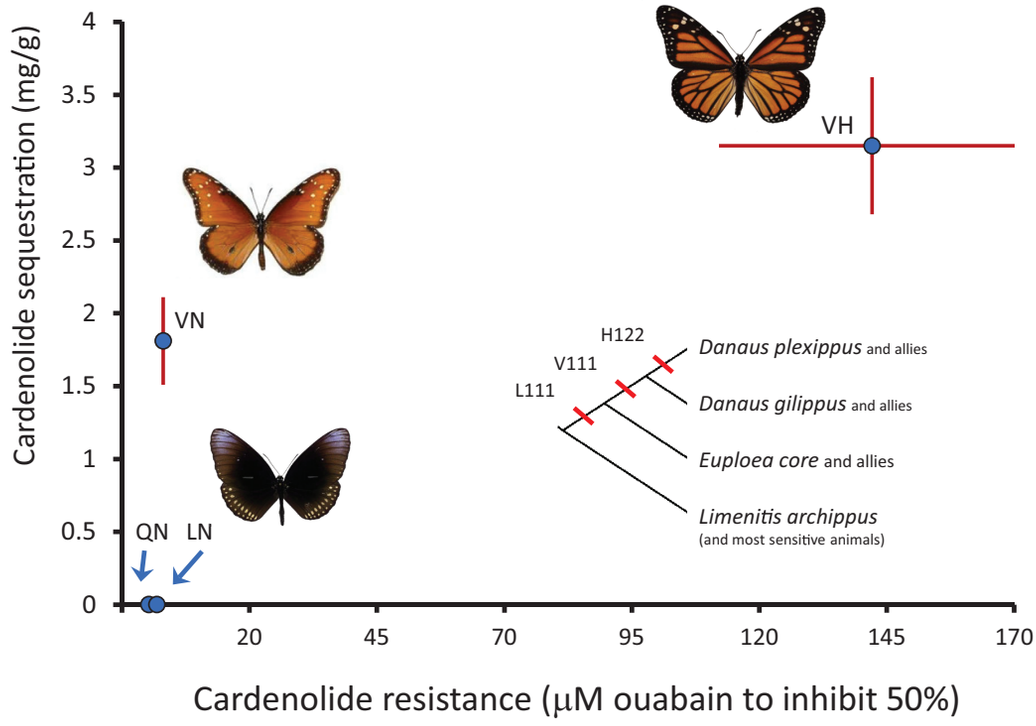


Figure 2: Monarch model for the evolutionary progression toward high levels of sequestration seen in the diversification of the milkweed butterflies (Danainae). Substitutions at amino acid position 111 preceded the more functionally important change at position 122 (ancestrally Q and N, respectively; based on Petschenka et al. 2013*a*; Karageorgi et al. 2019). In this scenario, early specialization of herbivores on cardenolide-containing plants occurs via modest resistance of the sodium pump (target site insensitivity) and presumably other adaptations that reduce physiological exposure to cardenolides. Sodium pump resistance to cardenolides is evolutionarily associated with increased ability to sequester them. On the *x*-axis, we present the concentration of a standard cardenolide toxin (ouabain) necessary to cause 50% inhibition of four genetically engineered *Drosophila* enzymes (IC_{50}; higher values indicate that the enzyme is more resistant; analyses based on data in Karageorgi et al. 2019); survival of transgenic *Drosophila* larvae and adults on diets with cardenolides follow the same pattern of sodium pump resistance (Karageorgi et al. 2019). The *y*-axis represents mean sequestration of cardenolides (in the caterpillar integument) when fed eight different species of *Asclepias* foliage (data from Petschenka and Agrawal 2015). Shown are least squares means ± SE and images for *Euploea core* (L111 N122), *Danaus gilippus* (V111 N122), and *D. plexippus* (V111 H122). Q111 N122 are shown as the ancestral amino acids, but no Q111 N122 Lepidopterans are known to sequester cardenolides.

specialists (Lepidoptera, Coleoptera, Diptera, Hemiptera, and Orthoptera; Petschenka et al. 2017; Karageorgi et al. 2019; Yang et al. 2019; fig. 3). Accordingly, for each group we evaluate the number of independent origins of cardenolide specialization (using natural history information and molecular phylogenies where available) and provide an assessment of sodium pump substitutions, sequestration, and aposematism using existing data and a substantial body of new sequencing and chemical analyses presented here. Where information is available, we include herbivores adapted to non-*Asclepias* members of Apocynaceae as well as plants from other botanical families that contain either cardenolides or bufadienolides. Thus, across ~28 origins of insect specialization on cardenolide plants, we ask the following questions.

1. How common is the sequestering specialist syndrome of coupled sodium pump substitutions (V111 and H122), sequestration, and aposematism? Are there other syndromes—repeated examples of particular combinations of specific amino acid substitutions, sequestration, and aposematism?

2. As predicted by the monarch model, is there a general evolutionary progression toward the sequestering specialist syndrome? Although we lack detailed phylogenetic information for most groups, we use taxonomic information and data on out-groups to infer preadaptations and the evolutionary pattern of syndrome evolution for the three sets of traits considered here.

3. Are there lineage-specific patterns in terms of the extent of these trait combinations or exceptions to them?

Given the existence of trait variation among cardenolide feeders, we anticipated that not all species would fall into the same syndrome, even when considering only the three sets of traits (sodium pump substitutions, sequestration,

Table 1: Resistance of engineered *Drosophila* sodium pumps (Na^+/K^+-ATPase)

Category	Amino acids at positions 111 and 122	IC_{50} μM ± SE (cell culture)	Representative insect species with this amino acid pattern
Susceptible wild type	QN	.093 ± .012[a]	*Drosophila melanogaster*,[b] *Euchaetes egle*
Modest resistance	LN	.468 ± .160[c]	*Tetraopes tetrophthalmus*, *Cycnia tenera*
Modest resistance	TN	1.448 ± .682	*Rhyssomatus lineaticollis* (Na^+/K^+-ATPase copy A)
Modest resistance	EN	1.085 ± .209	*Largus* spp., *Bemisia tabaci*[b]
Modest resistance	VN	1.591 ± .550	*Danaus gilippus*, *Leptinotarsa decemlineata*[b]
Medium resistance	QY	2.863 ± .368	*Rhyssomatus lineaticollis* (Na^+/K^+-ATPase copy B)
Medium resistance	QH	7.397 ± 1.411[a]	*Liriomyza asclepiadis*
High resistance	EY	12.557 ± .622	All sequenced Aphididae and *Myzocallis asclepiadis*
High resistance	TH	64.283 ± 7.239	*Oncopeltus fasciatus*, *Lygaeus kalmii*, *Monophadnus latus*
High resistance	HH	33.750 ± 1.510	*Phytomyza hellebori*
High resistance	VH	44.484 ± 3.645	*Danaus plexippus*, *Chrysochus auratus*, *Scelolyperus lecontii*

Note: Amino acids of interest were introduced to the *Drosophila* Na^+/K^+-ATPase, and the enzymes were produced in cell culture (all substitutions are in the same genetic background). Here we report the concentration of the standard cardenolide, ouabain, causing 50% inhibition of the enzyme (IC_{50}) in vitro. The effect of each amino acid substitution may be further enhanced by the genetic background of the specific species (Mohammadi et al. 2022; Agrawal and Hastings 2023).

[a] Data previously published in Dalla et al. (2017).

[b] Indicates non-cardenolide-adapted species.

[c] Data previously published in Karageorgi et al. (2019).

and aposematism). Thus, in figure 4 we present the 12 possible combinations of traits we examined. In addition to the sequestering specialist syndrome, we observed species with less than maximal sodium pump substitutions that nonetheless were sequesterers and aposematic. These species are predicted to use additional or alternative mechanisms to cope with cardenolides. Next, existing data indicated that not all aposematic species were sequesterers (Dobler et al. 2012), suggesting that some species may use other forms of defense or may be Batesian mimics. Finally, we predicted the lack of species with the following trait combinations (syndromes): (1) sequestering species without sodium pump substitutions (these are not expected because sequestering species bring toxic cardenolides into their bodies, which should require at least some protection of the Na^+/K^+-ATPase) and (2) sequestering species that lack aposematism (such species would not be expected to reap as many benefits of sequestration, as predators would not learn to avoid attack; Ruxton et al. 2019). Thus, in this study we evaluate the extent of convergence and frequency of the set of possible syndromes for herbivorous feeders on cardenolide plants (fig. 4).

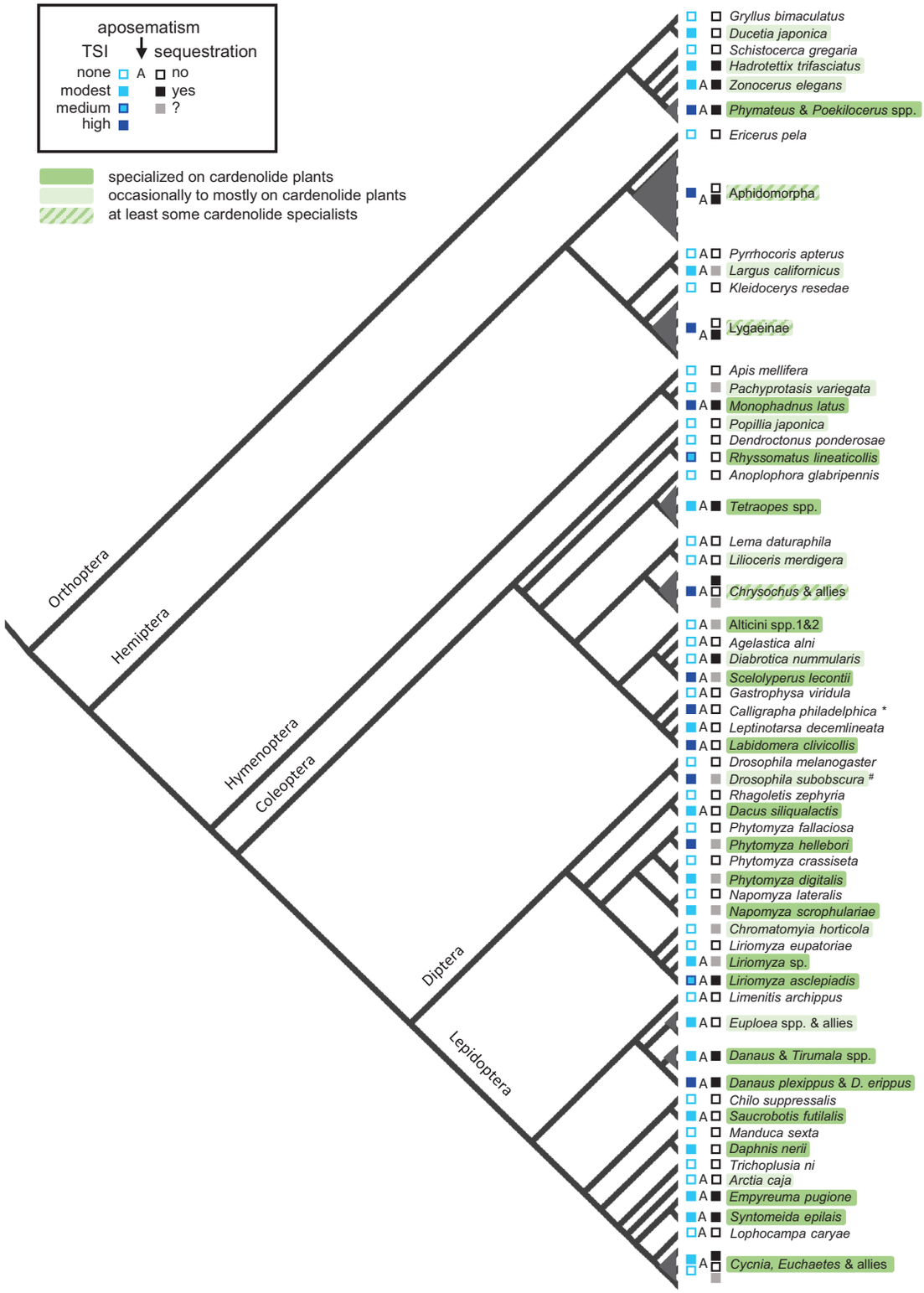
Material and Methods

To address the above questions, we used collecting and rearing methods to assess amino acid substitutions in the sodium pump and cardiac glycoside sequestration of previously known and newly identified specialist insect species feeding on cardiac glycoside plants (table S2). We focus on consumers of the common milkweed *Asclepias syriaca* (Apocynaceae) but also feeders of a co-occurring cardenolide-containing confamilial species (*Apocynum cannabinum*) that has its own specialists, and we also provide data for a hymenopteran, *Monophadnus latus*, on bufadienolide-containing *Helleborus* species (Ranunculaceae). Because several of the specialists were suspected not to sequester cardenolides, we were also specifically interested in cardenolide processing in sequesterers and nonsequesterers and used detailed chemical analysis of food, insect bodies, and frass to address this. In addition, we used a generalist/outgroup representative of Lepidoptera, Coleoptera, Orthoptera, and Hemipteran aphids that can feed and develop on milkweed, to assess their level of passive sequestration and cardenolide processing. We have also added amino acid sequences of the substitution-containing portion of ATPα1 for species not previously sequenced (table S3).

Collection localities for all insects are included in table S2. Some field-collected larvae and eggs or larvae from colonies were reared in the laboratory on cardenolide-containing plant tissue. Those insects were housed individually or in small groups in small, vented plastic containers with fresh plant leaves from the appropriate host plant until pupation. Frass from late-stage Lepidopteran larvae was collected and frozen for chemical analysis, along with the plant and insect tissues. Grasshoppers were reared on distinct host plants (from egg to adult) as indicated in table S2). Phylogenetic trees were constructed with Mesquite and edited in PowerPoint.

Chemistry Methods

Insect tissues were analyzed for cardenolides as in Petschenka et al. (2022*b*) on an Agilent 1100 high-performance liquid chromatography (HPLC) system or following the methods


aposematism
TSI
sequestration
none
modest
medium
high
A
no
yes
?
specialized on cardenolide plants
occasionally to mostly on cardenolide plants
at least some cardenolide specialists
Orthoptera
Hemiptera
Hymenoptera
Coleoptera
Diptera
Lepidoptera
Gryllus bimaculatus
Ducetia japonica
Schistocerca gregaria
Hadrotettix trifasciatus
Zonocerus elegans
Phymateus & Poekilocerus spp.
Ericerus pela
Aphidomorpha
Pyrrhocoris apterus
Largus californicus
Kleidocerys resedae
Lygaeinae
Apis mellifera
Pachyprotasis variegata
Monophadnus latus
Popillia japonica
Dendroctonus ponderosae
Rhyssomatus lineaticollis
Anoplophora glabripennis
Tetraopes spp.
Lema daturaphila
Lilioceris merdigera
Chrysochus & allies
Alticini spp.1&2
Agelastica alni
Diabrotica nummularis
Scelolyperus lecontii
Gastrophysa viridula
Calligrapha philadelphica *
Leptinotarsa decemlineata
Labidomera clivicollis
Drosophila melanogaster
Drosophila subobscura #
Rhagoletis zephyria
Dacus siliqualactis
Phytomyza fallaciosa
Phytomyza hellebori
Phytomyza crassiseta
Phytomyza digitalis
Napomyza lateralis
Napomyza scrophulariae
Chromatomyia horticola
Liriomyza eupatoriae
Liriomyza sp.
Liriomyza asclepiadis
Limenitis archippus
Euploea spp. & allies
Danaus & Tirumala spp.
Danaus plexippus & D. erippus
Chilo suppressalis
Saucrobotis futilalis
Manduca sexta
Daphnis nerii
Trichoplusia ni
Arctia caja
Empyreuma pugione
Syntomeida epilais
Lophocampa caryae
Cycnia, Euchaetes & allies

of Agrawal et al. (2022) by mass spectrometry on an Orbitrap Q-Exactive spectrometer. Detailed methods are provided in the supplemental PDF (see supplementary methods 1).

