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Plant chemical diversity enhances defense against herbivory

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Multiple hypotheses have been put forth to understand why defense chemistry in individual plants is so diverse. A major challenge has been teasing apart the importance of concentration vs. composition of defense compounds and resolving the mechanisms of diversity effects that determine plant resistance against herbivores. Accordingly, we first outline nonexclusive mechanisms by which phytochemical diversity may increase toxicity of a mixture compared to the average effect of each compound alone. We then leveraged independent in vitro, in vivo transgenic, and organismal experiments to test the effect of equimolar concentrations of purified milkweed toxins in isolation vs. mixtures on the specialist and sequestering monarch butterfly. We show that cardenolide toxin mixtures from milkweed plants enhance resistance against this herbivore compared to equal concentrations of single compounds. In mixtures, highly potent toxins dominated the inhibition of the monarch's target enzyme (Na^+/K^+-ATPase) in vitro, revealing toxin-specific affinity for the adapted enzyme in the absence of other physiological adaptations of the monarch. Mixtures also caused increased mortality in CRISPR-edited adult *Drosophila melanogaster* with the monarch enzyme in vivo, whereas wild-type flies showed lower survival regardless of mixture type. Finally, although experimentally administered mixtures were not more toxic to monarch caterpillars than single compounds overall, increasing caterpillar sequestration from mixtures resulted in an increasing burden for growth compared to single compounds. Phytochemical diversity likely provides an economical plant defense by acting on multiple aspects of herbivore physiology and may be particularly effective against sequestering specialist herbivores.

chemical ecology | coevolution | monarch butterfly | chemical diversity | cardenolide

Significance

Plants make a diversity of defensive substances, but we do not yet know the significance of such chemical diversity against herbivorous pests. We show that plant toxin mixtures from milkweeds act on multiple physiological mechanisms of the monarch butterfly to reduce insect performance. Toxin mixtures imposed stronger negative effects than individual compounds at three scales: 1) greater inhibition of an essential cellular enzyme (sodium–potassium pump), which was dominated by a highly potent toxin (labriformin) in mixtures, 2) mixtures induced higher mortality of transgenic flies expressing the monarch's enzyme but lacking sequestration abilities, and 3) costs of toxin sequestration for monarchs were stronger when fed mixtures. The evidence points to how chemical diversity provides an effective and economical defense against herbivores.

Chemical ecologists seek to understand the distribution, abundance, and diversity of chemical compounds produced by organisms. Plants have evolved an astonishing diversity of compounds that play myriad ecological roles in nature, ranging from fragrances that attract mutualists to chemical defenses that repel or poison herbivores (1). In the case of defense, the hypothesized role of phytochemical diversity in plant–herbivore interactions has been rising in prominence over the past decade (2–8), and multiple hypotheses have been formulated to understand its significance (9). Despite advances, the few studies that have addressed these hypotheses led to heterogeneous outcomes (2, 6, 10–12), likely because the mode of action of most compounds is unknown (13), and the underlying insect adaptations to cope with most plant toxins remain unresolved. Indeed, the mechanisms by which individual toxins may impact herbivores when occurring in plant mixtures have not been distinguished to date (Table 1). An additional challenge has been to tease apart the relative importance of concentration vs. composition of compounds and to test their effects experimentally with and without the adaptations of relevant herbivore species (9, 14, 15).

Here, we demonstrate a stronger negative effect of natural phytochemical mixtures from milkweed plants (*Asclepias* spp.) on their specialized herbivore, the monarch butterfly (*Danaus plexippus*), relative to single compounds. Milkweeds contain cardenolides, toxic plant secondary metabolites that inhibit the Na^+/K^+-ATPase (i.e., sodium pump) present in all animal cells, negatively impacting primary animal functions (19). Monarch butterflies have evolved cardenolide insensitivity through stepwise mutations in amino acid positions 111, 119, and 122 of the α-subunit of their sodium pump (20), and also the ability to accumulate (i.e., sequester) host plant toxins to reduce predation (21). Although sequestration is often viewed as the pinnacle of adaptation to toxic host plants, it also exposes these herbivores to toxins they bring into their bodies. Thus, this system presents a reciprocally adapted species pair (22), chemical compounds with a known mode of action (23), and animal targets that can be genetically engineered to address fundamental hypotheses (20).

We tested the effect of purified milkweed toxins in isolation vs. in mixtures on the monarch butterfly by leveraging independent in vitro and in vivo experiments that tease apart multiple adaptations of the herbivore to cope with plant defenses: 1) in vitro enzyme assays of the monarch's sodium pump and an unadapted species, allowing us to examine the potency of individual cardenolides and their mixtures on their physiological target in

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Table 1. Effects of phytochemical diversity may arise from several nonexclusive mechanisms, all of which result in a nonadditive effect of mixtures compared to single compounds in equal total concentration

Diversity mechanisms	Impacts on organisms
Pharmacodynamic synergism (16)	Individual compounds in mixtures may move through the animal body and exert their function in distinct ways, potentially on multiple targets, increasing the overall impact.
Pharmacokinetic synergism (16)	Some compounds in mixtures may facilitate the absorption or movement of other compounds through "solvent" or surfactant effects, acting as modulators of compound transport, permeation, and bioavailability.
Dominance effect	Low concentrations of some compounds may be sufficient to dominate the mixture's negative effect [analogous to the selection effect (17) in plant ecology]. While this mechanism does not strictly fit the definition of synergy, it nonetheless contributes to the enhanced effects observed in diverse mixtures compared to individual compounds.
Environmental diversity	Mixtures may change the relationship between the organism and environment (e.g., making the organism more sensitive to a stress). Unlike synergistic effects based on multiple targets, environmental synergy relies on specific conditions (e.g., changes in temperature or pH of the mixture containing the compounds) for its manifestation and may not always occur.

In chemical ecology, the term "synergy" was historically used for such nonadditive effects of mixtures impacting herbivore performance (12). Nonetheless, in nearly all organismal assays, the above alternative mechanisms for how phytochemical diversity impacts organisms are not distinguished (6, 9, 12, 18). Analyses at different scales (e.g., the combination of in vitro and in vivo approaches, or tests on specialists versus generalists) may help to distinguish the causes of phytochemical diversity.

the absence of other mechanisms of living organisms to handle toxins; 2) feeding and survival of wild-type and CRISPR-Cas9-edited *Drosophila melanogaster,* with the latter expressing the same three amino acid substitutions as the monarch's sodium pump (hereafter "monarch flies") but lacking the ability to transform, actively transport, and accumulate cardenolides compared to monarchs; and 3) cardenolide ingestion and accumulation in monarch caterpillars and impacts on their performance.

In each experiment, we tested for effects of mixtures by using three dominant leaf cardenolides from each of two different milkweed species: the main host for the herbivore, *Asclepias syriaca*, and a less often used host, *Asclepias perennis*, in which the cardenolide composition strongly differs from other milkweed species (24, 25) (*SI Appendix*, Fig. S1); our goal was to employ a dominant and a subordinate plant species that differ in their cardenolide profiles to increase the generality and inference from out work. To this end, we used equimolar solutions of each compound in isolation and as mixtures of the three compounds from each host species. Below we present effects of single toxins vs. their mixtures, accounting for plant species in analyses, with detailed species-specific effects reported in *SI Appendix*. Critically, this approach statistically blocks for the two groups of three compounds from each species, thereby maximizing general inference and power to detect the effects of phytochemical diversity. Our design integrates robust in vitro, in vivo transgenic, and organismal tests for the effects of phytochemical diversity against herbivores through their specific genetic adaptations, controlling for the overall toxin concentration.

Results and Discussion

Toxin-Specific Effect on Enzyme Activity. We examined the effect of purified individual toxins from milkweeds and their mixtures on the physiological target of cardenolides, the sodium pump, obtained from monarch neural tissues and the porcine kidney, the latter serving as a highly sensitive control enzyme (hereafter "unadapted enzyme") (Fig. 1). On average, the monarch enzyme was 55-fold less inhibited by cardenolides compared to the unadapted enzyme ($F_{1,67}$ = 5,059.24, $P < 0.001$, Fig. 1 and *SI Appendix*, Fig. S2; for milkweed species-specific effects, see *SI Appendix*, Fig. S3 and *Supporting Results*). This supports previous work in which amino acid substitutions of the adapted sodium pump have reduced cardenolide binding affinity through spatial and polarity changes of the target site (26, 27). In line with previous work (23, 28, 29), we found that individual cardenolides had much more variable effect (nearly 46-fold) on inhibition of the adapted monarch sodium pump compared to the 3.5-fold variation in inhibition of the unadapted enzyme (Fig. 1).

Toxin mixtures had stronger inhibitory effects on both the adapted monarch ($P < 0.001$, 50% higher inhibition) and unadapted ($P = 0.031$, 18% higher inhibition) enzymes than the average of single compounds (*SI Appendix*, Fig. S2), although enzyme inhibition by mixtures was not always stronger than each of their corresponding individual toxins (Fig. 1). These results indicate two key findings. First, the specialist herbivore's enzyme adaptations are not equally effective against all cardenolides. For instance, the highly toxic compound labriformin from *A. syriaca* was, by far, the most inhibitive against the monarch enzyme, whereas this same compound showed average inhibition of the unadapted enzyme (Fig. 1). Second, effects of phytochemical diversity at the physiological level of sodium pump inhibition are likely driven by toxin-specific affinity and competition for the target site, leading some compounds to dominate the target site, even if at low concentrations in mixtures (Fig. 1 and Table 1). Specifically, we found that the highly toxic labriformin reduced the overall amount of toxin required to inhibit the adapted enzyme by 50% (i.e., IC50) from 19.1×10^{-5} mol L^{-1} under the assumption of equal toxin-specific binding in mixtures, to 2.99×10^{-5} mol L^{-1} observed in the experimental mixture. Therefore, such highly potent compounds may help reduce substantial costs of defense production (especially of less potent compounds that could be needed in higher concentrations), supporting the notion that phytochemically diverse mixtures provide an economical means of plant defense. It is, thus far, unclear whether such dominance effects could scale up to impacts on whole organisms in vivo.