Categorizing Sequestration and Aposematism

We measured cardenolide/bufadienolide sequestration quantitatively for all insects (in the pupal or adult stage following juvenile feeding) but largely categorize species with the binary states of sequestering or not. Nonetheless, for the few species where we found cardenolides but in substantially lower concentrations than the bulk of sequesterers, we note this and interpret the data with caution, as it is unclear whether such sequestration is functional against predators. Similarly, we report species as aposematic or not based on the presence of bright and contrasting coloration visible to our vertebrate eyes. Where ambiguity exists, we highlight this in the text.

Sequencing of Cardenolide Feeders with Previously Unknown Sodium Pump Substitutions

For several insects, either the gene encoding the Na^+/K^+-ATPase alpha 1 subunit comprising the region of interest (111, 122) was amplified and sequenced using a reverse-transcription polymerase chain reaction (RT-PCR) approach or RNA extractions were sent off for transcriptom sequencing. Insects were collected in RNA*later* (*Diabrotica nummularis*, *Hadrotettix trifasciatus*, *Phymateus saxosus*, *Platycorynus peregrinus*, *Tetraopes femoratus*, *T. texanus*), frozen and freeze dried (*Aphis asclepiadis*, *Myzocallis asclepiadis*, *Popillia japonica*), or freshly frozen at −80°C (*Cycnia inopinatus*, *Lilioceris merdigera*, *Chrysochus asclepiadeus*) and then ground in liquid nitrogen using a mortar and pestle. RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI) or the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), reverse transcribed using Superscript III (Invitrogen, Carlsbad, CA), and subjected to RT-PCR using primers and methods described previously (Dobler et al. 2012). Alternatively, Illumina transcriptomes were generated (NextSeq 2000 or HiSeq 500; 150-bp paired-end reads) that were assembled with Trinity (Grabherr et al. 2011) using default parameters and searched with Blast (Altschul et al. 1997) for known sodium pump sequences of closely related species. All sequences have been deposited in GenBank (table S3).

Genome Searches to Understand the Origin of Aphid Resistance to Cardenolides

To trace the origin of the E111, Y122, and A797 (E glutamic acid, Y tyrosine, and A alanine) substitutions in the aphids, genomes of Aphidomorpha (aphids [12] and phylloxerans [2]), Aleyrodoidea (white flies [1]), Coccoidea (scale insects [6]), and Psylloidea (jumping plant lice [2]) available and online searchable as of October 2023 were searched by tblastn with the Na^+/K^+-ATPase alpha 1 amino acid sequence of *Aphis nerii* (GenBank accession number AFU25665.1), alpha 1 homologs identified, and the amino acid status at the relevant positions recorded.

Producing Na^+/K^+-ATPase Proteins in Cell Culture

To rigorously assess the impact on resistance of various amino acid substitutions at positions 111 and 122 of the Na^+/K^+-ATPase alpha 1 subunit in a single genetic background, we generated 11 constructs of combinations found in nature (for the respective two positions 111 and 122: QN, LN, TN, EN, VN, QY, QH, EY, TH, HH, and VH [L leucine and T threonine]). These were then tested for resistance to ouabain to categorize the enzymes as modest, medium, or highly resistant. We previously reported on a few of these constructs in Dalla et al. (2017) and Karageorgi et al. (2019), while the others were newly produced for the current study.

We started with a pFastBac Dual vector (Invitrogen) with the wild-type *Drosophila melanogaster* alpha subunit (GenBank accession number HE962487) under the polyhedrin promoter and the corresponding beta subunit Nrv2.2 (GenBank accession number NM_001273235) under the p10 promoter. Codons for amino acids at positions 111 or 122 (according to the numbering of the pig enzyme) were altered by site directed mutagenesis with the QuickChange XL kit (Invitrogen) to introduce amino acids of interest. Bacmids were produced according to the manufacturer's protocol and transfected into Sf9 cells to obtain a first stock of baculovirus that was used to infect a second

Figure 3: Composite phylogeny of the six taxonomic orders of insects known to have cardenolide-adapted herbivorous species based on available phylogenies. Shown is the level of specialization on cardenolide plants (highlighting over text), level of target site insensitivity (TSI) determined by the sodium pump sequence, aposematism, and evidence for sequestration of cardenolides. We focus on the ~28 independent origins of cardenolide specialization (including at least three origins in the aphids that are not shown individually). Nonadapted out-groups are without highlighting over the text. Thus, each time we have placed a non-cardenolide-adapted species on the phylogeny, the closest adapted feeder indicates a likely origination. Clear cases of aposematism (labeled A) are indicated for all species, including out-groups. Clades of multiple species are shown with triangles, somewhat proportional to the number of species in the group. If more than one character state is shown, this indicates variation among the species in the clade. An asterisk indicates a herbivorous species autogenously producing cardenolides. A pound sign indicates a species that is polymorphic for TSI and whose association with cardenolide plants is largely unexplored.

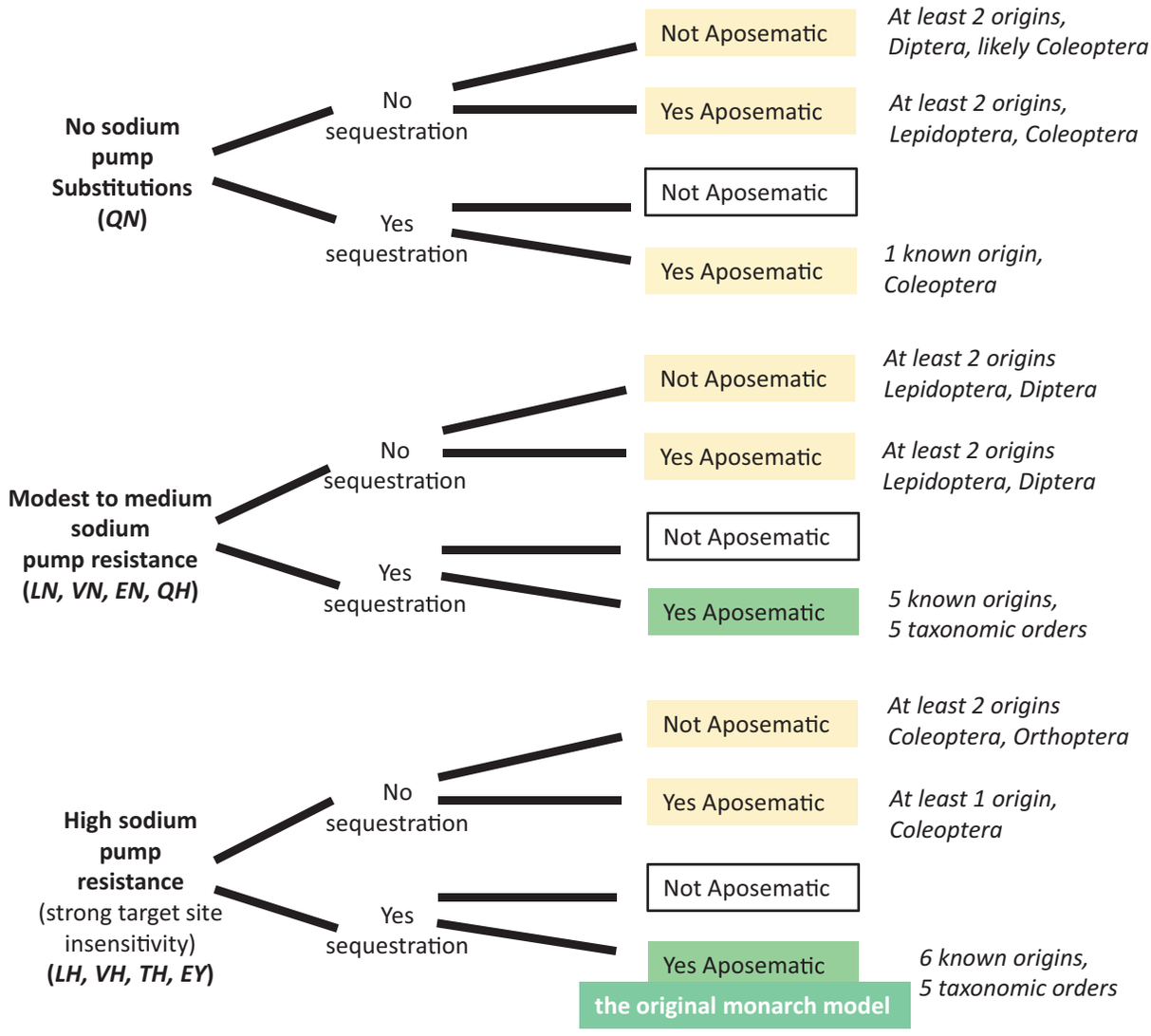


Figure 4: Herbivore syndromes for the milkweed insects are envisioned to have three axes (sodium pump substitutions, sequestration, and aposematism) and together characterize insect performance in the context of dietary resistance to cardenolides and adaptations to predators. The bifurcating lines generate 12 possible combinations of the three traits, but do not represent evolutionary order. Tallying the ~28 independent origins of cardenolide feeding, we see the following patterns. The two darker (green) boxes indicate several independent origins, at least five of each and in five taxonomic orders each. Lighter (yellow) boxes indicate one to two known origins of that trait combination, typically in more than one insect order. Some of the ~28 origins of cardenolide feeding do not have complete data, not allowing their categorization. The three shown in a clear box do not exist for cardenolide-adapted insects to our knowledge: all sequesterers are aposematic. Nonsequestering aposematic insects are potentially Batesian mimics. Additional mechanisms to cope with cardenolides certainly exist (use of transporters, blood-brain barriers, detoxification), but too little is known currently to expand this matrix.

charge of Sf9 cells. Proteins were harvested from these cells following our previously published protocols (Dalla et al. 2017) and assayed for their resistance against the cardenolide ouabain (Petschenka et al. 2013*a*; Dalla and Dobler 2016). In brief, equimolar aliquots of protein solution were subjected to a buffer regime with increasing molarity of ouabain (10^{-8} to 10^{-3} M) and their activity revealed as the amount of phosphate released. Background ATPase activity was determined in a reaction volume without K^+, in which no Na^+/K^+-ATPase activity can be observed and subtracted in all reactions. The relative activity of each reaction mix was expressed as percentage of an uninhibited aliquot without ouabain. The inhibitory concentration at which 50% of enzyme activity was abolished was determined

by fitting a four-parameter logistic function (nlme) to the percent activity values using R (4.3.1 GUI 1.79 BigSur ARM build; RBG ~ SSfpl(log.conc, A, B, xmid, scal)).

Results

General Patterns

There are 12 possible combinations for the three traits we considered (fig. 4): the level of TSI (no TSI—Q111 N122; modest to medium TSI—species with a single substitution at 111 or 122 [respectively LN, VN, TN, EN, QY, QH]; or strong TSI—species with substitutions at both 111 and 122 [LH, VH, TH, EY]; see table 1; fig. 2), sequestration (yes or no), and aposematism (yes or no). A list of the species for which we are adding new data is provided in table S2, whereas data on other species are provided with appropriate references in table S3 (all relevant sodium pump, ATPα1, and amino acid sequences). Overall and consistent with expectations, we did not find sequesterers that were not aposematic (fig. 4). In other words, among the most general patterns we observed was that insects that sequester cardenolides from their host plants are aposematic. Also consistent with expectations, most sequestering species (all but one, the leaf beetle *Diabrotica nummularis*) had at least modestly resistant sodium pumps (figs. 4, 5). One of the three milkweed aphid species (*Aphis asclepiadis*) has strong TSI and is sequestering, but it is cryptic and thus a potential exception to our general patterns (discussed below in the specific section on Hemiptera).

Convergence on the Sequestering Specialist Syndrome

Across the ~28 origins of specialization on cardenolide plants (fig. 3), the syndrome of strong TSI (i.e., V111 with H122, T111 with H122, or E111 with Y122; table 1), sequestration, and aposematism evolved together at least six times in five insect orders: in monarchs (Dobler et al. 2012; Zhen et al. 2012), *Chrysochus* beetles (Labeyrie and Dobler 2004), pyrgomorphid grasshoppers (Dobler et al. 2019; Yang et al. 2019), lygaeine bugs (Dobler et al. 2012; Bramer et al. 2015), aphids (Zhen et al. 2012; Züst and Agrawal 2016), and hymenopteran sawflies (fig. S1 [figs. S1–S6 are available online]; tables 1, 2). For the hymenopteran *Monophadnus latus* (Tenthredinidae), strong TSI is coupled with aposematism, but sequestration had not been previously tested. Here we report unambiguous sequestration of bufadienolides in their larval integument when feeding on *Helleborus* sp. (fig. S1). In each of these cases of the sequestering specialist syndrome, there are typically multiple descendant species with the combined traits, although reversions have sometimes been detected (discussed in the taxonomic summaries below).

Convergence on Other Strategies

The existence of multiple origins of cardenolide specialization within four out of six orders (all except Orthoptera and Hymenoptera) allows for testing for convergence at multiple scales (fig. 3). Among these, we found strong convergence for a second syndrome: insects with only modest to medium TSI that are nonetheless sequesterers and aposematic (tables 1, 2); this trait combination evolved in each of five insect orders (fig. 3), including Diptera (no Hymenopterans have been found). Thus, strong TSI is not needed for sequestration and aposematism. In these cases, species typically had a single substitution at amino acid position 111, to leucine, valine, or glutamic acid from the ancestral glutamine (at 111 and 122 they were respectively LN, VN, and EN, providing 5–17-fold resistance compared with QN; table 1). An example of this strategy is the aposematic sequestering arctiid moth *Empyreuma pugione*, with an only modestly resistant sodium pump (L111 N122; Dobler et al. 2015; Petschenka et al. 2012). *Liriomyza asclepiadis* (Diptera) in the Agromyzidae is a bit difficult to classify because of its tiny size (<2 mm in length; fig. 1) and low levels of sequestration, although it has a medium level of TSI (Q111 H122). Each of these species most likely maintain additional physiological tolerance to cardenolides, provided by the blood-brain barrier and efflux transporters (Petschenka et al. 2013*b*; Dobler et al. 2015; Birnbaum et al. 2017; Groen et al. 2017; Kowalski et al. 2020). As will be shown in the specific taxonomic sections below, this modestly resistant group includes species that sequester cardenolide levels as high as those in monarch butterflies (e.g., *Tetraopes* spp. and *Cycnia* spp.).

We found several aposematic cardenolide-feeding species that do not sequester cardenolides, some of which are likely Batesian mimics; it is possible that some of these species may also be advertising other chemical defenses (e.g., Pasteels 1993), but they are not sequestering cardenolides. This trait combination repeatedly evolved within the full range of sodium pump backgrounds, from no TSI to strong TSI (at positions 111 and 122 they were respectively QN, LN, VH, and QY; fig. 4; table 2). Nonetheless, within each of the backgrounds, the nonsequestering aposematic syndrome evolved only a couple of times (table 2; fig. 4).

In summary, the cardenolide-adapted insects show some general rules. Sequestration of plant toxins is common and was consistently associated with aposematism, nearly always with at least a modest level of sodium pump resistance to cardenolides. Nonetheless, there are two highly convergent groups of sequestering aposematic insects: those that have strong TSI (substitutions at amino acid positions 111 and 122) and those with modest to medium TSI (substitution at only 111). The latter group clearly requires

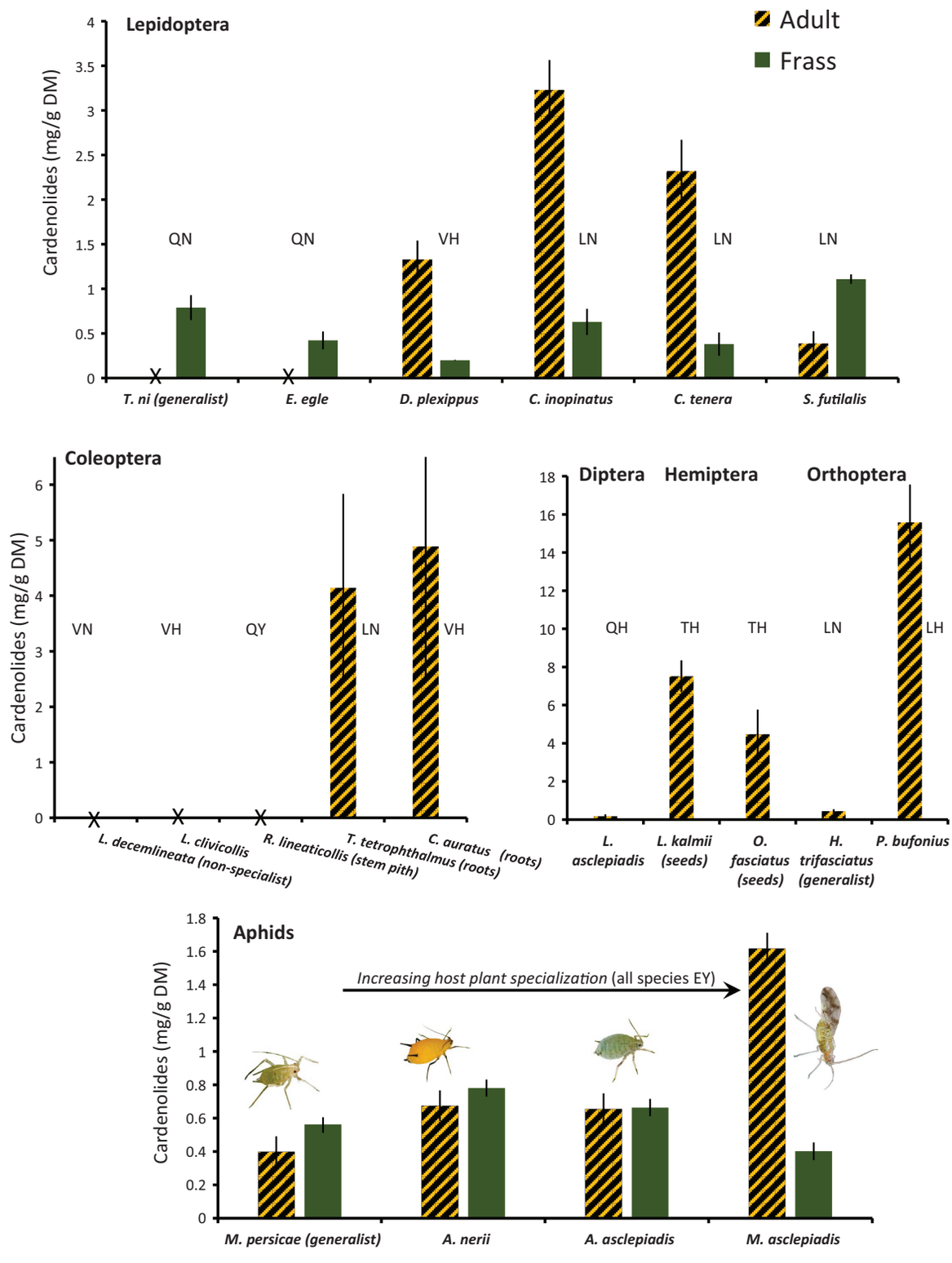


Figure 5: New data on sequestration of cardenolides on a dry mass (DM) basis in five orders of insects. Shown are means ± standard errors for adults and frass (an X along the *x*-axis indicates no cardenolides detected). The sodium pump phenotype at amino acid positions 111 and 122 is shown next to the bars. Values for *Liriomyza asclepiadis* are above zero but very low. Data for the four phloem-feeding aphid species are from Züst and Agrawal (2016; photos by Tobias Züst). The larval or nymphal food for other species is leaves except where noted next to the species name.

Table 2: Repeatedly evolved insect herbivore syndromes among the cardenolide feeders.

Herbivore strategy	Characteristics	Example species
Susceptible sodium pump feeders (QN)	Sensitive sodium pump, may or may not be aposematic; only one species known to sequester (*D. nummularis*); some, like *E. egle*, could be Batesian mimics	*Chromatomyia horticola* (Diptera); *Euchaetes egle* and likely *Cycnia oregonensis* (Lepidoptera); *Lilioceris merdigera* and *Diabrotica nummularis* (Coleoptera); *Pachyprotasis variegata* (Hymenoptera)
Nonsequestering, with modest to high TSI—diet-driven sodium pump substitutions (LN, VN, VH, QY)	Modest to highly insensitive sodium pump, nonsequestering, may or may not be aposematic; some are likely Batesian mimics	*Daphnis nerii* and *Euploea core* (Lepidoptera); *Dacus siliqualactis* (Diptera); *Labidomera clivicollis* and *Rhyssomatus lineaticollis* (Coleoptera); *Ducetia japonica* (Orthoptera); *Phytomyza hellebore* and some other Diptera could be in this group (sequestration unclear)
Sequestering without highly resistant sodium pumps (LN, VN, EN, QH)	Modest to medium insensitive sodium pump, yet sequestering and aposematic; these species likely employ other means to cope with cardenolides, including transporters, detoxification, and so on	*Syntomeida epilais*, *Cycnia tenera*, and other *Arctiids*; *Saucrobotys futilalis*, *Danaus gilippus* (Lepidoptera); *Tetraopes* spp. (Coleoptera); *Hadrotettix trifasciatus* and some Pyrgomorphidae (Orthoptera); possibly *Liriomyza asclepiadis* (low sequestration) and *Largus* spp. (sequestration needs to be investigated)
Sequestering specialist syndrome (VH, LH, TH)	Highly resistant sodium pumps, sequestering, and aposematic; the monarch model	*Danaus plexippus* (Lepidoptera); *Chrysochus auratus* and possibly *Scelolyperus lecontii* (Coleoptera); lygaeines and several aphids (Hemiptera); *Phymateus* and *Poekilocerus* spp. (Orthoptera); *Monophadnus latus* (Hymenoptera)

Note: These syndromes emerged from our analysis of the possible combinations between sodium pump substitutions, sequestration, and aposematism among the known cardenolide-adapted insect species. Below each herbivore strategy are representative sodium pump phenotypes, showing the amino acid abbreviations at positions 111 and 122, respectively. We do not include here species that autogenously produce cardenolides (e.g., *Calligrapha* spp.).

additional tolerance mechanisms, a research area that is ripe for study. Finally, nonsequestering insects on cardenolide plants are often aposematic, suggesting that some species may have evolved Batesian mimicry or are defended by other means.

Evolutionary Progressions and Gene Duplication

Perhaps based on intuition alone, we might hypothesize that the order of evolution of the three traits in the sequestering specialist syndrome would be sodium pump adaptation to toxins first (i.e., TSI), followed by sequestration and then aposematism. Nonetheless, the milkweed butterflies defy this expectation (Petschenka et al. 2013*a*). Although dietary tolerance, of course, has primacy in host plant specialization, there is more than one mechanism of this dietary tolerance. For milkweed butterflies, early-diverging species are highly tolerant of cardenolides in their diet (Petschenka and Agrawal 2015), despite having only modest sodium pump substitutions (the early-diverging danaine *Euploea core* is L111 N122; fig. 2). Accordingly, caterpillars of *E. core* suffer mortality if injected with cardenolides, demonstrating that its rather sensitive target site is normally protected from dietary toxins by preventing their uptake into the hemolymph. In the milkweed butterflies, additional sodium pump substitutions (V111 and H122) later evolved with sequestration (fig. 2; Petschenka and Agrawal 2015). A similar progression was observed in pyrgomorphid grasshoppers (Yang et al. 2019), where early-diverging species have modest sodium pump substitutions, a more generalized diet, and sequester some cardenolides, whereas the most derived clade has strong TSI and increased sequestration.

In other cases we lack the phenotypic variation or phylogenetic resolution within clades to observe the order of trait evolution (e.g., Diptera; Petschenka et al. 2017), although shifts away from cardenolide plants have shown how some of these species evolutionarily reverted in at least some of these traits (e.g., Hemiptera; Bramer et al. 2015). Although multiple origins of cardenolide feeding exist among Coleoptera and later-diverging Lepidoptera (i.e., moths), more detailed phylogenies and phenotyping is needed to assess

evolutionary progressions. Finally, those species that produce other forms of defense or that are potentially Batesian mimics necessarily defy the expected order, as they lack sequestration but may be derived from aposematic ancestors.

Several cardenolide-adapted herbivores have duplications of the relevant sodium pump gene (ATPα1), and this has been suggested as a means to deploy highly resistant copies where the animals are most exposed to toxins but to avoid the costs of TSI in other places (Zhen et al. 2012; Dalla and Dobler 2016; Herbertz et al. 2022). Among cardenolide specialists, gene duplication is present, but variable, in four of the six insect orders, but not in the Lepidoptera or Hymenoptera. Few generalities have emerged so far for predicting the presence of gene duplication of the sodium pump. One interesting observation is that lygaeid bugs (with four copies; Yang et al. 2019) maintain cardenolide tolerance even when they switch to other diets, although some have lost aposematism (e.g., Bramer et al. 2015; Petschenka et al. 2022*a*).

Where the evolutionary progression cannot be assessed, information about "preadaptations" may still be informative. In our previous work, we identified a constrained path of repeated molecular evolution among the milkweed-feeding herbivores (Karageorgi et al. 2019). In particular, changes at amino acid position 122 were almost universally preceded by substitutions at positions 111 and 119. We next more generally summarize preadaptations, or preexisting attributes of the sequestering specialist syndrome, in each of the taxonomic orders present before specialization on cardenolide plants. In other words, we ask whether sodium pump substitutions, sequestration, or aposematism existed in the clade prior to the evolution of cardenolide specialization. We interpret the existence of such preadaptations as likely to have facilitated the evolution of the syndrome. Some general patterns surface that we further elaborate on in the taxonomic sections.

In the Lepidoptera, the inferred ancestors of both the Danaini and milkweed-feeding Erebidae appear to often have L111 and aposematic coloration. Several earlier-diverging lineages are also sequesterers of alkaloids. In the Coleoptera, at least one lineage of milkweed feeders (*Labidomera*) has an early-diverging relative with V111 that is also aposematic (but feeding on Solanaceae). Among the tiny leaf-mining flies (Agromyzidae), the group appears to have an ancestor that is predicted to be aposematic (black and yellow), irrespective of host plant affiliation. Sodium pump gene duplication, strong TSI, and aposematism is ancestral in the Lygaeinae (Hemiptera), although this group is assumed to have a basal association with the Apocynaceae; further work on out-groups is warranted here. In the larger group comprising the aphids (Aphidomorpha), a resistant sodium pump gene (E111, Y122, and an additional strong TSI conferring substitution of alanine at position 797, A797) is apparently maintained in the whole clade, yet the reasons for its origin and whether this was associated with host plant adaptation remain obscure. Finally, aposematism, sequestration, and modest TSI (L111) are all present in earlier-diverging lineages of the Orthoptera that have specialized on cardenolide plants. Thus, it appears that in all major groups of cardenolide specialists, not only was there molecular preadaptation in terms of one sodium pump substitution, but aposematism or sequestration also frequently occurred in the lineage.

Lineage-Specific Patterns in Traits and Syndromes

Within-group lineage-specific patterns potentially reflect constraints, which we broadly define as biased outcomes over the course of evolution (Schwenk 1994/1995). Accordingly, we do not attempt to separate effects of differing natural selection versus differing genetic architecture in the biased outcomes seen in different lineages. Of course, this separation is an important goal, but it is not yet possible in this system.

As indicated above, sodium pump gene duplication has occurred in all orders except the Lepidoptera and Hymenoptera, thus apparently being constrained in these clades. Although H122 exists in cardenolide-specialized insects in all taxonomic orders, this substitution has apparently not appeared among the hundreds of species (and at least four independent origins of specialization) in the later-diverging moths (Sphingidae, Crambidae, and Erebidae). Opposing this pattern are the Coleoptera, where four of the seven origins of cardenolide specialization involve H122 (all Chrysomelidae), and a fifth (*Rhyssomatus* in the Curculionidae) has a different substitution at the same amino acid position (Y122). Only *Tetraopes* spp. (Cerambycidae) and the sensitive but sequestering *Diabrotica nummularis* (Chrysomelidae; fig. S3) retain the ancestral N at this site. Finally, although there are multiple origins of cardenolide feeding in the agromyzid flies, there appear to be no lineage-specific rules. Cardenolide feeders are Q, L, or H at position 111 and N or H at position 122, with gene duplication also variable among these species. With limited information on the number of cardenolide specialization origins in the Hemiptera, Orthoptera, and Hymenoptera, little can be inferred about lineage-specific patterns.

Taxonomic Summaries

Lepidoptera

Butterflies—Danaini (Nymphalidae). Among the Lepidoptera, there are at least five evolutionary origins of specialization on cardenolide plants. The earliest diverging of these is in the butterfly family Nymphalidae, in which the Danaini tribe (>150 species), or "milkweed butterflies,"

generated the model of a constrained adaptive walk yielding the sequestering specialist syndrome (Petschenka et al. 2013*a*; Karageorgi et al. 2019; figs. 2, 3). Part of this model is that the early-diverging group, including *Euploea* spp., can specialize on cardenolide plants but possess an L and N at amino acid positions 111 and 122, respectively (modestly resistant) and are not sequestering (Petschenka and Agrawnal 2015; fig. 2). Remarkably, the evolution of increased resistance to cardenolides via a V111 substitution in *Danaus chrysippus* and *D. gilippus* (VN; table 1) is coincident with a moderate level of sequestration, and the addition of H122 in the clade containing the monarch butterfly *D. plexippus* (VH) corresponds to high sequestration (Aardema et al. 2012; Smith 2014; Petschenka and Agrawal 2015; fig. 2). A key insight from this work is that H122, which provides the lion's share of sodium pump resistance to cardenolides, is not necessary for dietary specialization on the plants with these toxins. Early-diverging Danaini presumably rely on other protective mechanisms to shelter their sensitive sodium pumps from cardenolides (e.g., Petschenka et al. 2013*b*; Petschenka and Agrawal 2015; Dreisbach et al. 2023). Despite not sequestering cardenolides, early-diverging danaines, such a *Euploea core*, are aposematic, especially in the larval stage. The Ithomiinae, an out-group to the Danaini, contains many species (including *Mechanitis polymnia*) that are L111 N122 (Petschenka 2013*a*; Karageorgi et al. 2019), feed on Solanaceae or Apocynaceae, and are also aposematic.

Moths (Sphingidae, Crambidae, Erebidae). There are many species (>50 identified, hundreds likely) of later-diverging Lepidopteran moths in at least three families contributing to at least four additional origins of cardenolide specialists: *Daphnis nerii* (Sphingidae; Petschenka and Dobler 2009), *Saucrobotys futilalis* (Crambidae; Grant 2007; this study), *Syntomeida epilais* and *Empyreuma pugione* (in the subtribe Euchromiina of Erebidae), and *Lerina incarnata*, *Cycnia* spp., *Euchaetes* spp., *Pygarctia* spp., and other genera of milkweed feeders (in the subtribe Phaegopterina of the Erebidae; Conner 2008; Dowdy et al. 2020). These moths confirm that H122 is not necessary for dietary specialization and also demonstrate that it is not required for the highest levels of cardenolide sequestration (fig. S2, supplementary text 1). The insight revealed from this work is that all sequestering moth species are L111 N122 (Dobler et al. 2012, 2015).

Further details on sodium pump substitutions, sequestration, and aposematism of the moths are provided in the supplemental PDF (see supplementary text 1, fig. S2).

Coleoptera

Herbivorous beetles are likely the most diverse group of plant-feeding insects on the planet and contain many cardenolide feeders. Within this group, we are aware of at least 15 genera or single species of cardenolide feeders, although the number of independent colonizations, especially in the subfamily Eumolpinae (Jolivet and Verma 2008), may be lower. At least five beetle taxa have sodium pump substitutions at both positions 111 and 122. An additional incidence of sodium pump insensitivity in the Chrysomelidae is coupled with autogenous production of cardenolides as defense substances (*Calligrapha philadelphica*; fig. 3). Duplication of the sodium pump genes has been detected twice, once in a weevil and once in chrysomelids. Details on sodium pump substitutions, sequestration, and aposematism of the beetles are given in the supplemental PDF (see supplementary text 2, fig. S3).