Mixtures Increase Monarch Fly Mortality. We next compared genetically engineered *D. melanogaster* adults with the monarch's sodium pump substitutions (VSH at amino acid positions 111, 119, and 122, respectively) to wild-type flies (QAN, respectively) for survival and feeding rate after consuming solutions enriched either with single cardenolides or equimolar toxin mixtures. Monarch flies survived 34% longer ($F_{1,112}$ = 45.06, $P < 0.001$) and fed 35% more ($F_{1,112}$ = 41.23, $P < 0.001$) compared to the wild-type flies on cardenolide-enriched diets (but had equal survival and feeding

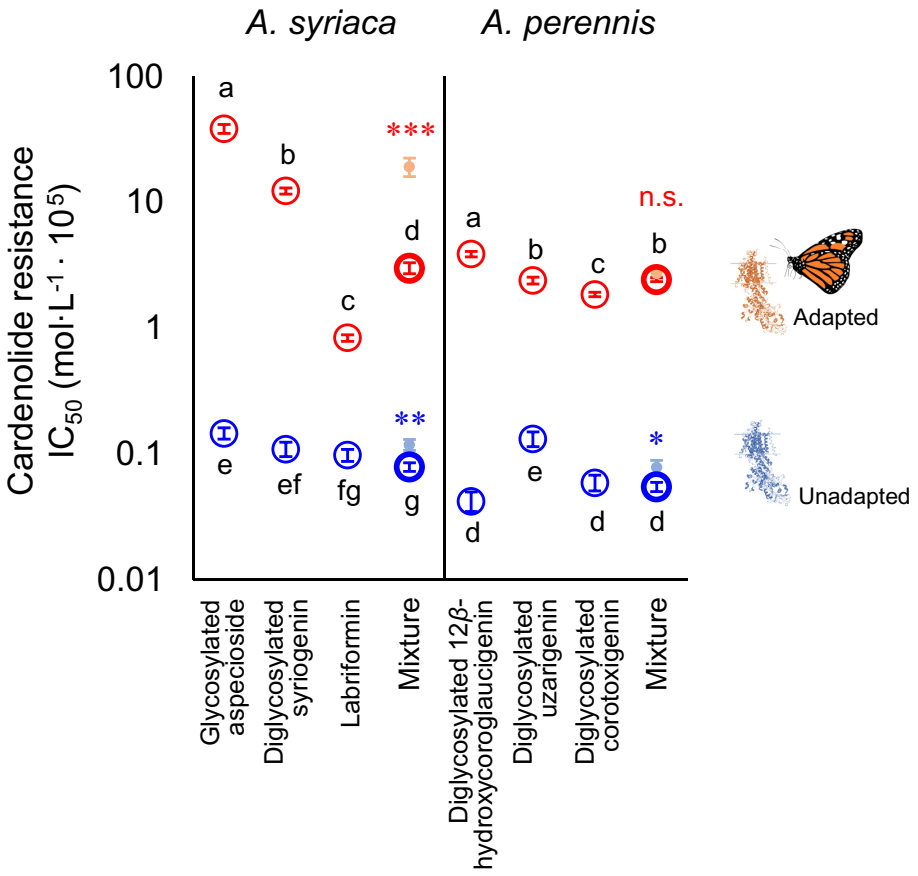

Fig. 1. Effects of individual cardenolides and mixtures from each milkweed species on resistance of the physiological target, the sodium pump (Na^+/K^+-ATPase). Sodium pump inhibition was toxin-specific for both unadapted and adapted organisms, especially with *A. syriaca* toxins. Serial dilutions of equimolar concentrations of purified cardenolides were used to examine the concentration at which the activity of the unadapted (blue) and monarch enzyme (red) reached 50% inhibition (IC_{50}) in vitro. Smaller, light-colored dots represent the expected values of mixtures under the null hypothesis of equal toxin binding to the physiological target. Different letters denote significant differences between toxin types and enzymes within each milkweed species ($P < 0.05$). Asterisks represent significant differences between the expected vs. observed values in mixtures (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; n.s. not significant). Shown are means ± SE (n = 3-6). Note the log scale on the *Y*-axis.

on control diets: *SI Appendix*, Fig. S4, $P_{survival} = 0.926$; $P_{feeding\ rate} = 0.982$). Nonetheless, when given toxin mixtures, monarch flies died 16% faster than when fed single toxins (Fig. 2; $P = 0.002$), whereas wild-type flies showed equivalently rapid mortality on both diet types (Fig. 2; $P = 0.785$). We found a similar pattern for feeding rate in both the monarch (17% lower in mixtures; $P = 0.003$) and wild-type fly lines (comparison of the lines, $P = 0.882$), suggesting that feeding on toxic diets impacted fly survival through accumulation of toxicity (for milkweed species-specific effects, see *SI Appendix*, Fig. S5 *A–D* and *Supporting Results*).

This result mechanistically demonstrates that CRISPR-edited flies benefit from the adapted sodium pump through greater survival when fed toxins, supporting prior work (20). However, the lack of additional physiological adaptations—present in monarchs—which reduce the toxic effects of multiple cardenolides (e.g., detoxification and transport) likely prevented survival of monarch flies upon ingesting toxin mixtures to be similar as to single compounds. Indeed, the survival of monarch flies was lower on mixtures compared to any of the individual compound treatments (*SI Appendix*, Fig. S4). This contrasted with the negligible, similar impact of single cardenolides and mixtures on monarch caterpillar survival (see *Materials and Methods* Section "*Monarch caterpillar feeding assay*"), suggesting that the adapted sodium pump is not sufficient as the only evolutionary innovation to deal with toxic cardenolides. In fact, cardenolide-adapted insects can convert, detoxify, or accumulate cardenolides in the gut lumen as part of their adaptations to retain toxins and cope with their toxicity (30, 31). Nonetheless, cardenolides may be transported differently and likely remain largely untransformed throughout the body of unadapted insects upon ingestion. Therefore, toxin mixtures increased mortality compared to singles in monarch flies likely due to negative, chemical diversity effects on the sodium pump enzyme in multiple parts of the insect's body upon movement of toxins after ingestion (Table 1). In fact, without active transport, cardenolides of different polarities may reach different tissues passively, simultaneously affecting different cell types in different parts of the body and also the kinetics of sodium pump inhibition in diverse ways (32). Conversely, the effect of any compound (either single or in mixture) resulted in high mortality due to their high toxicity for wild-type flies (Fig. 2).

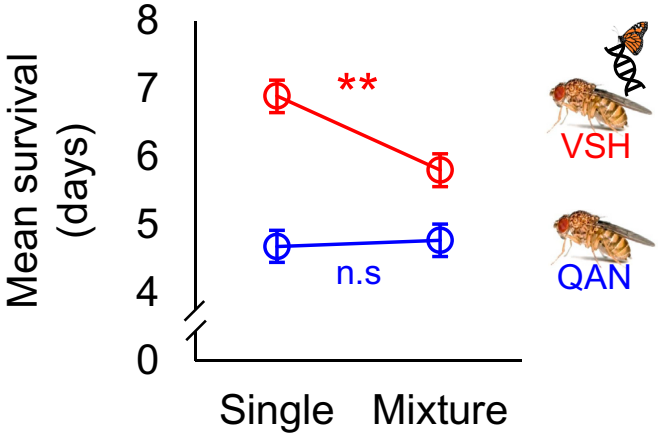

Fig. 2. Effects of single cardenolides vs. mixtures on survival of CRISPR-edited *D. melanogaster* with the monarch enzyme (VSH, red) and wild-type flies (QAN, blue). Monarch flies had higher survival overall but showed lower survival when fed toxin mixtures compared to single compounds. Fly diets were spiked with equimolar concentrations of purified cardenolide compounds for 12 d. Shown are means ± SE (n = 30 per fly line and mixture type). Asterisks denote significant differences ($P < 0.01$). Nonsignificant is denoted as "n.s." The vertical axis is truncated for ease of visualization.

Sequestering Mixtures Impairs Caterpillar Growth. Finally, we examined how toxin ingestion and accumulation in the monarch body differentially affected caterpillar growth when feeding on leaves of a very low-cardenolide milkweed species (*Asclepias incarnata*) laced with single toxins or mixtures. We found no net impacts of toxin ingestion from single cardenolides vs. mixtures on caterpillar growth overall (n = 30, β_{single} = 0.64, P = 0.111; n = 10, $\beta_{mixture}$ = 0.97, P = 0.204; no differences in their slopes t = 0.40, P = 0.688), and their effects did not differ from untreated control leaves (control vs. single, t = −1.57, P = 0.125; control vs. mixture, t = −1.56, P = 0.127). Additionally, monarch caterpillars sequestered equivalent cardenolide amounts, on average, from single and mixture treatments ($F_{1,36}$ = 0.17, P = 0.681, *SI Appendix*, Fig. S6). Nonetheless, despite overall sequestered concentrations being equivalent, the concentration range and relative composition of sequestered compounds differed strongly between treatments with the different single cardenolides or their mixtures (Compound: $F_{5,36}$ = 349.51, P < 0.001; Mixture type: $F_{1,36}$ = 113.11, P < 0.001; Compound × Mixture type: $F_{5,36}$ = 18.56, P < 0.001). This result suggests selective sequestration by caterpillars.

Despite no overall effect on growth, we found that increasing sequestration from leaves painted with toxin mixtures imposed a strong burden on caterpillar growth (n = 10, $\beta_{mixture}$ = −4.31, P = 0.011) compared to leaves painted with single compounds (n = 30, β_{single} = −0.43, P = 0.336) (difference in slopes of the two regressions: t = −2.55, P = 0.015) (Fig. 3; for milkweed species-specific effects of single compounds and mixtures, see *SI Appendix*, Fig. S7 and *Supporting Results*). To avoid potential biases from our original analysis, we restricted the original range of sequestration and experimental sample size of single compounds (i.e., n = 30, 0.01 to 11.7 nmol mg^{-1}) to be the same as in mixtures (i.e., n = 10, 0.5 to 6.7 nmol mg^{-1}) and reanalyzed the data with 10,000 simulations (see *Materials and Methods* Section "*Effects of cardenolide accumulation on monarch performance*"). We still found a greater burden when sequestrating more from mixtures (n = 10, $\beta_{mixture}$ = −6.27, P = 0.034) compared to single compounds (n = 10, β_{single} = −3.32, P = 0.078) (*SI Appendix*, Fig. S8). We interpret this result as cardenolide mixtures increasing the cost of sequestration for monarch caterpillars.