In summary, the Coleoptera, like the Lepidoptera, repeatedly colonized cardenolide plants and reached all levels of adaptation: some species, such as *Diabrotica nummularis* and *Lilioceris merdigera*, fall into a susceptible (more or less) specialist category even if they are clearly aposematic. A notable difference to the Lepidoptera consists in the frequent occurrence of autogenously produced defensive cardenolides (Blum 1981; Pasteels 1993) that could be the basis of honest warning signals independent of sequestered plant toxins. Most Coleoptera on cardenolide plants, however, evolved higher levels of sodium pump adaptation with at least moderate to medium resistance (at 111 and 122, respectively, LN, TN, VN, and QY; an exception is *D. nummularis*; fig. S4). As observed in some arctiids, *Tetraopes* falls in this category (L111 N122), although species in this genus sequester high levels of cardenolides. This implies that the perineurium (or other mechanisms) efficiently protect the sensitive nervous tissue in these species. The categories, including highly resistant sodium pumps (V111 H122), evolved repeatedly, mirroring the monarch model. Autogenous defenses alter the situation compared with the Lepidoptera, as in *Labidomera clivicollis* and *C. philadelphica*, where antipredator defense does not rely on sequestration but on production of noncardenolide defensive substances. In contrast to the Lepidoptera, insensitive sodium pumps twice go along with gene duplications and may, as we will see below for the Lygaeinae, enable the larger group containing *Chrysochus* to switch repeatedly away from and back to cardenolide plants.

Diptera

Agromyzidae. Tiny flies in the family Agromyzidae represent likely >3,000 species of typically host-specific leaf miners of herbaceous angiosperms (Winkler et al. 2009), of which six species in four genera have thus far been identified to feed on cardenolide-containing plants (this is almost certainly an underestimate; Dobler et al. 2012;

Petschenka et al. 2017). Remarkably, these flies have colonized plants that have themselves convergently evolved cardenolide toxins (i.e., only two fly species are currently known from milkweeds). Of the six cardenolide-adapted flies, five have amino acid substitutions at positions 111 or 122, while one generalist species, *Chromatomyia horticola*, has retained the ancestral Q111 N122 sodium pump, despite having the ability to feed on cardenolide-rich *Erysimum cheiranthoides* (Brassicaceae). Of the five other species, one shows gene duplication of the sodium pump (*Phytomyza hellebori*, feeding on *Helleborus foetidus*, Ranunculaceae, with the fly maintaining a highly resistant and susceptible copy of ATPα1; Petschenka et al. 2017). Three of the other species are L111 N122, conferring less than 10-fold insensitivity to cardenolides (Petschenka et al. 2017; fig. 2; table 1). Surprisingly, two *Liriomyza* spp. feeding on milkweeds in North America (*Liriomyza asclepiadis* and the other undescribed) maintain divergent sodium pumps (Q111 H122 vs. L111 N122, respectively), suggesting that these should be independent origins (Dobler et al. 2012; Petschenka et al. 2017).

Details of toxin sequestration and aposematism in agromyzids, as well as additional species in the Tephritidae and Drosophilidae, are presented in the supplemental PDF (see supplementary text 3, fig. S4). Although the existence of multiple independent origins of cardenolide feeding in the Diptera (especially Agromyzidae) is clear, more detail is needed to understand host shifts, the sodium pump genotypes of non-cardenolide-feeding close relatives of each origin, and any possible benefits of the low sequestration and aposematic coloration in this group. Thus far, this group seems to do it all by any means (e.g., may or may not have TSI or gene duplication). There appear to be no rules within the agromyzids with regard to the genetic basis of sodium pump resistance to cardenolides. Nonetheless, there is certainly a diversity of cardenolide feeders in the Diptera to be discovered, and these would be profitable for study and synthesis.

Hemiptera

Among the true bugs, taxa from at least three families have been found to be associated with cardenolide-containing plants. In order of decreasing knowledge about the association, we have seed-feeding Lygaeinae (Lygaeidae), phloem-sucking Aphididae, and leaf-damaging *Largus* spp. (Largidae).

Lygaeinae (Lygaeidae). The red and black seed-feeding Lygaeinae bugs, *Lygaeus kalmii* and *Oncopeltus fasciatus*, have both long been model systems for understanding the evolutionary ecology of sequestration and aposematism (Duffey and Scudder 1974; Isman et al. 1977; Berenbaum and Miliczky 1984; Sillén-Tullberg 1985; Moore and Scudder 1986; fig. 1). These species are among the top sequesterers of cardenolides known on milkweeds (fig. 5; Agrawal et al. 2012). It has been demonstrated that their extreme sodium pump insensitivity to cardenolides, due to four highly substituted copies (Moore and Scudder 1986; Yang et al. 2019; Herbertz et al. 2024), were basal adaptations in the subfamily, and they are present even for species not feeding on cardenolide-containing plants (Bramer et al. 2015). These Lygaeinae thus conform to the sequestering specialist syndrome, having coincident TSI, sequestration, and aposematism.

The Lygaeinae's insensitivity to cardenolides has apparently facilitated host shifts (and speciation) onto distantly related plants that themselves convergently evolved cardenolides (Petschenka et al. 2022*a*). In other words, preadaptations and convergent plant defense evolution have both contributed to the diversification of these seed bugs. Although this could be considered a classic case of "escape and radiate" coevolution (Ehrlich and Raven 1964), we unfortunately know little about the sequence of evolution for the traits relevant to the sequestering specialist syndrome in these bugs. In other words, TSI and sequestration of cardenolides appear on the same branch of the phylogeny (Bramer et al. 2015), and we lack replication for the evolution of these traits. Furthermore, this is a group that was ancestrally aposematic, and many early-diverging species feed on alternative host plant families. In addition, some species in the genus *Arocatus* have host shifted to non-cardenolide-producing trees and have lost the ability to sequester the compounds; they are brown and cryptic but nonetheless maintain resistant sodium pumps (Bramer et al. 2015).

Details on sodium pump substitutions, sequestration, and aposematism of the aphids and bordered plant bugs, *Largus* spp., are given in the supplemental PDF (see supplementary text 4, fig. S5). Overall, multiple patterns exist in the Hemiptera. Lygaeinae and several aphids conform to the sequestering specialist syndrome, while *Largus* spp. do not. The former appears to be a special case, potentially impacted by their feeding style and unusual sodium pumps, which are otherwise so highly conserved among animals but have four copies as well as basal substitutions. For *Largus* spp., more work is needed on their diet breadth and chemical ecology to decipher whether they will join the large group of sequestering species with modestly tolerant sodium pumps (table 2).

Orthoptera

Although orthopteran insects are often thought of as the quintessential generalist herbivores, specialism is not uncommon, and geographic and phylogenetic patterns exist

in terms of differential feeding strategies (Otte and Joern 1976).

Pyrgomorphidae. These species, in particular, are a group of about 500 largely old-world grasshoppers, known for feeding on toxic plants, sequestration of both cardenolides and pyrrolizidine alkaloids, and aposematic coloration. Indeed, these were among the first species for which sequestration in any animal was mechanistically studied (von Euw et al. 1967), and resistant sodium pumps were also discovered early on (table S1; Al-Robai et al. 1990, 1992). Nonetheless, the amino acid substitutions involved were not identified until recently (Dobler et al. 2019; Yang et al. 2019). Although some species (in the genera *Phymateus* and *Poekilocerus*) possess an L111 H122 form of the sodium pump (while also maintaining a relatively sensitive copy), several species in other genera (including *Zonocerus* spp.) have only the modest L111 yet appear to be aposematic cardenolide sequesterers (Yang et al. 2019). Additionally, the resistant sodium pump copies in *Phymateus* and *Poekilocerus* have two amino acid insertions that drastically increase resistance to cardenolides (Dobler et al. 2019). We add here that the rainbow milkweed locust (*Phymateus saxosus*) from Madagascar has the same highly resistant copy of the sodium pump as other *Phymateus* and *Poekilocerus* (tables S2, S3; fig. S6).

The pyrgomorphid grasshoppers loosely follow the sequestering specialist syndrome based on the monarch model. Based on images and data in Yang et al. (2019), all specialist sequesterers are aposematic, and three of four noncardenolide feeders are not aposematic. Nonetheless, L111 is the ancestral condition in the larger group. Even though there is a phylogenetic progression from L111 to H122, some of the L111 N122 species are aposematic and sequester cardenolides. Here we compared levels of sequestration of *Poekilocerus bufonius* (L111 H122; reared in Israel on *Calotropis procera*, Apocynaceae) to the potentially (modestly) aposematic generalist acridid *Hadrotettix trifasciatus*, reared on *Asclepias asperula* in Texas. *Hadrotettix trifasciatus* commonly feeds on *A. asperula* in the field (Lenhart 2014). We determined that *H. trifasciatus* was L111 N122 (tables S2, S3) and is a very modest sequesterer (0.4 mg/g dry mass; fig. 5). Additionally, *H. trifasciatus* excreted substantially more cardenolides (1.6 ± 0.1mg/g), but given that *A. asperula* leaves contained 3.3 ± 0.3 mg/g cardenolides, it appears that *H. trifasciatus* also metabolized some of the compounds. In comparison, not only did *P. bufonius* sequester a tremendous concentration of cardenolides (fig. 5), defensive gland secretions were much more concentrated than in the host plant (up to 40% cardenolides on a dry mass basis; table S4).

Overall, L111 seems to be common and likely the ancestral state in the Acrididae (Taverner et al. 2019), perhaps preadapting these generalists to tolerance of cardenolides. Some species have found their way to the sequestering specialist syndrome, but others have found a way without H122, joining members of several other orders of milkweed-feeding insects (fig. 4; table 2).

Tettigoniidae. A recent study has reported that *Ducetia japonica*, a generalist and omnivorous katydid (Tettigoniidae), has a duplicated copy of the sodium pump, as do early-diverging species in this group, which may be a preadaptation to feeding on cardenolide-containing plants (Wang et al. 2022). The authors report that this katydid has the E111 and V797 substitutions in its resistant sodium pump variant, which is expressed in the gut, and that the species feeds readily on cardenolide-rich oleander in the laboratory and field. Nonetheless, this insect was found not to sequester cardenolides.

Hymenoptera

There are a few known hymenopteran herbivores in the family Tenthredinidae feeding on cardenolide plants (*Pachyprotasis variegata* feeding on *Digitalis* spp., Plantaginaceae; and *Monophadnus latus* feeding on, *Helleborus* spp. in Ranunculaceae with related bufadienolides; Steyn and van Heerden 1998; Dobler et al. 2015). Despite encountering cardenolides, *P. variegata* maintains a sensitive (Q111 N122) sodium pump, while *M. latus* is T111 H122 (Dobler et al. 2015). These species show "easy bleeding" as larvae (i.e., minor damage to the integument causes exudation of hemolymph). As we have shown, *M. latus* sequesters bufadienolides in their larval integument, although they have not been studied in the hemolymph or adults (fig. S1). Although likely aposematic as larvae and possibly as adults, this has not been well studied (fig. S1). Other *Monophadnus* spp. feeding on *Helleborus* spp. were found to have deterrent compounds in hemolymph, but bufadienolides were not studied (Prieto et al. 2007).

Discussion and Conclusion

Twenty years ago, Labeyrie and Dobler (2004) reported the first evidence of molecular convergent evolution (via TSI) in this system (table S1). Nonetheless, it took nearly another decade to reveal a wider pattern of convergence (Dobler et al. 2012; Zhen et al. 2012). This work further suggested the notion that TSI was essential for sequestration of toxins among the cardenolide feeders. Here we have broadly evaluated this pattern, along with the association between the mechanisms of strong TSI (substitutions in ATPα1 at positions 111 and 122), sequestration, and aposematic coloration—representing what we termed the

"sequestering specialist syndrome." Using the ~28 known origins of cardenolide feeding across six taxonomic orders of insects, we find that association originating at least six times across five of six taxonomic orders (all except Diptera; figs. 3, 4).

Despite this high level of multivariate convergence, we also report numerous origins of sequestration and aposematism with only modest to medium TSI (i.e., 5–80-fold higher resistance than a sensitive sodium pump; tables 1, 2; fig. 4). The commonness of this combination of traits in five of six orders (all except Hymenoptera) has several important implications. First, strong TSI is not required for sequestration of toxins into the body. Indeed, we even report a single sequestering aposematic species (*Diabrotica nummularis*, Coleoptera) with a sensitive enzyme. Second, this result suggests the likely common use of physiological strategies to tolerate cardenolides other than TSI. Thus, at least two syndromes exist for cardenolide feeders, and substantial work remains in (1) discovering additional species and their traits and (2) exploring the evolution of those alternate (non-TSI) mechanisms to tolerate cardenolides.

Is there a cheating syndrome? We reported here three species (*Euchaetes egle*, *Labidomera clivicollis*, and *Lilioceris merdigera*) that feed on cardenolide plants and display aposematism yet are not sequestering. There are surely many other such species. This important result has several possible non-mutually-exclusive interpretations that will require further study. First, for the species in this group that has high sodium pump resistance (*L. clivicollis*), it appears that these substitutions were driven by diet, not sequestration. Among the danaine butterflies that provided the model for the sequestering specialist syndrome, it was sequestration that was the apparent driver of sodium pump substitutions; early-diverging species were tolerant of cardenolides despite having a largely sensitive enzyme (Petschenka and Agrawal 2015). Second, these species may be mimics of the sequestering species in the cardenolide-feeding community, which may well be an option for the arctiid moths. Although we have speculated that these species may be Batesian mimics because of their lack of sequestered cardenolides, two other possibilities exist. These species may be Müllerian mimics because of noncardenolide toxins (presumably autogenously produced) or not mimics at all (species that make their own toxins and evolved aposematism independently, as is likely for the leaf beetles). Thus, it is premature to conclude that there is a cheating syndrome in this system, although hopefully time will tell.

How Syndromes Evolve

In our previous work, we identified a repeated constrained molecular path toward strong TSI (Karageorgi et al. 2019). Although not universal, H122 was statistically much more likely to evolve following a phylogenetically earlier substitution at position 111 (Karageorgi et al. 2019). This progression was clearly identified among the danaine butterflies, pyrgomorphid grasshoppers, and some beetles. In the case of our multivariate assessment of syndrome evolution (including sequestration and aposematism), although we have not been able to phylogenetically reconstruct trait evolution, we have shown that in nearly all cases there is evidence of preadaptation in each lineage evolving cardenolide specialization. We interpret this result simply as evidence that cardenolide specialization typically consists of a consortium of traits, and the shift to cardenolide feeding typically arises in lineages with some of those traits already in place.

Despite the similarities in cardenolide-adapted insects spanning 350 million years of independent evolution, we have also shown here convergence on alternative trait combinations as well as lineage-specific outcomes. It is tempting to speculate that this is the way life leads to biodiversity. In the realm of this ecological microcosm, there are some rules, or at least repeated patterns. And some of these repeated patterns are almost certainly the result of constraint. For example, the sodium pump is among the most conserved animal enzymes, and accordingly it is not surprising that relatively few specific amino acid substitutions provide resistance to cardenolide toxins while maintaining high functionality. Although not a constraint per se, it is also not surprising that the association between sequestration and aposematism is very strong and highly repeated; the latter reinforces the fitness benefits of the former. Other patterns that are lineage specific have a less clear mechanistic basis for their convergence. That no Lepidopterans show Na^+/K^+-ATPase gene duplication and that none of the later-diverging moths that have repeatedly evolved cardenolide feeding show H122 suggest constraint. However, another possibility is that selection has been distinct in these species compared with other lineages. Distinguishing between differential constraint versus selection, while desirable, is not likely going to be easy. And perhaps it would not be as liberating as we might hope.

The herbivores feeding on cardenolide-containing plants appear to have diversified across the insect tree of life, taking advantage of common paths as well as alternative strategies. Some of this diversity has been allowed by the differences among groups, likely in feeding style, genetic architecture, and contingent historical events. Yet, of course, natural selection on the diversity of cardenolide feeders is not identical, even within a taxonomic group. For now, we will celebrate their similarities and differences, sometimes understanding where traits repeat but often not knowing their precise causes.