Higher accumulation of plant toxins typically benefits insects by reducing predation from the third trophic level (35, 36). Nonetheless, sequestration is a highly regulated process with toxin concentrations plateauing in the body (37, 38), eventually imposing metabolic costs at higher concentrations (21) that are often difficult to detect in typical relationships between insect growth and defense concentrations from the plant (28). This pattern of a burden or cost of cardenolide sequestration has been previously reported on whole plants (28, 35), yet here, we have revealed that phytochemical diversity intensifies this effect after controlling for the amount of toxin consumed. Our findings indicate that the negative effects of mixtures on insect growth upon sequestration are stronger than upon ingestion, most likely due to multiple nonexclusive effects of detoxification and transport occurring in the insect's body.

Although monarchs sequester some cardenolides intact, they typically convert toxins through distinct physiological mechanisms such as deglycosylation or hydrolysis of the thiazoline heterocycle of particular cardenolides, such as the nitrogen-containing labriformin (*SI Appendix*, Fig. S1) (29, 30). In addition, monarch caterpillars selectively sequester more compounds that are less toxic to themselves while maintaining toxicity against their predators (25, 30). Concordantly, we found that monarch caterpillars selectively sequestered compounds that are less toxic for their target enzyme from mixtures compared to single compounds, especially from the major host *A. syriaca* (*SI Appendix*, Fig. S7).

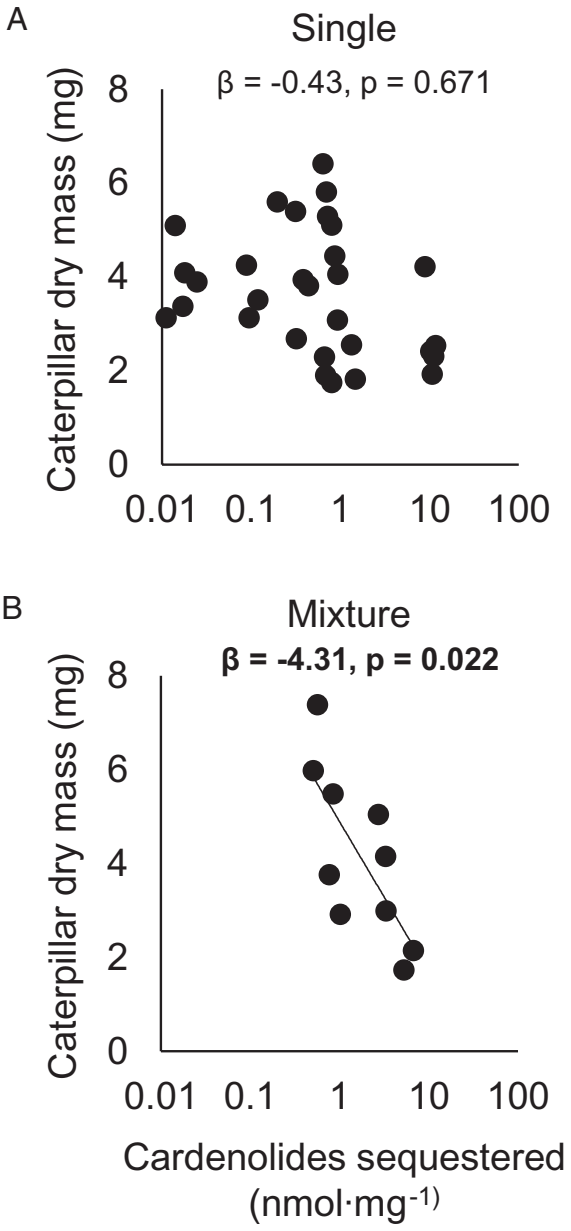

Fig. 3. Effect of single vs. mixtures of cardenolides on the relationship between toxin accumulation in the caterpillar body and insect growth. Shown are the effects of (*A*) single compounds (n = 30) or (*B*) mixtures of the same compounds (n = 10, solid line). Sequestration from cardenolide mixtures imposed a reduction in caterpillar growth. Equimolar concentrations of purified cardenolide compounds were painted on leaf disks of *A. incarnata*, a species with nearly nil cardenolides (33). Significance of regressions was corrected for nonindependence of both variables using caterpillar mass with the Monte Carlo procedure as described (34). Note the log scale on the *X*-axis of both panels.

Together, our findings suggest that phytochemical mixtures impose a greater cost than single compounds due to the greater importance of how toxins are metabolically handled by the insect and their effects inside their bodies, rather than how much toxin the insect ingests. The distinct pattern of sequestration of each toxin and their difference in toxicity appear to interact and cause a stronger burden for caterpillar growth when in mixtures. This result suggests that negative impacts of phytochemically diverse mixtures via multiple mechanisms may be proceeding in the insect's body (Table 1) (28, 29, 39, 40).

Toxin Mixtures Enhance Plant Defense through Impacts on Multiple Herbivore Adaptations. Our results bear on the long-standing mystery of why plants make so many secondary compounds. We have demonstrated a *raison d'être* of plant defense diversity on key herbivore adaptations. From the plant's perspective, producing chemically and functionally diverse defense mixtures against the herbivore community can have important ecological benefits. For instance, investing in low amounts of highly toxic, and likely more costly, compounds within mixtures may impose a great challenge to specialist herbivores, especially those that accumulate these compounds in their bodies. In turn, plants remain well defended against more generalized herbivores at potentially reduced costs. There may well be other benefits of phytochemical mixtures for plants than enhanced toxicity. For example, selection by different herbivores on distinct plant tissues may spur the evolution of diverse defense mixtures between organs within a plant (15, 41, 42).

Advances using targeted and untargeted metabolomics have opened promising avenues to link the evolution of plant chemical diversity to herbivore pressure in natural environments (43, 44), and recent work points toward potential benefits of greater chemical diversity against herbivores in tropical plant communities (3, 5, 45). Yet, critical to advancing our understanding is making the direct functional link between chemical diversity and performance of herbivores through impacts on their multiple adaptations while controlling for the concentration of plant compounds. In our case, specialized and sequestering monarchs are not only highly tolerant of cardenolides, but have distinct mechanisms to cope with individual compounds (28, 29, 39, 40). By leveraging three independent experiments aimed to tease apart the distinct monarch adaptations to cope with plant defenses, our study points toward multiple mechanisms operating simultaneously.

First, the in vitro enzyme assay demonstrated that, in absence of other physiological adaptations, mixture effects can be driven by differences in toxin-specific affinity for the physiological target. Specifically, labriformin, a compound repeatedly reported as highly toxic for specialist herbivores (24, 29), contributed the most to the inhibition of the monarch enzyme through dominance effects when in mixture, even at lower concentrations. This phytochemical diversity effect is unlikely due to synergism per se (Table 1).

Second, our in vivo assay of flies revealed that toxin- and species-specific patterns were modified compared to the enzyme assay, suggesting that the effect of toxins in living organisms depends on additional physiological factors apart from their affinity for their physiological target. Moreover, in this particular experiment, we used one-tenth of the original concentration of labriformin, both in singles and in mixtures due to its insolubility in the aqueous fly diet, underestimating its effects on monarch fly mortality and feeding rate (*SI Appendix*, Fig. S4). Therefore, unlike the in vitro assay, the stronger diversity effects of mixtures compared to any single toxin on monarch flies, especially from *A. syriaca*, could have occurred due to lack of other physiological mechanisms, such as toxin-specific transport and conversion to less toxic compounds in the insect's body (30, 31, 46). In other words, synergism may be at play in this in vivo analysis, but further evidence for the mechanisms of the mixture effect is needed.

Finally, the caterpillar bioassay showed that a negative impact of toxin mixtures on monarch performance can be much more difficult to detect, yet are likely explained through the costs of sequestration. Whereas mixture impacts from *A. perennis* on caterpillar growth were driven by diversity effects of the three toxins sequestered in similar amounts (i.e., lower selectivity in sequestration), the high selectivity for the least toxic glycosylated aspecioside (GA) from the major host, *A. syriaca* caused a hyperaccumulation in the insect's body that reduced caterpillar growth on average when consumed singly (27% reduction compared to the mean of all single toxins). On the other hand, the highly toxic labriformin was 20-fold less sequestered than GA in isolation and is processed differently before sequestration (30, 36, 47). Nonetheless, small deviations in how much labriformin was processed and sequestered in the caterpillar caused a strong reduction in its performance comparable to GA at the highest sequestered amounts (*SI Appendix*, Fig. S7).

In mixtures, selectivity patterns of individual *A. syriaca* toxins mirrored those when consumed singly, but the stronger burden of sequestration from mixtures on caterpillar growth suggests that diversity effects of distinct toxins on the insect, rather than their individual effects within the mixture, may be at play. Selective sequestration, distinct enzymatic machinery needed to convert strong toxins prior accumulation, and the high binding affinity of certain cardenolides for the sodium pump seem to act simultaneously on reducing caterpillar performance. Whether these mechanisms of diverse toxin mixtures acting on herbivores are strictly synergistic is still unclear (Table 1).

It is possible that the lack of a stronger pattern for *A. syriaca* mixtures on monarch caterpillars has to do with the different coevolutionary histories between the two milkweed species and this specialist herbivore. The multiple adaptations present in the monarch seem to cope better with the diversity of cardenolides from *A. syriaca* compared to *A. perennis*. Indeed, 90% of all migratory monarchs that reach the Mexican overwintering grounds have fed on *A. syriaca* for at least two generations (22), likely favoring selection for greater overall sequestration of *A. syriaca* compounds and greater selectivity for the most abundant compound glycosylated aspecioside (24, 29, 41) (*SI Appendix*, Figs. S6 and S7). On the other hand, major host plants for the monarch (i.e., *A. syriaca*) have evolved a diverse chemical arsenal aimed to impact its multiple adaptations in distinct ways, hence reducing insect performance. Both the plant and insect seem to keep up in this arms race (22), in which phytochemical diversity of compounds seems to play a major role. Regardless, we show that chemically diverse mixtures may benefit plants through independent experiments testing for impacts of toxins on multiple adaptations of this sequestering specialist herbivore.

Materials and Methods

Cardenolide Isolation and Purification. Three compounds each from *A. syriaca* (glycosylated aspecioside, diglycosylated syriogenin, labriformin) and *A. perennis* (diglycosylated 12-β-hydroxycoroglaucigenin, diglycosylated corotoxigenin, diglycosylated uzarigenin) were isolated and purified from bulk seed tissue using a preparative HPLC fractionation method as in Agrawal et al. (28) and determined to be at least 95% pure based on NMR spectrometry. These compounds were selected because they are dominant in leaf tissue (>70% and 40% of total concentration of cardenolides in *A. syriaca* and *A. perennis*, respectively), and also represent diversity in terms of structure and polarity.

Na^+/K^+-ATPase In Vitro Assay. We quantified the biological activity of the purified cardenolides in isolation or in mixtures using the Na^+/K^+-ATPase of neural tissue from dissected brains of monarch butterflies (adapted, highly resistant to cardenolides) and from porcine kidney tissue (nonadapted, highly sensitive to cardenolides; Sigma-Aldrich, St. Louis, MO) following methods of Petschenka et al. (48). Briefly, field-collected adult monarchs (Ithaca, NY) were frozen alive and their brains were dissected, homogenized in a ratio of 0.75 brains per 2 mL of Millipore water, and stored in aliquots at −80 °C until use. Na^+/K^+-ATPase activity was measured as the amount of inorganic phosphate enzymatically released from ATP in the presence of K^+ (Na^+/K^+-ATPase active) minus the amount of phosphate released in the absence of K^+ (Na^+/K^+-ATPase inactive). Powder of purified cardenolides was resuspended in 20% DMSO in deionized water to reach 1 mM concentrations and sonicated for 5 min. Mixtures were prepared by drawing 100 µL from each resuspended single toxin and combining their amounts for each milkweed species. We then prepared five serial dilutions (starting for both singles and mixtures with 1 mM and 0.1 mM

for monarch and pig enzyme, respectively, and then 1:10 of each previous dilution) to produce a six-point inhibition curve for each of the six single compounds or the two mixtures of three toxins from each milkweed species and incubated each with the adapted or the unadapted enzyme (8 compound types × 6 dilutions × 2 enzymes × 3–6 technical replicates = 450 reactions). Reactions were performed in 96- well microplates on a BioShake Iq microplate shaker (Quantifoil Instruments) at 200 rpm and 37 °C for 20 min and quantified photometrically at 700 nm following Taussky et al. (49). We estimated the sigmoid dose–response curve from the residual enzymatic activity of each dilution (difference between absorbances of the reaction of each dilution level minus that of completely inhibited reaction) using a four-parameter logistic function using the function *nlme* with *SSfpl* from the nlme package v3.1- 152 in R v4.3.0 following Züst et al. (50). From each function, we extracted the relative dilution at the inflection point (IC50), representing the concentration (in mM) of the toxin needed to inhibit the enzyme by 50%.

CRISPR-Engineered *D. melanogaster* Feeding Assay. We used the genetically edited fly lines VSH and QAN that Karageorgi et al. (20) generated with CRISPR–Cas9 coupled with Homology Directed Repair (HDR). We followed their fly husbandry protocol for fly population maintenance. To test the effect of toxin mixtures on fly survival and consumption rate, we used the CApillary FEeder (CAFE) assay as in Groen et al. (40), which was adapted from Ja et al. (51). Briefly, three mated female flies at 2 to 4 d posteclosion were placed in an empty clear vial. Each vial has a 7.4 μL capillary placed through the cotton stopper filled with 5% sucrose + 0.1% Tween solution in water laced with 1 mM of toxins. Each vial was treated as an experimental replicate. Each fly line received nine diet treatment: six individual compounds (three from each milkweed species), two mixtures (each one from the combination of three toxins from each milkweed species), and one blank control without toxins (only with 5% sucrose + 0.1% Tween). Treatments using toxin mixtures comprised a total of 15 replicates (i.e., a vial with three mated female flies each) × 2 fly lines × 2 milkweed species = 60 samples; treatments using individual toxins comprised a total of 5 replicates × 6 compounds × 2 fly lines = 60 samples for singles. Another treatment of control samples comprised 5 replicates × 2 fly lines = 10 controls. Flies were kept at 25 °C, roughly 50% humidity, 12 h:12 h day/night cycle. Capillaries were refilled every day with fresh solution and toxins when appropriate to ensure ad libitum food availability. We recorded fly survival and measured daily feeding rates once per day.

Due to solubility issues of the nonpolar labriformin in water, we used 0.1 mM when single and one third of that concentration in mixture. This modification was only made for the *Drosophila* feeding assays. In this experiment labriformin's toxic effects were among the strongest, suggesting that our results regarding this compound are more conservative than expected (*SI Appendix*, Fig. S4).

Monarch Caterpillar Feeding Assay. Monarch eggs were obtained from a colony (Cornell University, with fewer than 5 generations in the laboratory) and kept in a growth chamber set at 27 °C/24 °C day/night until hatching. Neonate caterpillars were reared on locally collected swamp milkweed (*A. incarnata*) leaf tissue until molting to the second instar. Swamp milkweed was chosen for rearing monarchs because it is virtually devoid of cardenolides in leaves (33). Each freshly molted second instar caterpillar was weighed on a microbalance and then placed in a container containing a fresh *A. incarnata* leaf punch (11 mm diameter) laced with one of the toxin treatments (see below), as well as two small pieces of moistened cotton, and covered with a tight-fitting lid.

Nine toxin treatments were employed, with 5 replicates each (45 caterpillars total): six individual cardenolide compounds, three from *A. syriaca* and three from *A. perennis* (see above in "Cardenolide isolation and purification"), two equimolar mixtures of the 3 compounds from each species, and a control with only ethanol. The toxin treatments used standardized realistic concentrations of cardenolides that monarch caterpillars may encounter in leaf tissues. Briefly, total cardenolide concentrations in all treatments involving toxins were estimated to be 6 μg per mg of dry tissue (69.7 ± 0.47 nmol cardenolide mg^{-1} dw leaf tissue, depending on cardenolide molecular mass). To achieve the desired concentrations, individual cardenolides were analyzed using HPLC, and concentration was estimated from a standard curve based on digitoxin. All treatment solutions were made up in 95% ethanol and pipetted evenly onto the top of the leaf punch surface. Ethanol was dried off in a fume hood prior to adding the caterpillar to each container.

Caterpillars were allowed to feed on their treated leaf discs, and a second treated disc was added to each cup after 22 h. Caterpillars continued to feed for an additional 33 h (55 h total) and were then promptly weighed and removed to a container with fresh *A. incarnata* leaf tissue. All remaining tissue from the two treated leaf punches was collected and photographed, for subsequent estimation of the amount of tissue (and cardenolide) consumed, using LeafByte (52). On average, each caterpillar consumed 84% of the treated leaf tissue and 40.1 ± 0.49 μg of cardenolides (with no difference between leaf disks laced with single cardenolides or mixtures; $F_{1,36} = 1.52$, $P = 0.226$). Caterpillars were fed untreated *A. incarnata* leaf tissue for an additional 36 h, allowing them to molt into 3rd instar. At this time, caterpillars were frozen at −80 °C for subsequent chemical analysis of accumulated cardenolides. Three caterpillars out of 45 died during the experiment in three different toxin treatments and they were also analyzed for cardenolide sequestration (see below).

Frozen caterpillars were freeze-dried (Labconco, Kansas City, MO) weighed on a microbalance, and then ground with a 3 mm stainless steel bead, using a tissue grinder (MixerMill; Retsch, Haan, Germany). Each sample was then extracted in 1 mL methanol, with ~30 silica-zirconia beads, using a FastPrep homogenizer (MP Biomedicals, Irvine, CA) twice for 45 s at 6.5 m/s. Extracts were centrifuged for 12 min at 14,000 rpm and 0.7 mL supernatant was transferred to a fresh tube. Extracts were then defatted by adding an equal volume of hexanes, vortexing three times for 10 s each, incubating at room temp for 5 to 10 min in between each vortex, centrifuging for 15 min at 14,000 rpm, and discarding the top hexane layer. Defatted samples were taken to dryness at 35 °C in a speedvac (LabConco, Kansas City, MO) and then resuspended in 0.2 mL methanol by agitation at 1,000 rpm on a BioShake Iq microplate shaker (Quantifoil Instruments), filtered using hydrophobic syringe filters (Restek), into glass vials for LCMS analysis.

Cardenolide Sequestration Analysis. The chemical structures of purified cardenolides from the two milkweed species were characterized by NMR and quantified by high-resolution mass spectroscopy (HRMS) following Agrawal et al. (29). The chemical structures of sequestered transformed products were characterized by HRMS and MS^2 from monarch wings. The corresponding genins were not detected in monarch wings. For the chemical analysis, 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 × 2.1 mm, particle size 1.8 μm) maintained at 40 °C with a flow rate of 0.5 mL/min. MS^1 and MS^2 spectra of each sample were analyzed in positive electrospray ionization mode with *m/z* ranges 70 to 1,000. MS^2 spectra were obtained via Excalibur software (ThermoFisher Scientific). LC-MS data were analyzed using MZmine software (53). The acquired LC-MS data files were converted to mzXML files using the ProteoWizard MSconvert tool. LC-MS data were then preprocessed 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. perennis* or sequestered in monarch caterpillars, and confirmed their structure by comparing MS^2 fragmentation spectra and retention time with pure isolated standards if available in our in-house library.

The relative concentration (semiquantification) based on ion counts for cardenolides found in monarch caterpillar bodies that fed on laced and nonlaced (control) *A. incarnata* leaves was determined using calibration curves of glycosylated aspecioside (for glycosylated aspecioside, aspecioside, and syriobioside), diglycosylated syriogenin (for diglycosylated, glycosylated syriogenin, and the *A. incarnata* gofruside found in control caterpillars), diglycosylated 12-β-hydroxycoroglaucigenin (for diglycosylated and glycosylated 12β-hydroxycoroglaucigenin, and glycosylated uzarigenin), and diglycosylated corotoxigenin (for diglycosylated and glycosylated corotoxigenin). Cardenolide accumulation in caterpillar bodies was then estimated as nmol of cardenolide per mg dry mass of insect. To facilitate interpretation, especially on the detailed species- and toxin-specific findings, results of cardenolides sequestered from monarchs were referred to the original milkweed compounds and not to the transformed products in the insect's body. The percentage of cardenolides sequestered from *A. incarnata* leaves was <1%, calculated from the average concentrations of control leaves (i.e., nonlaced with toxins) divided by the mean concentration of single toxins or by the mean total concentration of toxins in each mixture from each milkweed species.

Statistical Analysis.