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Statement of Authorship

A.A.A., A.P.H., and S.D. led the conceptualization, data collection, and analysis presented here. A.A.A. wrote the first draft of the manuscript with strong input from A.P.H. and S.D. P.A.L., M.B., and D.H. collected specimens and conducted growth trials with grasshoppers. V.W. produced and assayed most of the genetically engineered sodium pump constructs. C.D. conducted mass spectrometry for chemical characterization of sequestration. G.P. analyzed the bufadienolides in sawflies.

Data and Code Availability

All data files have been deposited in the Dryad Digital Repository (https://doi.org/10.5061/dryad.1zcrjdg0n), and raw sequence reads can be found in BioProject (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1071063).

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Supplementary materials for:

Convergence and divergence among herbivorous insects specialized on toxic plants: revealing syndromes among the cardenolide-feeders across the insect tree of life

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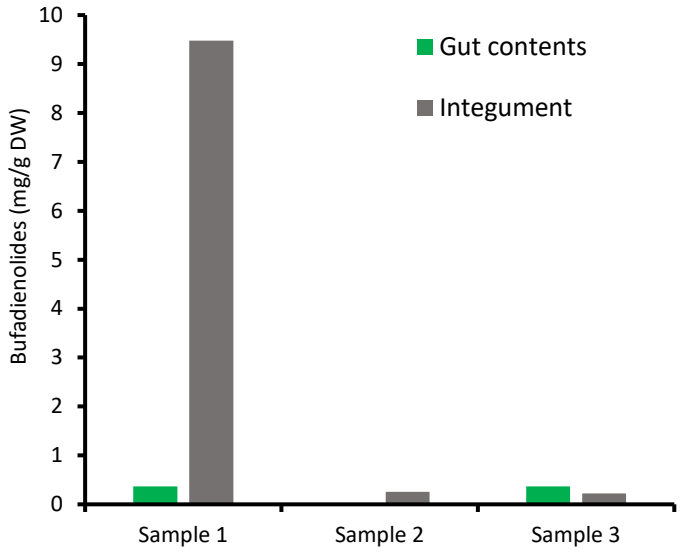

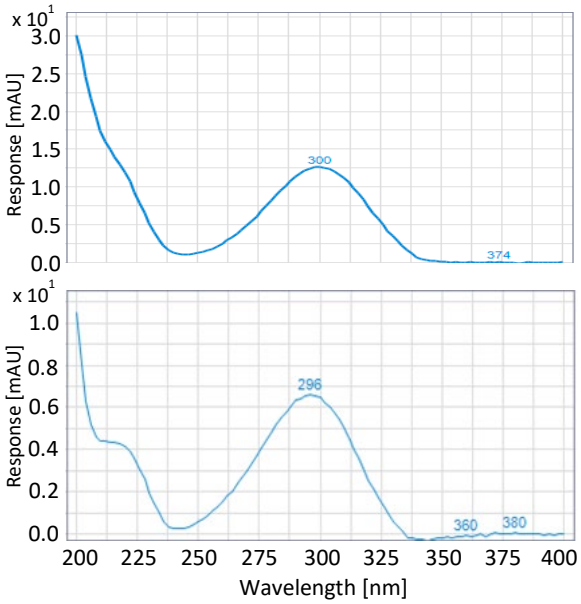

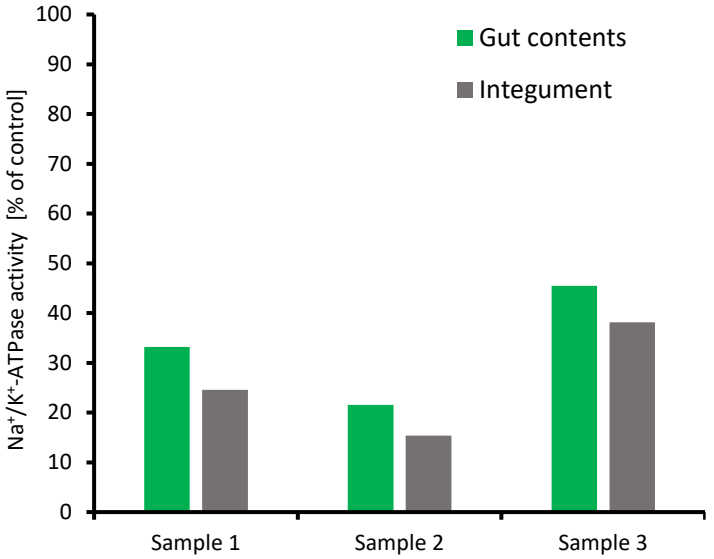

Figure S1. Aposematism and sequestration by larval *Monophadnus latus*. Bufadienolides were quantified in three samples by HPLC-UV (upper right) from larvae feeding on a *Helleborus* sp. Also shown is the UV spectrum of hellebrin (top) and a representative UV spectrum from the *Monophadnus* integument. Bufadienolides were confirmed by dosing the extracts on the porcine sodium potassium ATPase (bottom right, data shown are from undiluted samples). Note that lower bars for inhibition of the Na^+/K^+ ATPase indicate greater toxicity. Photo credit: André Mégroz.

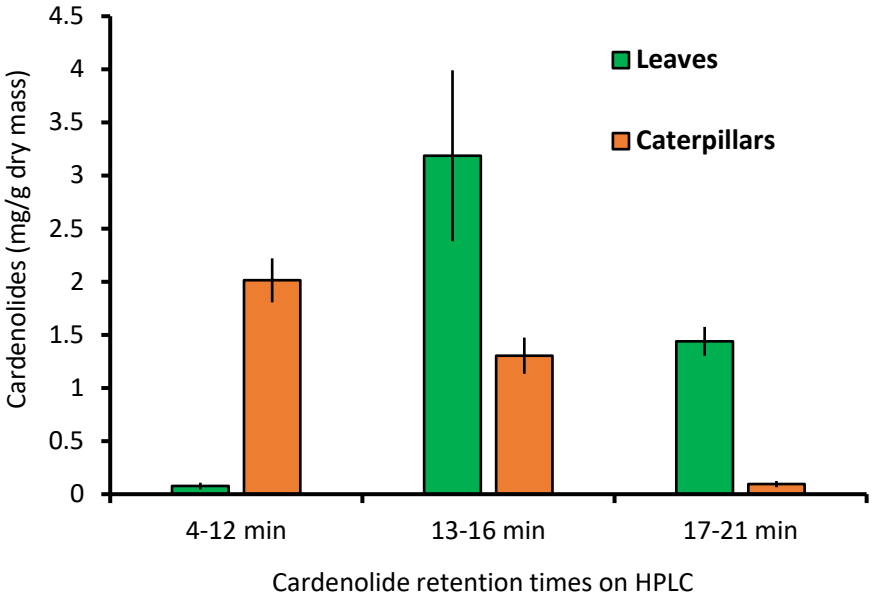

Figure S2. Leaf and caterpillar-sequestered cardenolides for *Asclepias linaria* and *Lerina incarnata* (Erebidae), respectively. *L. incarnata* are LN at amino acid positions 111 and 122. Data are from caterpillars collected in Molino Basin near Tucson, Arizona (USA). Note that total cardenolide concentrations are somewhat (38%) lower in caterpillars compared to leaves on a dry mass basis. Although leaves are dominated by mid-to-late retention time cardenolides (typically considered non-polar), most caterpillar cardenolides were polar compounds, suggesting detoxification as has been found in other milkweed insects. Shown are means ± SE. Photo credit: Anurag Agrawal.

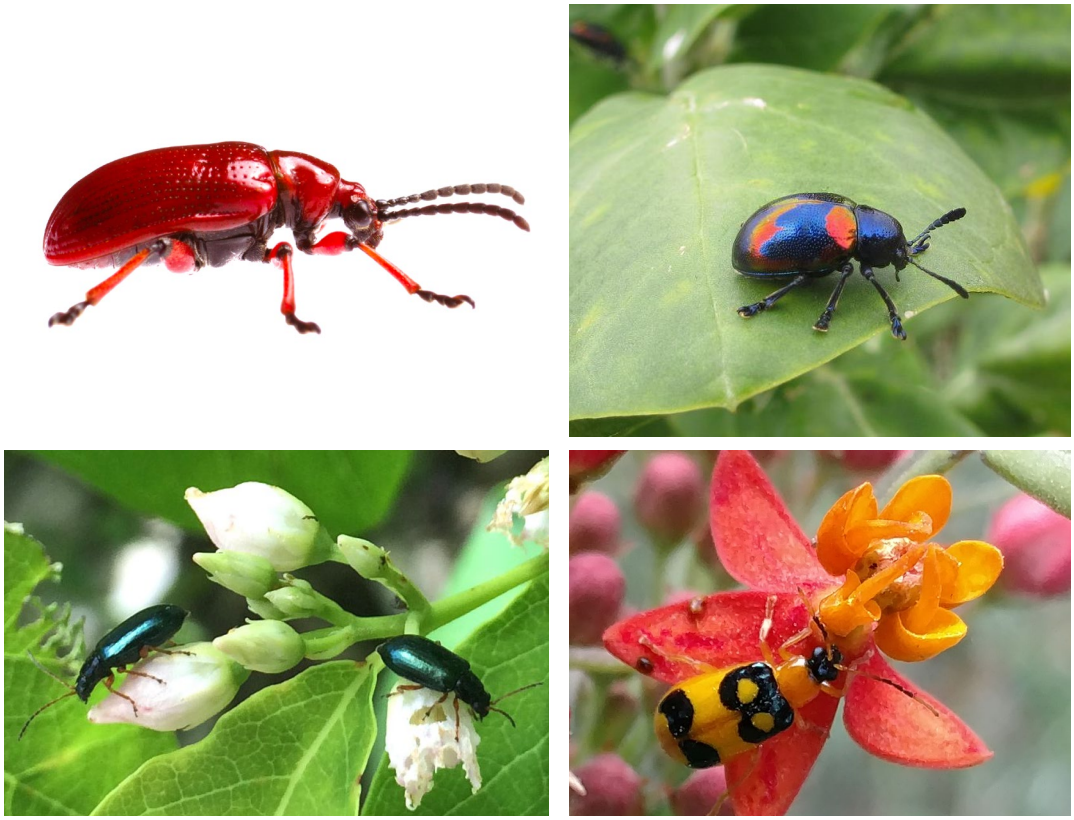

Figure S3. Cardenolide-adapted chrysomelid beetles for which additional research would be profitable. *Lilioceris merdigera* (top left, photo credit: Samuel Waldron) is neither a sequesterer nor has sodium pump substitutions but can be abundant on the cardenolide-containing Lilly of the Valley (*Convallaria majalis*). *Platycorynus undatus* (top right, photo credit: Thomas Ihle) feeding on *Calotropis gigantea* in Ban Phon Khen, Laos, belongs to the presumed sister genus to *Chrysochus* (Labeyrie and Dobler 2004). Gene duplication, target site insensitivity, and sequestration may have originated in *Platycorynus.* *Scelolyperus lecontii* (syn. *Pseudoluperus lecontii*, bottom left, photo credit: David Selytin) a V111 H122 species feeding on *Apocynum androsaemifolium* in Grove Creek, UT, USA. *Diabrotica nummularis* adult feeding on *Asclepias curassavica* (bottom right, photo credit: Anurag Agrawal) in Oaxaca, Mexico. *D. nummularis* is Q111 N122 and data on sequestered cardenolides in the elytra are presented in Figure S4. This is the only QN sequestering insect known.

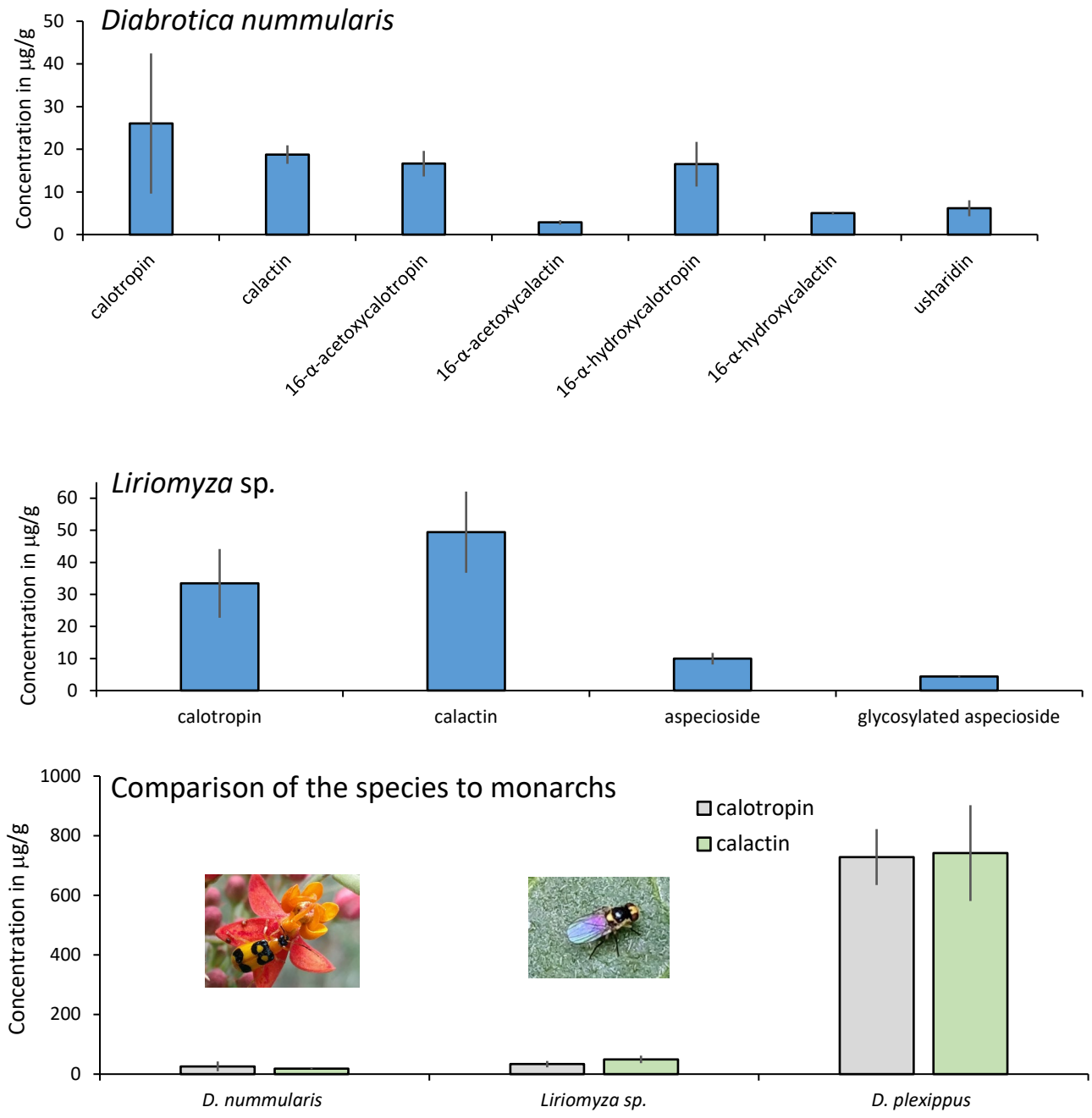

Figure S4. High resolution mass spectrometry reveals sequestration of specific cardenolides in *D. nummularis* (n=3, collected off *Asclepias curassavica* at two sites in Oaxaca, Mexico, only elytra were extracted and analyzed) and in adults of an undescribed species of *Liriomyza* collected off *A. oenotheroides* also in Oaxaca (n=2). The structures match MS/MS fragmentation spectrometric data from our library of isolated and characterized compounds. The lower panel shows sequestration of two dominant compounds in these species compared to monarchs (*Danaus plexippus*) reared on *A. curassavica*. Shown are means ± SE on a dry mass (DM) basis. Photo credits: Anurag Agrawal.