Resistance of Na^+/K^+-ATPase to cardenolides. Resistance of adapted (monarch) and unadapted (porcine) enzymes to cardenolides (hereafter "cardenolide resistance," mol L^{-1} 10^5) was compared between singles and toxin mixtures,

as well as between individual toxins using linear models using PROC MIXED in SAS v9.4. For the comparison between the overall effect of single toxins and mixtures, cardenolide resistance was the response variable, and insect enzyme (monarch or porcine), milkweed species (*A. syriaca* or *A. perennis*), mixture type (single or mixture), and all their possible interactions were included as fixed factors. For the comparison between the effect of individual toxins (6) and mixtures (2 experimental mixtures) from each milkweed species, cardenolide resistance was the response variable, insect enzyme, toxin type, and their interaction were considered as fixed factors. Since individual toxins in mixtures may have distinct affinity for the same physiological target and dominate patterns of sodium pump inhibition, we tested this under the null assumption of equal toxin binding for the physiological target while in mixtures. To this end, we estimated the average IC50 of the three toxins tested in isolation from each milkweed species and enzyme, obtaining an expected IC50 value under no competition between toxins for the binding site. We included the expected mixtures (4 to 6 replicates per enzyme and milkweed species) in the dataset and conducted tests comparing their effects to their corresponding observed values from experimental mixtures on the sodium pump. For both models, heterogeneous variance was allowed for each enzyme by individual toxin/mixture combination because it improved model fit. Cardenolide resistance was log-transformed or transformed to the fifth root to meet assumptions of normality in the first and second models, respectively. The latter transformation allows residuals to fit better into a normal distribution compared to more conventional transformations in certain datasets (15, 54).

Fly survival and feeding rate. We compared the effect of single compounds and mixtures on fly survival and feeding rate between the CRISPR-edited *Drosophila* with the monarch enzyme (VSH, "monarch" fly) and the wild-type (QAN) by performing generalized linear models with a binomial distribution and logit-link function using PROC GLIMMIX in SAS v9.4. For each variable, we first estimated the proportion of living flies or volume consumed in each day relative to their maximum for each vial (3 flies or maximum volume consumed in a given day, respectively) over the 12-d period of the experiment. The response variables, proportion of surviving flies and proportion of volume consumed, were analyzed separately, and fly line (line), milkweed species (species), mixture type (mixture), and all possible interactions were considered fixed factors. Day was included as a repeated measure by using the option RESIDUAL in the RANDOM statement, and vial was used as subject.

We also compared survival and feeding rate between the monarch and wild-type flies in toxin-free diets in separate analysis to test for intrinsic differences in performance after the CRISPR-editing process. Both wild-type and monarch flies showed the highest survival and feeding rate in toxin-free diets and both fly lines performed similarly (*SI Appendix*, Fig. S2, $P_{survival} = 0.926$; $P_{feed\ rate} = 0.982$), demonstrating no intrinsic differences in performance after the CRISPR-editing of the sodium pump enzyme.

Effects of cardenolide ingestion on monarch performance. We compared the overall effect of nonlaced (control) leaves, those laced with single compounds and with mixtures, and also the effect of individual toxins from each milkweed species on the relationship between feeding damage and caterpillar growth by performing linear regressions using PROC GLM in SAS v9.4. For the overall effect of control, singles, and mixtures, caterpillar dry mass was the response variable, and mixture type and its interaction with leaf damage were considered fixed factors. The interaction term was included to test for different effects of single toxins or mixtures (i.e., different slopes) in the covariation between caterpillar growth and feeding damage. To account for the effect of different toxins from each milkweed species in the analysis, mixture type (control, single, and mixture) was nested within species as single factor and in the interaction. For the effect of individual toxins from each milkweed species, caterpillar dry mass was the response variable, and toxin type (6 individual toxins, 2 mixtures, and 1 control) and its interaction with leaf damage were considered fixed factors. Then, we tested for differences from zero and between slopes for the effect of single compounds and mixtures (considering both species together and separately) and for differences from zero in the slope of each individual toxin from each species using the ESTIMATE statement in PROC GLM. Leaf damage from caterpillars was square root-transformed, and both caterpillar dry mass and leaf damage variables were standardized before the analysis.

Effects of cardenolide accumulation on monarch performance. We tested the difference between total cardenolide accumulation by monarch caterpillars from single compounds and toxin mixtures performing linear models using PROC MIXED in SAS v9.4. We first summed the concentrations of individual compounds accumulated on each caterpillar that fed on mixtures to estimate total cardenolides accumulated on that treatment. Total cardenolides accumulated in caterpillar bodies was the response variable and milkweed species (*A. syriaca* or *A. perennis*), mixture type (single or mixture), and their interaction were considered fixed factors. A heterogeneous variance model for the milkweed species by mixture type interaction was used because it improved model fit. We also tested for how individual cardenolide accumulation by the caterpillars differs between compounds and when consumed singly or in mixtures. Individual cardenolide concentration accumulated in caterpillar bodies was the response variable and compound identity (six toxins), mixture type (single or mixture), and their interaction were considered fixed factors. A heterogeneous variance model for the compound identity was used because it improved model fit. For this last model, cardenolide concentration was cubic root-transformed to satisfy the Shapiro–Wilk normality test.

We compared the overall effect of single compounds and mixtures, and also the effect of individual toxins from each milkweed species on the burden of cardenolide accumulation on caterpillar growth by performing linear regressions. To avoid spurious negative relationships between cardenolide accumulation in caterpillar bodies and caterpillar dry mass due to their nonindependence (i.e., cardenolide accumulation derives from caterpillar dry mass), we performed 10,000 Monte Carlo simulations for each regression analysis using PROC GLM into SAS v9.4, adapting the procedure of Morris et al. (34). For the overall effect of singles vs. mixtures, caterpillar dry mass was the response variable, and mixture type and its interaction with cardenolide accumulation were considered fixed factors. To account for the effect of different toxins from each milkweed species in the analysis, mixture type was nested within species as single factor and in the interaction. For the effect of individual toxins from each milkweed species, caterpillar dry mass was the response variable, and toxin type and its interaction with cardenolide accumulation were considered fixed factors. The interaction term in each model was included to test for different effects of mixture types or toxins (i.e., different slopes) in the covariation between caterpillar growth and cardenolide accumulation. Then, we tested for differences from zero and between slopes for the effect of single compounds and mixtures (considering both species together and separately), and for differences from zero in the slope of each individual toxin from each species using the ESTIMATE statement in PROC GLM. Cardenolide accumulation in insect bodies was log-transformed, and both caterpillar dry mass and cardenolide accumulation variables were standardized before the analysis on each simulation. Significance of the effects, now from independent response and predictor variables, were estimated from the 5th percentile of the resulting distribution of regression slopes (34). To test for biases in our regression analysis due to different sample sizes (n = 30 for singles, n = 10 for mixtures) and ranges of cardenolide concentrations sequestered by caterpillars (0.01 to 11.7 nmol mg^{-1} for singles, 0.51 to 6.70 nmol mg^{-1} for mixtures), we repeated our simulation procedure by restricting the data of simulated singles between the minimum and maximum of the simulated cardenolide concentrations from mixtures in each simulation. Then, for each simulation, we randomly selected 10 single toxin datapoints to be analyzed alongside the 10 simulated mixture datapoints (with their corresponding simulated caterpillar dry mass values).

We performed an identical regression analysis as above between caterpillar dry mass and the accumulation of each individual toxin within mixtures from each milkweed species. The purpose of this analysis was to qualitatively compare the patterns of sequestration of each toxin and their impact on caterpillar growth when consumed from a mixture and when consumed singly. Nonetheless, due to statistical dependency between interacting toxins impacting caterpillars when fed mixtures, statistical comparisons between individual toxins when sequestered singly and from mixtures cannot be made.

Data, Materials, and Software Availability. Chemistry, growth, sodium pump inhibition data have been deposited in zenodo (https://doi.org/10.5281/zenodo.10262932) (55).

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Supporting Information for
Plant chemical diversity enhances defense against herbivory

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Supporting Results

Effect of singles and mixtures of cardenolides from each milkweed species on the sodium pump

The monarch enzyme was strongly inhibited by *A. syriaca* mixtures compared to singles (76% greater inhibition, $p < 0.001$), whereas a similar and strong inhibition was found for both singles and mixtures from *A. perennis* toxins ($p = 0.460$) (Fig. S3A). A similar pattern was found for the unadapted enzyme for both milkweed species (Fig. S3B), although the reduction in enzyme activity due the mixture effect in *A. syriaca* was weaker than in the monarch (31% reduction, $p_{syriaca} = 0.002$; $p_{perennis} = 0.735$) because of strong enzyme inhibition overall from milkweed toxins.

Effects of singles and mixtures of cardenolides from each milkweed species on fly survival and feeding rate

When exploring milkweed species-specific effects, monarch flies died 1.36 days faster (20%, p=0.006) and tended to die 0.86 days faster (12%, p=0.091) when fed on *A. syriaca* or *A. perennis* mixtures, respectively (Fig. S5A), compared to single toxins. Similarly, mixtures from both *A. syriaca* and *A. perennis* significantly and marginally decreased, respectively, monarch flies feeding rate relative to single toxins (Fig. S5C; $p_{syriaca} = 0.028$; $p_{perennis} = 0.058$). Wild-type fly survival and feeding rate were strongly and negatively impacted by toxins overall, and both were similarly affected by single toxins and mixtures from either milkweed species (Survival: Fig. S5B; $p_{syriaca} = 0.636$; $p_{perennis} = 0.926$; Feeding rate: Fig. S5D; $p_{syriaca} = 0.882$; $p_{perennis} = 0.951$).

Monarch feeding and sequestration bioassay from singles and mixtures of cardenolides from each milkweed species

When exploring milkweed species-specific toxin effects on ingestion, we found no significant effects of individual toxins and mixtures on the relationship between feeding damage and caterpillar growth (*A. perennis*: $\beta_{D12BH} = 0.11$, $p = 0.756$; $\beta_{DC} = 1.01$, $p = 0.311$; $\beta_{DU} = 1.04$, $p = 0.367$; $\beta_{mixture} = 0.25$, $p = 0.513$; *A. syriaca*: $\beta_{GA} = 0.44$, $p = 0.352$; $\beta_{DS} = 0.14$, $p = 0.878$; $\beta_{LAB} = 0.33$, $p = 0.516$; $\beta_{mixture} = 0.73$, $p = 0.323$), nor differences in their slopes for all possible pairwise combinations (including controls) (all $p > 0.05$).

Regarding milkweed species-specific effects on toxin accumulation, we first checked that the contribution of *A. incarnata* leaf cardenolides to total toxin sequestration by the caterpillars in painted leaves was negligible (< 1% of the total, Fig. S6, estimated from non-laced, control leaves), as found in other studies[32]. Caterpillars sequestered 5.7 more cardenolides per unit dry mass from their main host, *A. syriaca*, compared to *A. perennis* ($F_{1,36} = 18.79$, $p < 0.001$), but there were no differences in sequestration between single toxins and mixtures for any milkweed species ($p_{syriaca} = 0.719$, $p_{perennis} = 0.581$) (Fig. S6). Sequestration of single toxins from each milkweed species did not show significant trends overall with insect growth (*A. perennis*: $\beta = -0.58$, $p = 0.724$, $n = 15$; *A. syriaca*: $\beta = -0.28$, $p = 0.843$, $n = 15$), although both were negative and had similar slopes ($t = -0.44$, $p = 0.66$). For each species' mixture, both showed stronger negative trends than single toxins although only the association with *A. perennis* mixture was significant on its own ($\beta_{perennis} = -6.49$, $p = 0.038$, $n = 5$) (Fig. S7B, E). Regarding regressions between each single toxin and insect growth, only the associations involving *A. perennis* diglycosylated 12 β-hydroxycoroglaucigenin, diglycosylated corotoxigenin, and the converted products of labriformin (present in the host *A. syriaca* only) were significant ($\beta_{D12BH} = -9.31$, $p = 0.004$, $n = 5$; $\beta_{DC} = -3.35$, $p = 0.044$, $n = 5$; $\beta_{LAB} = -5.98$, $p = 0.011$, $n = 5$), whereas this effect was not observed for the three other compounds (Fig. S7A, D). On the other hand, only the association between diglycosylated corotoxigenin with caterpillar growth showed a significant effect when exploring the effect of each toxin within mixtures ($\beta_{DC} = -6.30$, $p = 0.033$, $n = 5$). We recommend, nonetheless, caution when interpreting the associations between each toxin from mixtures with caterpillar growth because of non-independent effects of toxins in presence of others (e.g., how the ability of insects in handling each toxin may change when present in mixtures).

Supporting Figures

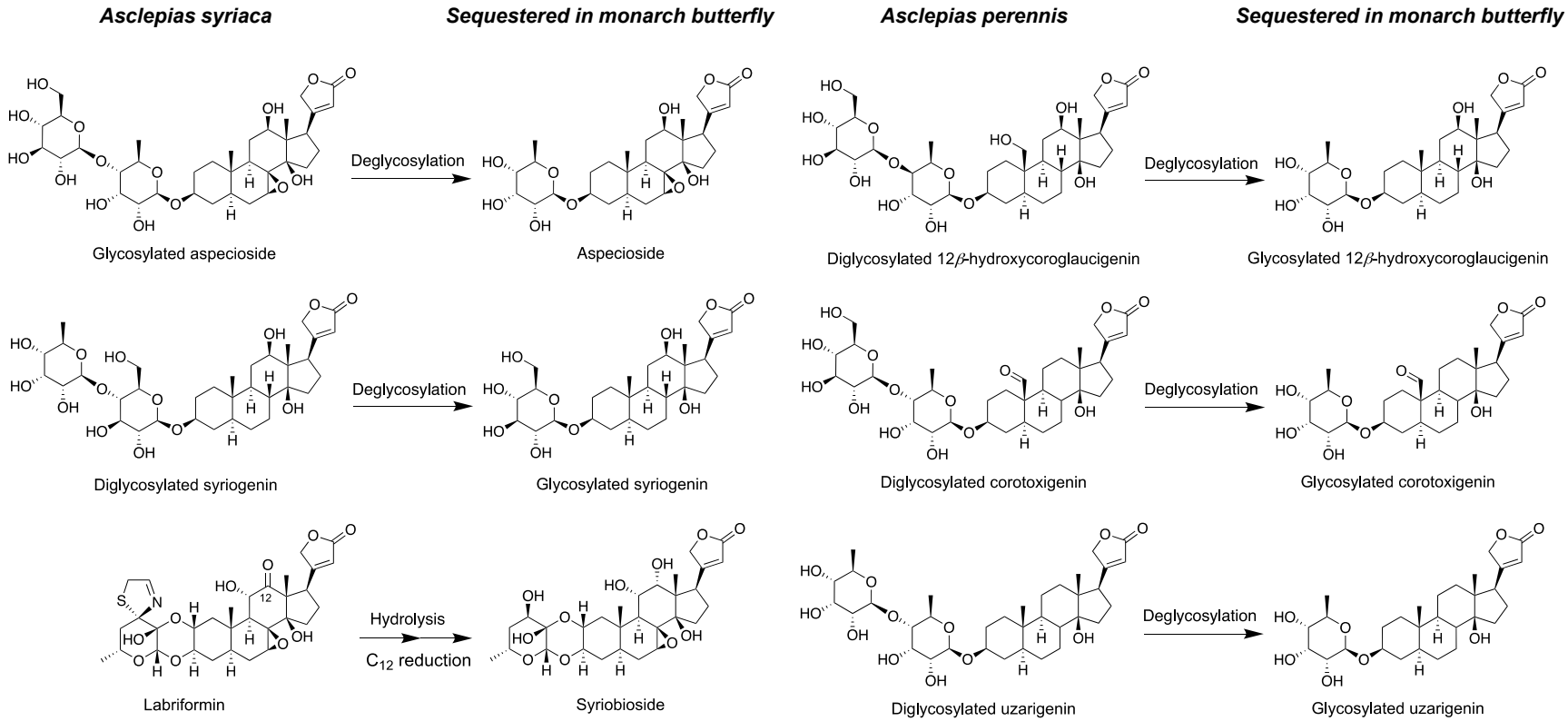

Fig. S1. Chemical structures of the three dominant cardenolide compounds from each of *Asclepias syriaca* and *A. perennis* isolated and purified in this study and the corresponding sequestered cardenolides after biotransformation by monarch caterpillars. The structure of the genin from diglycosylated and glycosylated uzarigenin is tentative and will require characterization by NMR to confirm its identity. The three compounds from *A. syriaca* and *A. perennis* typically comprise >70% and ~40% of the total leaf cardenolide concentration, respectively.

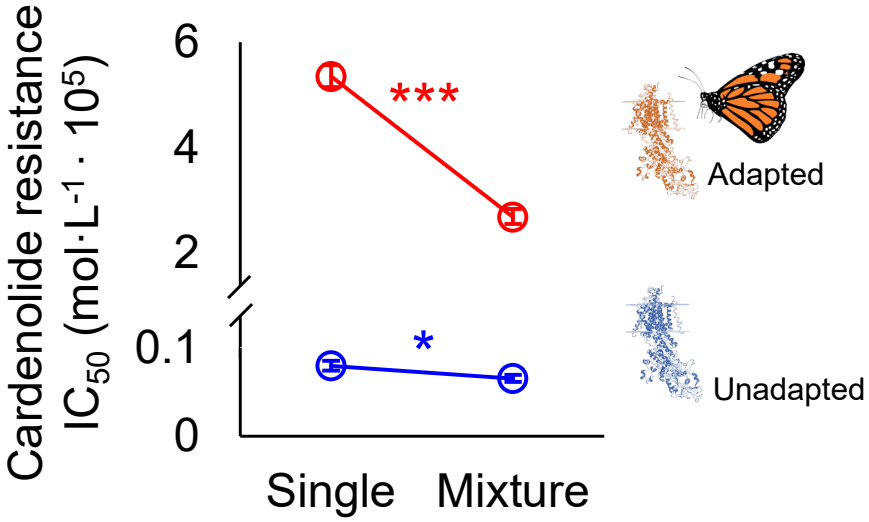

Fig. S2. Mean effects of single cardenolides and mixtures on resistance of the physiological target, the sodium pump (Na+/K+-ATPase), for both unadapted and adapted organisms. Serial dilutions of equimolar concentrations of purified cardenolides were used to examine the concentration at which the activity of the unadapted (blue) and monarch enzyme (red) reached 50% inhibition (IC50) *in vitro*. These means are derived from the data on the six compounds and two groups of mixtures presented in the main text (Figure 1). Asterisks denote significant differences between toxin types for each enzyme (*** < 0.001, * < 0.05), accounting for milkweed species in the analysis. Shown are means ± SE (n = 8-33 for each circle). Vertical axis is shown truncated for ease of visualization.

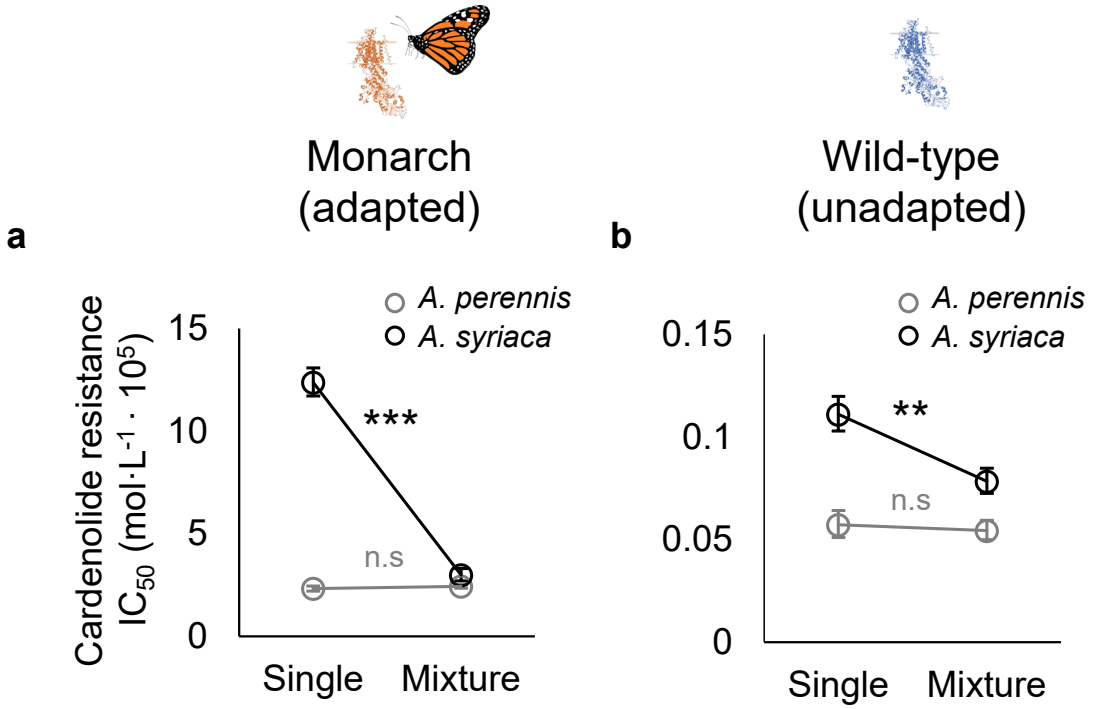

Fig. S3. Effect of singles and mixtures of equimolar concentrations of cardenolides from each milkweed species on resistance of the physiological target, the sodium pump (Na^+/K^+-ATPase), of adapted (monarch) (a) and unadapted (wild-type) (b) organisms. These means are derived from the data presented in the main text (Figure 1). Asterisks denote significant (*** < 0.001, ** < 0.01). Non-significant tests are denoted as 'n.s'. Shown are means ± SE (n = 4-18 for each circle).