Figure S5. *Largus cinctus* (Largidae) adults and nymphs feeding on *Asclepias oenotheroides* in Oaxaca, Mexico. *Largus* spp. are LN at amino acid positions 111 and 122 and are often reported on toxic plants (including plants with cardenolides and alkaloids). Additional research on host plant associations and sequestration would be valuable. Photo credit: Anurag Agrawal.

Figure S6. The Rainbow Milkweed Locust *Phymateus saxosus* (Pyrgomorphidae) on *Gomphocarpus fruticosus* in Madagascar. This species is LH at amino acid positions 111 and 122 in its resistant copy of the sodium pump, but it maintains a more sensitive copy as well. Photo credit: Anurag Agrawal.

Table S1. A non-exhaustive history of advances in understanding convergent evolution in the specialized milkweed herbivores. Here we focus on the physiological and genetic basis of insect tolerance to plant-produced toxins (cardenolides), which has now been identified in six taxonomic orders (Lepidoptera, Coleoptera, Hemiptera, Diptera, Orthoptera, and Hymenoptera).

patterns and early physiology		
1960s-70s	Diverse milkweed insects sequester toxins and are aposematic	(Brower 1969; Duffey and Scudder 1972; Reichstein et al. 1968; Rothschild et al. 1970; Rothschild et al. 1973; Von Euw et al. 1967)
1977	Monarch butterfly has a highly resistant sodium pump	(Vaughan and Jungreis 1977)
1986	*Oncopeltus* bugs have a highly resistant sodium pump	(Moore and Scudder 1986)
1990	*Poekilocerus* grasshopper has a highly resistant sodium pump	(Al-Robai et al. 1990, 1992)
genetics, function, and convergence		
1990s	Monarch butterfly has a functionally important genetic substitution in ATPα1 at amino acid position 122	(Holzinger et al. 1992; Holzinger and Wink 1996)
2004	*Chrysochus* beetles have same substitution at amino acid position 122	(Labeyrie and Dobler 2004)
2012	Substitutions at position 122 found in more species, including two more taxonomic orders of insects (clearly convergent); gene duplication reported in several species	(Dobler et al. 2012; Zhen et al. 2012)
2015	Substitution at position 122 found in a sawfly (Hymenoptera) feeding on *Helleborus* which contains bufadienolides	(Dobler et al. 2015)
phylogenetic patterns and evolution		
2013	Progressive phylogenetic pattern of genetic adaptation to cardenolides reported in the Danainae butterflies	(Petschenka et al. 2013a)
2017	"All at once" phylogenetic pattern of genetic adaptation to cardenolides reported in the Lygaeinae	(Bramer et al. 2015)
2017	Little phylogenetic pattern and consistency found in in the genetic adaptation of multiple leaf-mining flies (Agromyzidae) to cardenolide-containing host plants	(Petschenka et al. 2017)
2019	Progressive phylogenetic pattern of genetic adaptation and function in Pyrgomorphidae grasshoppers	(Yang et al. 2019)
2019	Function of specific substitutions demonstrated *in vivo*; convergent path of molecular evolution elucidated across taxa (substitutions at position 111 typically preceding substitutions at position 122)	(Karageorgi et al. 2019; Taverner et al. 2019)
2021	Functional sodium pump substitutions reported from four animal predators of milkweed insects (including a bird, rodents, nematode, and parasitic wasp)	(Groen and Whiteman 2021)

Table S2. Insects for which new data was collected in this study. Columns 111 and 122 refer to the amino acids at those positions in the α1-subunit of Na^+/K^+-ATPase. †Indicates plant and insect tissues were assessed for cardenolides in this study. *indicates generalists not typically feeding on milkweed. + indicates a species with Na^+/K^+-ATPase gene duplication; the amino acids are shown for only the most resistant copy of sodium pump. Diet is leaves unless otherwise noted. Data on amino acids of these and other insect species are given in Table S3.

Species	Order	Family	Diet (or collected from)	111	122	Collection	Locality	Latitude / Longitude
Rhyssomatus lineaticollis†	Coleoptera	Curculionidae	*A. syriaca* stem	Q	Y+	field	New York	42.001680, -73.875839
Tetraopes tetrophthalmus†	Coleoptera	Cerambycidae	*A. syriaca root*	L	N	field	New York	42.4553582, -76.3961842
T. femoratus	Coleoptera	Cerambycidae	*A. latifolia*	L	N	field	Utah	38.593022, -109.565509
T. texanus	Coleoptera	Cerambycidae	*A. asperula*	L	N	field	Alabama	32.309503, -86.286892
Labidomera clivicollis†	Coleoptera	Chrysomelidae	*A. syriaca* / *A. curassavica*	V	H	reared	New York	42.4339002, -76.4743532
Chrysochus auratus†	Coleoptera	Chrysomelidae	*A. cannabinum* root	V	H+	field	New York	42.436648, -76.397816
Leptinotarsa decemlineata†	Coleoptera	Chrysomelidae	**A. syriaca*	V	N	reared	New York	42.519037, -76.334084
Lilioceris merdigera	Coleoptera	Chrysomelidae	*Convallaria majalis*	Q	N	field	Germany	53.715311, 10.140596
Scelolyperus lecontii	Coleoptera	Chrysomelidae	*A. cannabinum*	V	H	field	Colorado	40.108452, -105.333179
Diabrotica nummularis†	Coleoptera	Chrysomelidae	*A. curassavica*	Q	N	field	Mexico	17.199666, -96.756894
Popillia japonica	Coleoptera	Scarabaeidae	**A. syriaca* (occasional feeder)	Q	N	field	New York	42.454944, -76.397188
Liriomyza asclepiadis†	Diptera	Agromyzidae	*A. syriaca*	Q	H	field	New York	42.4339002, -76.4743532

Liriomyza sp.[†]	Diptera	Agromyzidae	*A. oenotheroides*	L	N	Field	Mexico	17.0809361133, -96.717697694
Poekilocerus bufonius[†]	Orthoptera	Pyrgomorphidae	*Calotropis procera*	L	H+	reared	Israel	31.457224, 35.379182
Phymateus saxosus	Orthoptera	Pyrgomorphidae	*Gomphocarpus fruticosus*	L	H+	field	Madagascar	-22.068316, 46.783374
Hadrotettix trifasciatus[†]	Orthoptera	Acrididae	**A. asperula*	L	N	reared	Texas	30.614, -98.071
Lygaeus kalmii[†]	Hemiptera	Lygaeidae	*A. syriaca* seed	T	H+	field	New York	42.4339002, -76.4743532
Oncopeltus fasciatus[†]	Hemiptera	Lygaeidae	*A. syriaca* seed	T	H+	field	New York	42.452041, -76.462867
Aphis asclepiadis	Hemiptera	Aphidinae (Aphididae)	*A. syriaca* phloem	E	Y	field	New York	42.454944, -76.397188
Myzocallis asclepiadis	Hemiptera	Calaphidinae (Aphididae)	*A. syriaca* phloem	E	Y	field	New York	42.434482, -76.475509
Danaus plexippus[†]	Lepidoptera	Nymphalidae	*A. syriaca*	V	H	reared	New York	42.452347, -76.47768
Lerina incarnata[†]	Lepidoptera	Erebidae	*A. linaria*	L	N	field	Arizona	32.334420, -110.680371
Cycnia tenera[†]	Lepidoptera	Erebidae	*A. cannabinum*	L	N	reared	New York	42.385732, -76.398724
Cycnia inopinatus[†]	Lepidoptera	Erebidae	*A. syriaca*	L	N	reared	Michigan	42.643198, -83.553181
Euchaetes egle[†]	Lepidoptera	Erebidae	*A. syriaca / A. curassavica*	Q	N	reared	New York	42.385732, -76.398724
Saucrobotys futilalis[†]	Lepidoptera	Crambidae	*A. cannabinum*	L	N	reared	New York	42.4339002, -76.4743532
Trichoplusia ni[†]	Lepidoptera	Noctuidae	**A. syriaca*	Q	N	reared	colony	
Monophadnus latus[†]	Hymenoptera	Tenthredinidae	*Helleborus spp.*	T	H	Field	Switzerland	47.441289, 9.408203

Table S3. Sodium pump amino acid sequences (positions 111-122, 797) for species in the phylogeny (Figure 3). Highlighted are new sequences presented in the current study.

Species	AA 111-122, 797	Reference or BioProject	Genbank Acc. No.
Orthoptera			
Gryllus bimaculatus (Gryllidae)	QASTVEDPSDDN, T	Ylla et al. 2021	GLG98691.1
Ducetia japonica B (Tettigoniidae)	ETTQEESPKNDN, V	Wang et al. 2022	OP712496.1
Schistocerca gregaria (Acrididae)	LASTVEEPSDDN, T	Dobler et al. 2019	LR535710.1
Hadrotettix trifasciatus (Oedipodinae)	LASTVEEPSDDN, T	this study, RT-PCR	OR983313
Zonocerus elegans (Pyrgomorphidae)	LASTVEEPSDDN, T	Yang et al. 2019	MK294072.1
Phymateus saxosus A (Pyrgomorphidae)	LASTTEESADSENH, -	this study, RT-PCR	OR983314
Phymateus aegrotus A (Pyrgomorphidae)	LASTTEESADSENH, T	Dobler et al. 2019	LR535713.1
Phymateus leprosus A (Pyrgomorphidae)	LASTTEESTDTENH, T	Yang et al. 2019	MK294075.1
Poekilocerus bufonius A (Pyrgomorphidae)	LASTTEESADSDDH, T	Dobler et al. 2019	LR535715.1
Poekilocerus pictus A (Pyrgomorphidae)	LASTTEESADSDDH, T	Yang et al. 2019	MK294076.1
Hemiptera			
Ericerus pela (Coccidae)	QATTVEDPADDN, T	this study, genome search	GCA_011428145.1
Aphis asclepiadis (Aphidae, Aphidinae)	ETSTTEDPNDDY, A	this study, RT-PCR	OR983315
Aphis nerii (Aphidae, Aphidinae)	ETSTTEDPNDDY, A	Zhen et al. 2012	JQ771496.1
Myzus persicae (Aphidae, Aphidinae)	ETSTTEDPNDDY, A	PRJNA397782	XM_022311823.1
Myzocallis asclepiadis (Aphidae, Calaphidinae)	ETSTSGDPNDDY, A	this study, RT-PCR	OR983316
Therioaphis trifolii (Aphididae, Calaphidinae)	ETSTSGDTNDDY, A	this study, genome search	GCA_027580255.1
Cinara cedri (Aphididae, Lachninae)	ETSTTEDPNDDY, A	this study, genome search	GCA_902439185.1
Tuberolachnus salignus (Aphididae, Lachninae)	ETSTTEDPNDDY, A	this study, genome search	GCA_956483605.1
Drepanosiphum platanoidis (Aphidinae, Drepanosiphinae)	ETSTTEDPNDDY, A	this study, genome search	GCA_948098885.1
Sipha flava (Aphididae, Chaitophorinae)	ETSTTEDPNDDY, A	this study, genome search	GCA_003268045.1
Pemphigus obesinymphae (Aphididae, Eriosomatinae)	ETSTTEDPNDDY, A	this study, genome search	GCA_025469235.1
Chaetogeoica ovagalla (Aphididae, Eriosomatinae)	ETSTTEDPNDDY, A	this study, genome search	GCA_032441825.1

Eriosoma lanigerum (Aphididae, Eriosomatinae)	ETSTTEDPNDDY, A	this study, genome search	GCA_013282895.1
Tetraneura akinire (Aphididae, Eriosomatinae)	ETSTTEDPNDDY, A	this study, genome search	GCA_021307375.1
Schlechtendahlia chinensis (Aphididae, Eriosomatinae)	ETSTTEDPNDDY, A	this study, genome search	GCA_019022885.1
Hormaphis cornu (Hormaphididae)	ETSTTEDPNDDY, A	this study, genome search	GCA_017140985.1
Daktulosphaira vitifoliae (Phylloxeridae)	ETSTTVNSNDDY, A	this study, genome search	GCA_025091365.1
Adelges cooleyi (Adelgidae)	ETSTTNDANDDY, A	this study, genome search	GCA_025091365.1
Pyrrhocoris apterus (Pyrrhocoridae)	QASTVEEPSDDN, T	Dobler et al. 2012	HE956739.1
Largus spec. (Largidae)	ETSTVEEPSDDN, T	Dobler et al. 2012	HE956738.1
Kleidocerys resedae (Lygaeidae, Ischnorrhynchinae)	QASTVEEPSDDN, T	this study, RT_PCR	OR983317
Cosmopleurus fulvipes A (Lygaeidae, Lygaeinae)	TSNTVEEASDNH, A	this study, transcriptome	SRX23623216
Arocatus melanocephalus A (Lygaeidae, Lygaeinae)		this study, transcriptome	SRX23623217
Tropidothorax leucopterus A (Lygaeidae, Lygaeinae)	TSTTVEEASDNH, A	this study, RT-PCR	OR983318
Oncopeltus fasciatus A (Lygaeidae, Lygaeinae)	TSTTVEEASDNH, A	Zhen et al. 2012	JQ771520.1
Lygaeus kalmii A (Lygaeidae, Lygaeinae)	TSTTVEEASDNH, A	Zhen et al. 2012	JQ771513.1
Hymenoptera			
Apis mellifera (Apidae)	QASTSEDPNDDN, T	Karageorgi et al. 2019	XM_006564160.3
Pachyprotasis variegata	QATTVEDPNDDN, T	Dobler et al. 2015	LN736263.1
Monophadnus latus (Tenthredinidae)	TASTLEESNDDH, T	Dobler et al. 2015; Taverner et al. 2019	LN736262.1; MK959031.1
Coleoptera			
Popillia japonica (Scarabaeidae)	QASTEEPSDDN, T	this study, RT-PCR	OR983319
Dendroctonus ponderosae (Curculionidae)	QASTVEEPNDDN, T	PRJNA846874	XM_019913697.2
Rhyssomatus lineaticollis B (Curculionidae)	QASTVEEPSDDY, T	Zhen et al. 2012	JQ771523.1
Anoplophora glabripennis (Cerambycidae)	QASTVEEPADDN, T	PRJNA348318	XM_018706533.2
Tetraopes tetrophthalmus (Cerambycidae)	LASTVEEPSDDN, T	Dobler et al. 2012; Zhen et al. 2012	HE956745.1; JQ771526.1
Tetraopes femoratus (Cerambycidae)	LASTVEEPSDDN, T	this study, RT-PCR	OR983320
Tetraopes texanus (Cerambycidae)	LASTVEEPSDDN, T	this study, RT-PCR	OR983321

Lema daturaphila (Chrysomelidae, Criocerinae)	QASTVEEPADDN, T	this study, RT-PCR	OR983322
Lilioceris merdigera (Chrysomelidae, Criocerinae)	QASTIEEPSDDN, T	this study, RT-PCR	OR983323
Chrysochus asclepiadeus B (Chrysomelidae, Eumolpinae)	VVSTVEEASDDH, T	this study, transcriptome	SRX23623219
Chrysochus auratus B (Chrysomelidae, Eumolpinae)	VVSTVEEASDDH, T	Dobler et al. 2012; Zhen et al. 2012	HE956741.1; JQ771501.1
Chrysochus cobaltinus B (Chrysomelidae, Eumolpinae)	VVSTVEEASDDH, T	Labeyrie & Dobler 2004; Yang et al. 2019	AJ617745.1; MK765672.1
Platycorynus peregrinus B (Chrysomelidae, Eumolpinae)	VVSTVEEASDDH, -	this study, RT-PCR	OR983324
Platycorynus peregrinus A (Chrysomelidae, Eumolpinae)	LASTVEEPSDDN, T	this study, RT-PCR	OR983325
Alticini sp.1 (Chrysomelidae, Alticinae)	QASTVEEPADDN, T	Dobler et al. 2012	HE956742.1
Alticini sp.2 (Chrysomelidae, Alticinae)	QATTVEEPADDN, -	this study, RT-PCR	OR983326
Agelastica alni (Chrysomelidae, Galerucinae)	QASTVEEPADDN, T	this study, genome search	GCA_950111635.1
Diabrotica nummularis (Chrysomelidae, Galerucinae)	QASTVEEPSDDN, -	this study, RT-PCR	OR983327
Scelolyperus lecontii (Chrysomelidae, Galerucinae)	VASTVEEPSDDH, T	this study, transcriptome	SRX23623218
Gastrophysa viridula (Chrysomelidae, Chrysomelinae)	QASTVEEPADDN, T	Dobler et al. 2012	HE956744.1
Calligrapha philadelphica (Chrysomelidae, Chrysomelinae)	VASTVEEPSDDH, T	Taverner et al. 2019	ERR1333730
Leptinotarsa decemlineata (Chrysomelidae, Chrysomelinae)	VASTVEEPNDDN, T	PRJNA420356	XM_023161024.1
Labidomera clivicollis (Chrysomelidae, Chrysomelinae)	VASTAEEPNDDH, T	Dobler et al. 2012	HE956743.1
Diptera			
Drosophila melanogaster (Drosophilidae)	QASTSEEPADDN, T	Sun et al. 1998; Dobler et al. 2012	AF044974.1; HE962487.1
Drosophila subobscura O3 4 7 (Drosophilidae)	VASTSEEPSDDH, -	Pegueroles et al. 2016	KT318959.1
Rhagoletis zephyria (Tephritidae)	QASTSEEPSDDN, T	PRJNA331175	XM_017619496.1
Dacus siliqualactis (Tephritidae)	VASTTEEPSDDN, -	Schneider et al. 2017	n.a.
Phytomyza fallaciosa (Agromyzidae)	QASTSEEPSDDN, T	Petschenka et al. 2017	LT795082.1
Phytomyza hellebori A (Agromyzidae)	HASTSEEPSDDH, T	Petschenka et al. 2017	LT795110.1
Phytomyza crassiseta (Agromyzidae)	QASTSEEPSDDN, T	Petschenka et al. 2017	LT795078.1
Phytomyza digitalis (Agromyzidae)	LASTSEEPSDDN, T	Petschenka et al. 2017	LT795083.1
Napomyza lateralis (Agromyzidae)	LASTEEEPSDDN, T	Petschenka et al. 2017	LT795079.1
Napomyza scrophulariae (Agromyzidae)	LASTEEEPSDDN, T	Petschenka et al. 2017	LT795080.1

Chromatomyia horticola (Agromyzidae)	QASTSEEPSDDN, T	Petschenka et al. 2017	LT795081.1
Liriomyza eupatoriae (Agromyzidae)	QASTSEEPSDDN, T	Petschenka et al. 2017	LT795109.1
Liriomyza spp. 1 Mexico (Agromyzidae)	LASTTEEPSDDN, T	Dobler et al. 2012	HE956748.1
Liriomyza spp. 2 Mexico (Agromyzidae)	LASTTEEPSDDN, T	this study, RT-PCR	OR983328
Liriomyza asclepiadis (Agromyzidae)	QASTSEEPSDDH, T	Dobler et al. 2012; Yang et al. 2019	HE956747.1; MK294077
Lepidoptera			
Limenitis archippus	QASTVEEPADDN	Zhen et al. 2012	JQ771509.1
Euploea phaenareta (Nymphalidae)	LASTVEEPSDDN, T	Petschenka et al. 2013a	HF945456
Euploea core (Nymphalidae)	LASTVEEPSDDN, T	Yang et al. 2019	MK294079
Idea leuconoe (Nymphalidae)	LASTVEEPSDDN, T	Petschenka et al. 2013a	HF945457.1
Tirumala septentrionis (Nymphalidae)	VASTVEEPSDDN, T	Petschenka et al. 2013a	HF945464.1
Tirumala petitverana (Nymphalidae)	VASTVEEPSDDN, T	Petschenka et al. 2013a	HF945463.1
Danaus chrysippus (Nymphalidae)	VASTVEEPSDDN, T	Petschenka et al. 2013a	HF945452.1
Danaus genutia (Nymphalidae)	VASTVEEPSDDN, T	Petschenka et al. 2013a	HF945453.1
Danaus erippus (Nymphalidae)	VASTVEEPSDDH, T	Petschenka et al. 2013a	HF945451.1
Danaus plexippus (Nymphalidae)	VASTVEEPSDDH, T	Dobler et al. 2012; Zhen et al. 2012	HE956755.1; JQ771507.1
Chilo suppressalis (Crambidae)	QASTVEEPADDN, T	Karageorgi et al. 2019, genome search	GCA_902850365.2
Saucrobotys futilalis (Crambidae)	LASTVEEPSDDN, T	Dobler et al. 2012	HE956749.1
Manduca sexta (Sphingidae)	QASTVEEPSDDN, T	PRJNA674349	XM_030176828.2
Daphnis nerii (Sphingidae)	LASTVEEPSDDN, T	Dobler et al. 2015	LN736265.1
Trichoplusia ni (Erebidae)	QASTVEEPADDN, T	Karageorgi et al. 2019, genome search	GCF_003590095.1
Arctia caja (Erebidae)	QASTVEEPADDN, T	this study, RT-PCR	OR983329
Empyreuma pugione (Erebidae)	LASTVEEPADDN, T	Dobler et al. 2015	LN736266.1
Syntomeida epilais (Erebidae)	LASTEEEPSDDN, T	Taverner et al. 2019	SRR9586629
Lophocampa caryae (Erebidae)	LASTVEEPADDN, T	Zhen et al. 2012	JQ771510.1
Cycnia oregonensis (Erebidae)	QASTVEEPADDN, T	Dobler et al. 2012	HE956750.1

Cycnia inopinatus (Erebidae)	LASTVEEPSDDN, -	this study, RT-PCR	OR983330
Cycnia tenera (Erebidae)	LASTVEEPSDDN, -	Dobler et al. 2012	HE956751.1
Euchaetes egle (Erebidae)	QASTVEEPADDN, T	Dobler et al. 2012; Zhen et al. 2012	HE956752.1; JQ771508.1
Lerina incarnata (Erebidae)	LASTVEEPSDDN, T	Dobler et al. 2012	HE956754.1
Pygoctenucha terminalis (Erebidae)	LASTVEEPSDDN, T	Dobler et al. 2012	HE956753.1

Table S4. Cardenolide content of *Poekilocerus bufonius* (Pyrgomorphidae) defensive secretions (four samples) from grasshoppers reared on *Caloptropis procera*. Calotropin and calactin made up 62-87% of the total cardenolides in secretions and overall, the secretions were a remarkable 23-42% cardenolide on a dry mass basis. Shown are compounds that made up >2% of the total, with HPLC retention times (minutes) of each compound.

sex	secretion (mg dry)	9.4 (mg/g)	10.8 (mg/g)	11.8 (mg/g)	13.8 (mg/g) (calotropin)	14.8 (mg/g) (calactin)	total cardenolides (mg/g)
male	0.27	8.72	5.17	3.82	108.47	147.76	292.30
male	0.492	13.52	4.67	16.64	141.82	191.72	413.26
female	3.549	14.67	12.67	16.36	68.81	76.76	234.31
female	0.169	7.88	13.72	15.37	137.40	213.92	420.09

Supplementary Methods 1. HPLC and Mass Spectrometry methods for cardenolide analysis of insect tissues and sequestration.

Plant and insect tissues were freeze-dried and ground to a fine powder. For plant samples, 50 mg dried, ground tissue was used for extraction, while insect samples varied in mass. All insects were weighed (dry) individually, and then pooled where appropriate to reach a target mass of 20-50 mg, except for the very small insects (*Liriomyza*, pooled but only reached 6-8 mg total). Fats from all samples were first extracted with 1.5 mL petroleum ether by vortexing for 10s, incubating at room temperature for 10 min, and repeating for 3 cycles, then centrifuging at 16,100 g for 10 min and discarding the solvent. After allowing any remaining solvent to dry off, samples were extracted in 1 mL methanol, spiked with 15 μg hydrocortisone as an internal standard, using a Fast Prep homogenizer and zirconia/silica beads (MP Biomedicals, Irvine, CA, USA), as in Petschenka et al. (2022b). Solids were then pelleted with a 12 min centrifugation at 12,600g and supernatants taken to dryness in a rotary evaporator (Labconco Corp., Kansas City, MO, USA).

Most insects were reared on *Asclepias* spp. However, for the subset of samples consisting of *Apocynum* tissues and *Apocynum*-reared insects, supernatants were subjected to an additional lead acetate cleanup procedure prior to drying. To each of those samples, 50 μL of a 30% lead acetate solution was added and the sample was incubated at room temp for several minutes, before a 12 min centrifugation at 12,600g to remove precipitates. Supernatants were then treated with 0.2 mL 5% sodium sulfate and centrifuged as above to precipitate free lead ions, before cardenolides were extracted with chloroform and taken to dryness. Residues were resuspended in 0.2 mL 16% acetonitrile in water and compounds brought into solution by shaking at 1000 rpm (BioShake, QInstruments, Jena, Germany) for 10 min. Samples were then filtered using 0.45 μm hydrophilic PTFE filter plates (Millipore Sigma, Burlington, MA, USA), prior to HPLC analysis.

Samples were analyzed, as in Petschenka et al. (2022b) on an Agilent 1100 HPLC (Agilent, Santa Clara, CA, USA) using a Gemini C18 reversed-phase column (3 μm, 150 x 4.6 mm, Phenomenex, Torrance CA, USA) and a constant flow of 0.7 mL/min of the following gradient of acetonitrile and Millipore-filtered water: 0–2 min at 16% acetonitrile; 2–25 min from 16% to 70% acetonitrile; 25–30 min from 70% to 95% acetonitrile; 30–35 min at 95% acetonitrile; followed by 10 min reconditioning at 16% acetonitrile. 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 yield a concentration of each cardenolide (and total cardenolide) in units of μg per mg dry tissue.

Larvae of the hymenopteran sawfly *Monophadnus latus* were collected from an ornamental *Helleborus* hybrid in St. Gallen, Switzerland, in June 2023 and reared on cut leaves. Actively feeding larvae were anesthetized on ice, and the guts were dissected under ice-cold PBS (leaving the head and anus attached to prevent spillage of gut contents into body tissues). Adhering buffer was removed by gently pulling the tissues over the edge of the dissection dish, and samples were stored at -80°C, freeze-dried and extracted in 1 ml of methanol and centrifuged as described above. Fifteen μl of each filtered extract was injected into an Agilent Infinity 1260 II HPLC system (Agilent Technologies, USA) equipped with an EC 150/4.6 NUCLEODUR C18 Gravity column (3 μm particle size, 150 mm × 4.6 mm, Macherey-Nagel, Düren, Germany). Samples were chromatographed at a constant flow rate of 0.7 ml/min using a water/acetonitrile gradient as follows: 0-2 min 16% acetonitrile; 25 min 70% acetonitrile; 30-35 min 95%

acetonitrile. Peaks were detected at 300 ± 8 nm using a diode array detector (DAD). Absorbance spectra were recorded between 200 and 400 nm. Peaks with a characteristic absorption maximum around 300 nm similar to the absorption spectrum of hellebrin were classified as bufadienolides (Petschenka et al. 2022, Am Nat). Bufadienolides were quantified at 300 ± 8 nm using a hellebrin calibration curve (5, 10, 25 50, 100 µg/ml). All putative bufadienolide peaks in one sample were summed up and divided by the tissue dry weight to calculate total bufadienolide concentrations. As a complementary approach to test for the presence of bufadienolides, we measured the inhibitory potency of our tissue extracts on porcine Na^+/K^+-ATPase according to Petschenka et al (2022).

Mass spectrometry to assess sequestration. We used high resolution liquid chromatography coupled with Mass spectrometry to assess cardenolides in three samples of a previously unstudied beetle *Diobrotica nummularis* and two samples of *Liriomyza* sp. *D. nummularis* were collected from two sites in Oaxaca, Mexico (from *A. curassavica*) and were preserved in RNA-later. Elytra were extracted as above and analyzed for cardenolides. The adult *Lyriomyza* sp. were similarly collected (from *A. oenotheroides*), and preserved, and individually extracted and analyzed. We used reversed-phase chromatography in a Dionex 3000 LC coupled to an Orbitrap Q-Exactive mass spectrometer controlled by Xcalibur software (ThermoFisher Scientific). Methanolic extracts were separated on an Agilent Zorbax Eclipse XDB-C18 column (150 mm x 2.1 mm, particle size 1.8 µm) maintained at 40 °C with a flow rate of 0.5 mL/min. Each sample was analyzed in positive electrospray ionization mode with *m/z* ranges 70-1000 for both MS1 and MS2 spectra. LC-MS data was then processed with the open-source MZmine 2 software and consisted of peak detection, removal of isotopes, alignment, filtering, and peak filling. We mined the generated feature table to retrieve cardenolide ion adducts known to be present in *A. syriaca* and *A. curassavica.* We confirmed their structure by comparing MS2 fragmentation spectra and retention time with pure isolated standards if available in our in-house library. The relative concentration (semi-quantification) based on ion counts for all cardenolides was determined using the calibration curve of aspecioside.

Supplementary text 1 – Taxonomic details on Lepidoptera. Further details on moth sodium pump substitutions, sequestration, and aposematism.

Published reports of sequestration in *Syntomeida epilais* and *Empyreuma pugione* (Petschenka et al. 2012; Rothschild et al. 1973; von Nickisch-Rosenegk et al. 1990) are complemented by the data we present here for *Saucrobotys futilalis*, *Cycnia tenera* and *C. inopinatus* (Figure 5), and *Lerina incarnata* (Figure S2). Indeed, the two *Cycnia* spp. studied here reveal higher levels of sequestration than those reported for monarchs (Figures 2, 5). Cardenolide specialization within the Phaegopterina appears to be pre-adapted with the L111 N122 sodium pump, which also occurs in the non-cardenolide-plant-feeding *Lophocampa caryae* (Taverner et al. 2019). Unlike these moths, *Daphnis nerii* (*LN*) does not sequester toxins (Petschenka and Dobler 2009, Petschenka et al. 2013), given that adynerin, the single compound reported in small amounts in their bodies (Abe et al. 1996), does not inhibit the sodium pump (Petschenka et al. 2018). However, *D. nerii* caterpillars survived injection of relatively large amounts of the cardenolide ouabain unharmed, suggesting that the sensitive sodium pump present, almost exclusively in the nervous tissue, is protected by a strong blood-brain barrier (Petschenka et al. 2013b). All sequestering milkweed moths are aposematic in at least one of their life-stages, while *D. nerii* is cryptic.

The specialist milkweed tussock moth *Euchaetes egle* is one of the few known milkweed feeders with a sensitive sodium pump (Q111 N122) and it does not sequester any cardenolides (Figure 5). We confirmed the lack of sequestration in *E. egle* by feeding several replicates foliage from both *Asclepias syriaca* (Figure 5) as well as *A. curassavica*, the latter having 3-fold higher cardenolide concentrations; no cardenolides were found in their pupae. As a "control" generalist moth species, we found that *Trichoplusia ni* (Noctuiidae) (Q111 N122) readily developed on cut leaves of *A. syriaca* (although it refuses to feed on intact plants with pressurized latex). Since cardenolides occur in all tissues (including cut leaves), the mechanism by which *T. ni* is able to cope with cardenolides is unclear (as is the case for *E. egle*). *T. ni* too lacked any cardenolides in the pupae. Perhaps most moths have strong blood-brain barriers or the ability to prevent uptake of cardenolides into the hemolymph. Given these findings, the aposematic *E. egle* caterpillars could be a Batesian mimic of adult monarchs (Figure 1). Nonetheless, it may possess other means of unpalatability, dependent on host plants, as was demonstrated in an assay with bat predators (Hristov and Conner 2005).