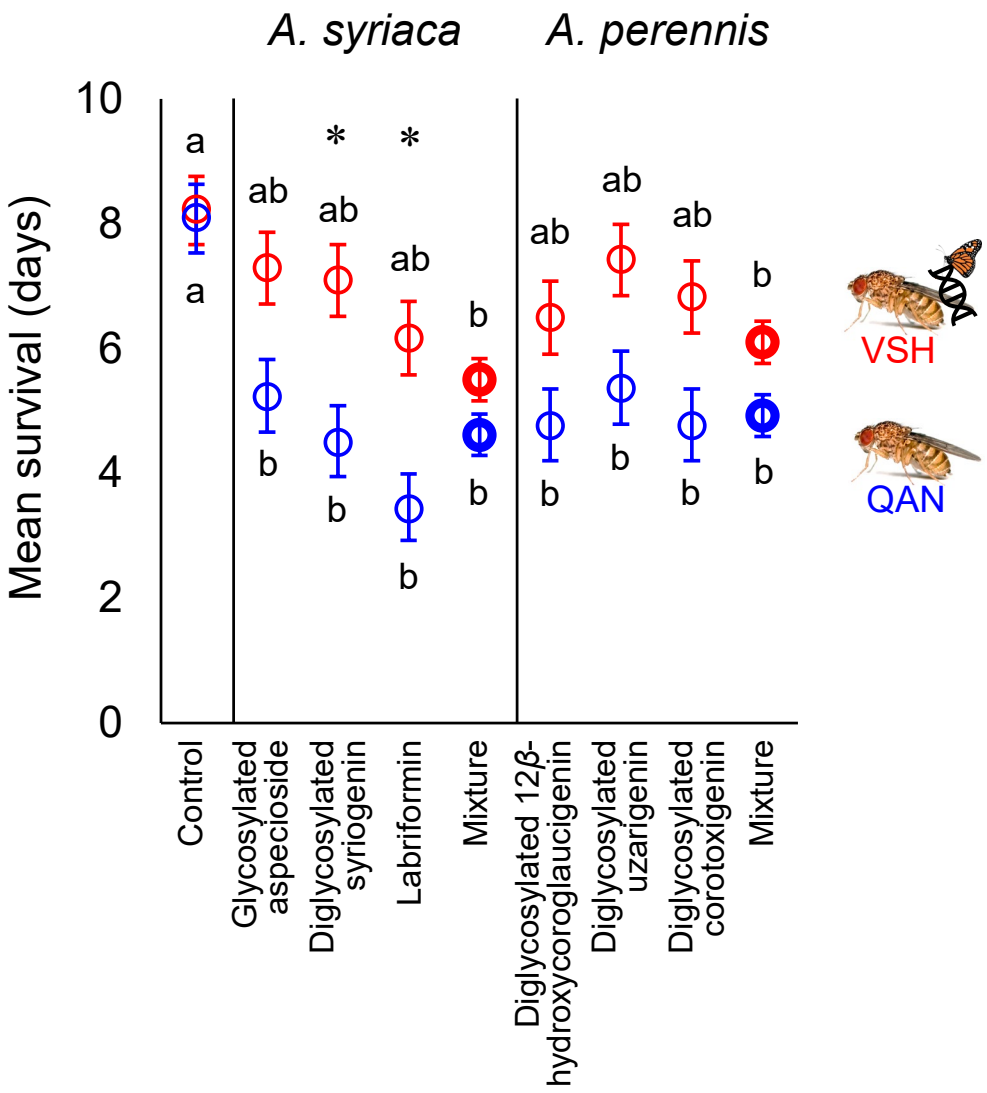

Fig. S4. Effects of individual toxins and mixtures of cardenolides from each milkweed species on survival of wild-type *Drosophila melanogaster* (QAN, blue) and CRISPR-edited flies with the monarch enzyme (VSH, red). Monarch flies had higher survival overall but showed lower survival when fed toxin mixtures compared to each individual compound. Fly diets were spiked with equimolar concentrations of purified cardenolide compounds for 12 days. Different letters denote significant differences between toxin types for each fly line within each milkweed species ($p < 0.05$). Asterisks denote significant differences between fly lines for a given toxin ($p < 0.05$). Shown are means ± SE (n = 5 for single toxins and n = 15 for mixtures per fly line and milkweed species).

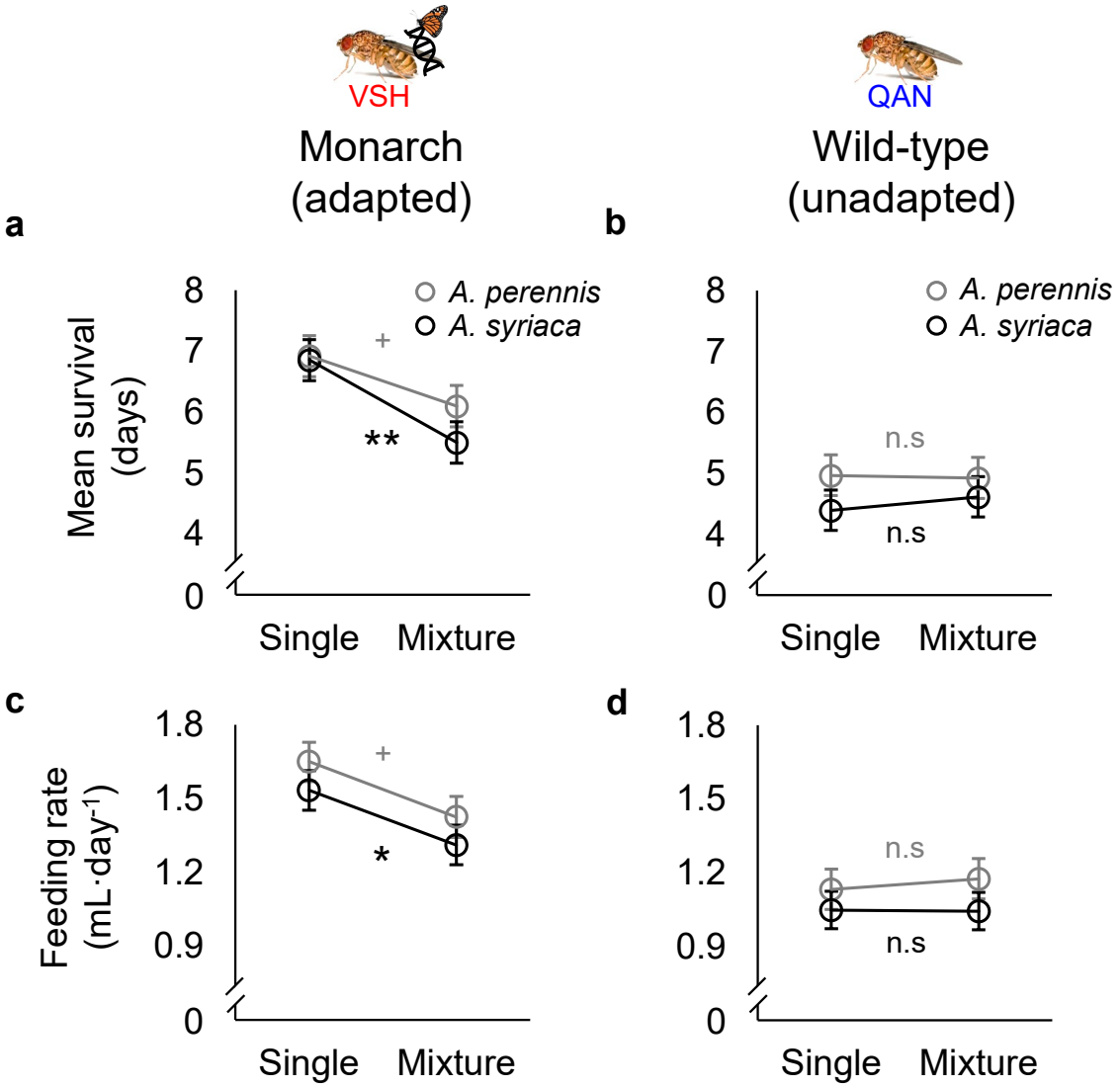

Fig. S5. Effect of singles and mixtures of equimolar concentrations of cardenolides from each milkweed species on fly survival (a, b) and fly feeding rate (c, d) of adapted (monarch) and unadapted (wild-type) phenotypes. Asterisks and plus symbol denote significant (** < 0.01, * < 0.05) and marginal differences (+ p < 0.1), respectively. Non-significant are denoted as 'n.s'. Shown are means ± SE (n = 5 for single toxins and n = 15 for mixtures per fly line and milkweed species). Vertical axes in all panels are truncated for ease of visualization.