We note several additional points regarding cardenolide-feeding lepidopterans. First, no gene duplication of the sodium pump has been observed among the several origins of cardenolide-feeding. Second, the ancestral sensitive sodium pump at these two positions (Q111 N122) is sufficient for species to specialized on cardenolide-containing plants (such as *Euchaetes egle*, although it does not sequester). Multiple origins of L111 in moths are associated with sequestration, which contrasts with L111 N122 Danaini butterflies lacking sequestration). Additionally, *Cycnia oregonensis* is *QN* and associated with cardenolide-rich *Apocynum* spp. (DiTommaso et al. 2009), suggesting that this species may be a non-sequesterer (but this has not been tested). Finally, there are at least 15 additional *Euchaetes* spp. that are associated with Apocynaceae, but have not been tested for sequestration and their ATPα1 sequences have yet to be analyzed (Conner 2008; Dowdy et al. 2020).

Supplementary text 2 – Taxonomic details on Coleoptera. Further details on beetle sodium pump substitutions, sequestration, and aposematism.

Chrysomelidae. Intriguingly, the exact same substitutions as in the Monarch, V111 N122, arose four times independently in the Chrysomelidae, in *Chrysochus* and its relatives (Eumolpinae) (Labeyrie and Dobler 2004; this study), *Scelolyperus lecontii* (Galerucinae) (this study), *Labiodomera clivicollis* (Chrysomelinae) (Dobler et al. 2012; Zhen et al. 2012) and independently in *C. philadelphica* (Chrysomelinae) (Taverner et al. 2019), yet while all these species are aposematic (Figure 1, Figure 5) not all are sequestering cardenolides. This discovery was first made in the sequestering and aposematic Eumolpinae *Chrysochus auratus* (Figures 1, 3) and *C. cobaltinus* (Labeyrie and Dobler 2004). In these species a duplicated copy of the sodium pump gene (ATPα1) carries the V111 H122 substitutions while the ancestral copy is L111 N122 (Zhen et al. 2012). Our recent data show that the gene duplication and TSI go back further in this group, as the congeneric but non-cardenolide adapted *C. asclepiadeus* also has both gene copies, as does the presumed sister genus to *Chrysochus, Platycorynus* (Table S2, Table S3, Figure 5)*,* which feeds on cardenolide-containing *Calotropis* spp. (Jolivet and Verma 2008). Previously only the L111 N122 gene copy was found here, but the resistant gene copy must have been missed in the original attempts to identify the sodium pump via DNA based PCR, cloning and sequencing (Labeyrie and Dobler 2004). Thus, gene duplication along with strong TSI (V111 H122) go further back in the Eumolpinae.

Scelolyperus lecontii (syn. *Pseudoluperus lecontii*) beetles are iridescent bluish-black, which may be considered aposematic (Fabricant et al. 2014) but it is not yet known whether they sequester cardenolides from their host plants. Thus, *C. auratus* and likely *S. lecontii* conform to the sequestering specialist syndrome while the swamp milkweed beetle *Labidomera clivicollis* is specialized on *Asclepias* species and does not sequester cardenolides (even when fed cardenolide-rich *A. curassavica*) (Figure 5). A close relative of *L. clivicollis*, the largely Solanaceae-feeding Colorado Potato Beetle (*Leptinotarsa decemlineata*) is V111 N122, and like *T. ni* (discussed above), will readily develop on cut leaves of *A. syriaca*, but shows no sign of sequestration (Figure 5). Both *L. clivicollis* and *L. decemlineata* are defended by autogenously produced non-proteinogenic amino acids (Pasteels 1993; Timmermans et al. 1992).

C. philadelphica represents a special case as it relies on autogenous cardenolide production (Timmermans et al. 1992). *C. philadelphica*, which feeds on dogwood (*Cornus* spp.), produces cardenolides itself but does not sequester them, and is *VH* (Taverner et al. 2019; Timmermans et al. 1992). A species of Galerucinae, *Diabrotica nummularis* has been found repeatedly feeding on *Asclepias curassavica* in Mexico, from which it sequesters cardenolides into the elytra (Figures 5 and S3), yet this species is Q111 N122 and devoid of other conspicuous resistance conferring substitutions (Table S3). *Lilioceris merdigera* (Chrysomelidae, Criocerinae) is likewise aposematic and feeds on cardenolide-containing *Convallaria majalis* (Liliaceae) with the sensitive Q111 N122 state yet only makes use of the plant cardenolides in fecal shields of the larvae (it does not sequester the compounds) (Baum and Dobler 2024).

Curculionidae. Two of the other speciose families of herbivorous beetles, Curculionidae and Cerambycidae, provide some evolutionary replication for milkweed feeders and evaluation of the specialist sequestering syndrome (Figures 1, 3). The stem-feeding weevil, *Rhyssomatus lineaticollis* (Curculionidae*),* has a duplication of the sodium pump, with one copy being T111 N122 and the other

Q111 Y122 (Zhen et al. 2012). Both the T111 (Dobler et al. 2012) and the Y122 have been functionally validated and yield medium resistance to cardenolides (Table 1). Despite this resistance, *R. lineaticollis* does not sequester cardenolides (Figure 5) and its coloration is open to interpretation. Although the solid black weevil stands out to our vertebrate eyes on milkweed leaves and stems, it quickly drops to the soil when approached, where it is cryptic. Accordingly, *R. lineaticollis* appears to join *L. clivicollis* and *L. merdigera* in the non-sequestering group that is presumably primarily diet-adapted (Figure 4, Table 2). Additional species of *Rhyssomatus* feed on *Asclepias* spp. and require further investigation (Arcila Hernández et al. 2020). One other curculionid, the Aak weevil *Paramecops farinosus*, is a known cardenolide specialist, found on *Calotropis* spp., not aposematic, but has yet to be investigated for TSI and sequestration (Wijeweera et al. 2022). We expect it to be a non-sequesterer.

Cerambycidae. Four-eyed beetles in the genus *Tetraopes* (Cerambycidae) are a radiation of ≥25 species, most of which are aposematic and associated with North American *Asclepias* spp. and feed on roots as larvae (Agrawal 2004b; Farrell 2001), (Figure 1). Because the sodium pump of the four-eyed red milkweed beetle *T. tetrophthalmus* is L111 N122 (Figure 3), along with congeners *T. femoratus* and *T. texanus* (Table S2, Table S3) we assume that all *Tetraopes* spp. share this enzyme phenotype. The few species that have been studied sequester high levels of cardenolides, comparable to monarchs and other species with much more resistant sodium pumps (Ali and Agrawal 2017) (Figure 5), although there is much to be learned about variation within the genus. At this stage, data from a handful of *Tetraopes* spp. suggest that these species join several lepidopterans as sequesterers with modestly resistant sodium pumps (Figure 4, Table 2). The neotropical sister genus *Phaea* (Farrell 2001) has 57 species some of which are reported to feed on *Asclepias* and *Thevetia* species (Apocynaceaea) (Chemsak 1999) with latex and cardenolides but nothing is known about sequestration or TSI in these species.

Scarabaeidae. The Japanese Beetle (*Popillia japonica*) is a generalist that has been repeatedly found feeding on *Asclepias* species (Baker and Potter 2018; AAA, personal observations), yet it has a sensitive sodium pump (Q111 N122) and likely does not sequester cardenolides (Table S2, Table S3).

Supplementary text 3 – Taxonomic details on Diptera. Fly toxin sequestration and aposematism, as well as additional species in the Tephritidae and Drosophilidae

Leaf-mining flies were previously mostly considered from the perspective of dietary tolerance to cardenolides. In this study, we provide the first evidence that two species (*Liriomyza asclepiadis* feeding on *A. syriaca* and the undescribed species feeding on *A. oenotheroides*) sequester small amounts of cardenolides (Figure 5 and S4). Although several cardenolides were detected among multiple independent samples of pupae, the level of sequestration is 95% lower on a dry mass basis than in other insect sequesterers on *A. syriaca* (Figure 5). As such, it is unclear whether these low levels could be effective against a predator. We note that undescribed specimens of *Liriomyza* have been collected in different regions of Mexico on different *Asclepias* host plants. Although they have identical sodium pump sequences, a sequence divergence of 0.061 for CO1 (genbank Acc. No HE862404 and No. PP396814) suggests that they may belong to two separate species. Although *L. asclepiadis* and the undescribed species are aposematically colored (Figure 1), all *Liriomyza* species seem to share black and yellow coloration irrespective of host plant, while the other cardenolide-feeding leaf miners (*Chromatomyia horticola, Napomyza scrophulariae, P. digitalis* and *P. hellebori*) show less evidence for aposematism. These findings don't negate the inclusion of *L. asclepiadis* in the specialist sequestering syndrome, but complicate the interpretation of the evolution of these cardenolide specialists. While none of these agromyzid flies can be said to fully adhere to the sequestering specialist syndrome, *L. asclepiadis* could fit if its cardenolide sequestration and aposematic coloration are defensive. Both *L. asclepiadis* and *L.* sp. sequester small amounts of cardenolides from their host plants (Figure 5 and Figure S4). Other leaf mining Diptera have found unknown ways to tackle cardenolide toxins.

Tephritidae. One additional fly, *Dacus siliqualactis* (Tephritidae) commonly feeds on cardenolide-containing *Gomphocarpus* seeds in east Africa, is V111 N122, and does not sequester cardenolides (Schneider et al. 2017). This could be another case of a modestly tolerant non-sequesterer (Table 2); its congeners that do and don't feed on cardenolide-containing plants would be well-worth investigating.

Drosophilidae. Finally, there is the intriguing case of *Drosophila subobscura* from Europe, which was found to be polymorphic for its sodium pump, with some populations that are V111 H122 (Pegueroles et al. 2016). Although typically consuming rotting fruits, *D. subobscura* has been reared from compost with a cardenolide-producing *Digitalis* sp. (Shorrocks 1982), suggesting that its sodium pump polymorphism may be functional.

Supplementary text 4 – Taxonomic details on additional Hemiptera, aphids and *Largus* spp: sodium pump substitutions, toxin sequestration and aposematism.

Aphididae. Common milkweed (*A. syriaca*) has four species of aphids, three in the subfamily Aphidinae and a fourth in the earlier diverging Calaphidinae (*Myzocallis asclepiadis*) that can be found feeding and reproducing on plants in natural populations in eastern North America, even at a single site: *Myzus persicae* (a broad generalist), *Aphis nerii* (which has hundreds of hosts, mostly in the Apocynaceae), *A. asclepiadis* (<10 host species, Apocynaceae), and *M. asclepiadis* (apparently restricted to *A. syriaca*) (Züst and Agrawal 2016) (Figure 5). All species have E111 and Y122, and also A797 (Zhen et al. 2012) (Table S2, Table S3). The latter mutation was among the first shown to provide very strong TSI (Croyle et al. 1997) and is as effective as H122 (Croyle et al. 1997; Dalla et al. 2013). As enzyme assays with mutated sodium pump genes of *D. melanogaster* expressed in cell culture show, the combination of E111 and Y122 also provides high resistance to cardenolides while the individual mutations provide only moderate resistance (Table 1). A search of the currently available genomes revealed that not only all aphids, but the whole clade of Aphidomorpha (including the pine and spruce aphids and the phylloxerans) possess the combination of E111, Y122, A797 in their Na^+/K^+-ATPaseα1 genes and only in the Coccoidea (scale insects, e.g. in *Icerya purchasi*, *Ericerus pela* or *Phenacoccus solenopsis*), is the ancestral Q111 N122 T797 combination for these amino acids present. Accordingly, like the Lygaeinae, all known Aphididae, independent of host plant, apparently are insensitive to cardenolides (although the Lygaeinae can be expected to be much more resistant, Table 1).

Given these early sodium pump substitutions, it is perhaps surprising that there are not more aphids on cardenolide-producing plants. Among the four species investigated, there is variation in tolerance to, and sequestration of, cardenolides. For example, there is some evidence for negative effects of cardenolides on *A. nerii* (Agrawal 2004a; Birnbaum et al. 2017; Züst et al. 2018) and *M. asclepiadis*, but not the other species (Züst and Agrawal 2016; Züst and Agrawal 2017). Increasing specialization is associated with greater sequestration, but not with aposematism. The most obviously aposematic species (*A. nerii*) is a relatively low sequesterer of cardenolides, at least on some host plant species (Züst and Agrawal 2016), but this sequestration can negatively affect predators (Malcolm 1989; Züst et al. 2018).

Sequestration in the aphids seems decidedly different than for the chewing insects. Firstly, mostly non-polar cardenolides accumulate in aphid bodies, whereas the reverse is true for monarchs and other chewing insects (Malcolm 1990; Züst and Agrawal 2016; Züst et al. 2018). This suggests a more passive basis for sequestration in aphids, as non-polar compounds more easily cross animal membranes (Züst and Agrawal 2016), although some cardenolide modification also appears to occur in *A. nerii* (Züst et al. 2018). Passive accumulation of cardenolides in aphids (even in the generalist species) is consistent with the basal sodium pump resistance to cardenolides in the Aphididae – in other words, perhaps there has been little selection for modification and storage of cardenolides, as there has been in other species (e.g., monarchs and Lygaeinae Agrawal et al. 2021; Agrawal et al. 2022). Accumulation of cardenolides in the highest sequestering and most specialized aphid, *M. asclepiadis*, rivals that of monarchs (both accumulate over 1 mg/g dry mass cardenolides when feeding on *A. syriaca*) (Züst and Agrawal 2016). The other three species of aphids sequestered less than half that, but were relatively similar in concentration and composition of cardenolides between species (Züst and Agrawal 2016).

Among the three specialist aphids on milkweed, only *A. nerii* is unambiguously aposematic (Figure 5). *Myzocallis asclepiadis* could be considered aposematic as adults (Figure 5), and the nymphs

exhibit bright coloration (variable, but often spotted pink), although this does not fall in the classic characterization of aposematism. And finally, *A. asclepiadis* appears unambiguously cryptic. At the risk of over-speculation, we posit that *A. asclepiadis* breaks the mold in an interesting and explainable way. By being tolerant to cardenolides, passively sequestering, and nearly universally tended and protected by ants in the field (Mooney and Agrawal 2008; Züst and Agrawal 2017), we speculate that selection for aposematism was relaxed in this aphid species.

In summary, our knowledge to date is that all aphids (and the whole clade of the Aphidomorpha) are physiologically resistant to cardenolides, although the ancestral selective agent for the sodium pump substitutions is unclear. Despite this resistance, most aphids will simply not feed on cardenolide-containing plants. Variation among species in sequestration appears to be more strongly associated with the level of host plant specialization than sodium pump substitutions. Finally, although many other aphid species feed on other plants with cardenolides (e.g., foxglove) (Christmann et al. 1993), little work has been done on aspects of their sodium pump resistance, sequestration, and aposematism.

Largidae. Bordered plant bugs, *Largus* spp., have been reported associated with several species of *Asclepias* in California (Booth 1990) and in Northern Sonora (Mexico) associated with *A. angustifolia* (Dobler et al. 2012) and Oaxaca (Mexico) associated with *A. oenotheroides* and *A. glaucescens* (AAA, personal observations, Figure S5). Observations reported on iNaturalist confirm these associations (e.g., https://www.inaturalist.org/observations/57395458). In Oaxaca, substantial leaf damage was repeatedly observed by *L. cinctus* on *A. oenotheroides* (Figure S5). Nymphs are clearly aposematic, while adults are ambiguously so (Figure S5). *Largus* spp. are known to be sequesterers of pyrrolizidine alkaloids on other host plants (Klitzke and Trigo 2000). Although it is somewhat unclear, *Largus* spp. appear to be toxic plant generalists, not unlike several species in the Lygaeinae (Carlson and Agrawal 2023). In both cases, the bugs show strong associations with milkweeds (or other cardenolide plants), but appear to feed on many species outside this group, and also have shifts onto other toxic plant species (Petschenka et al. 2022a). *Largus* exhibits E111 N122, similar to the Aphidomorpha and at least one beetle (*Rhyssomatus lineaticollis*), while most other Hemipterans have the ancestral Q111 (Karageorgi et al. 2019; Taverner et al. 2019). Tests with engineered enzymes show that E111 provides modest levels of insensitivity to cardenolides (similar to V111) (Table 1). Further study on the extent of *Largus* spp. diversity, their associations with cardenolide-containing plants, and cardenolide sequestration are in order.