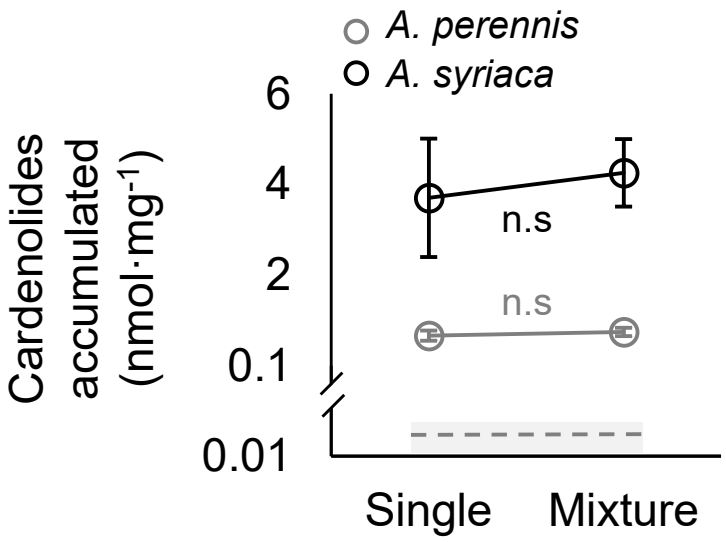

Fig. S6. Effects of single vs. mixtures of equimolar concentrations of cardenolide compounds from each milkweed species on the amount of total cardenolides found in monarch caterpillar bodies after feeding on painted *Asclepias incarnata* leaf disks with purified cardenolides. Maximum contribution of *Asclepias incarnata* cardenolides to overall sequestration (estimated from non-laced control leaves) is represented with a dashed line and grey area (mean ± SE). Non-significant tests are denoted as 'n.s'. Shown are means ± SE (n = 15 for singles and n = 5 for mixtures for each milkweed species). Vertical axis is truncated for ease of visualization.

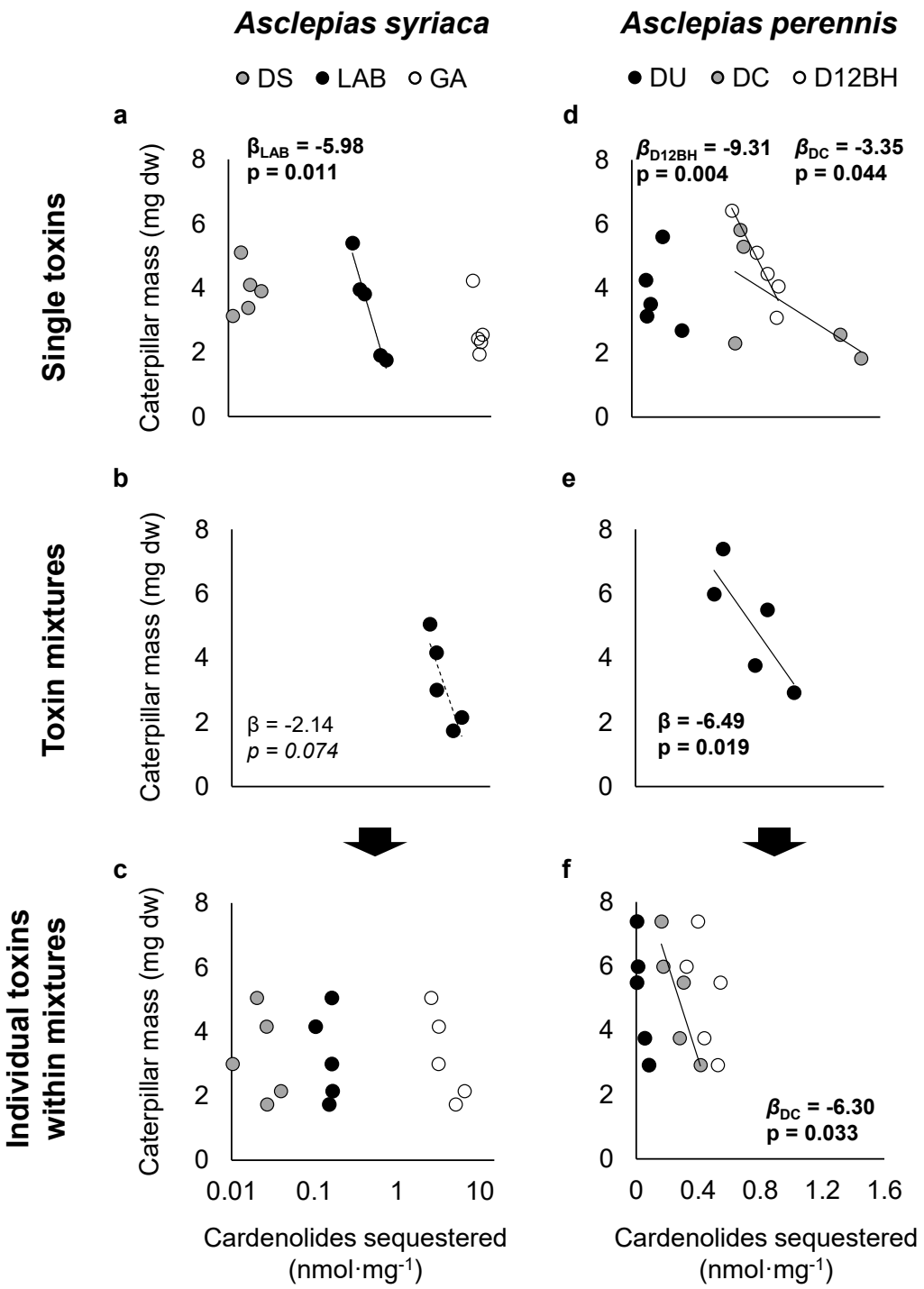

Fig. S7. Relationships between cardenolides sequestered and caterpillar growth in single toxins (a, b) and mixtures (c, d) from *A. syriaca* (a, c, respectively) and *A. perennis* (b, d, respectively). Solid lines and bold values indicate significant associations corrected for caterpillar mass being used on both axes using a modification of the Monte Carlo procedure (10,000 simulations) as described(1). DS = diglycosylated syriogenin, LAB = labriformin, GA = glycosylated aspecioside, DU = diglycosylated uzarigenin, DC = diglycosylated corotoxigenin, D12BH = diglycosylated 12*β*-hydroxycoroglaucigenin (see Fig. SS1). n = 5 for each toxin.

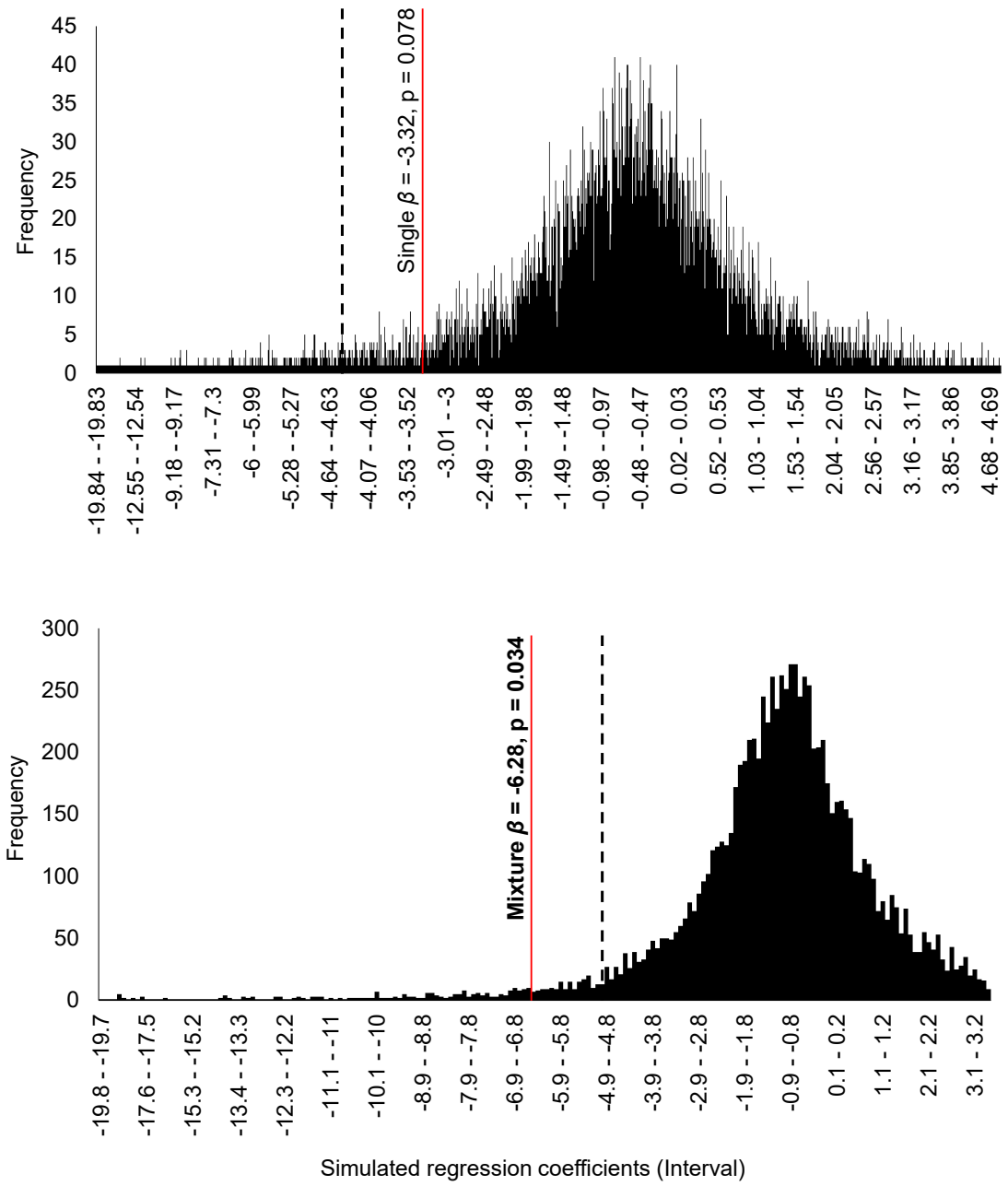

Fig. S8. Distribution of 10,000 simulated regression slopes between cardenolides sequestered and caterpillar growth for single toxins and mixtures, each mixture type constrained to the same range values and sample sizes (n = 10). For each simulation using a modification of the Monte Carlo procedure as described (1), simulated single toxin values were restricted within the maximum and minimum of simulated mixture values. Then 10 datapoints of single toxins from that range were randomly selected to be analyzed alongside the 10 simulated mixture datapoints. The distribution represents the absolute frequency (Y-axis) of regression parameter slopes obtained through simulations that fall within the intervals of regression slopes on the X-axis. Significant observed regression values (red lines) that fall on the left side of the 5th percentile of the simulated regressions (dashed black line) –now corrected for caterpillar mass being used on both variables– are indicated in bold.

SI References

1. W. F. Morris, M. B. Traw, J. Bergelson, On testing for a tradeoff between constitutive and induced resistance. *Oikos* **112**, 102-110 (2006).