



Cardenolide Intake, Sequestration, and Excretion by the Monarch Butterfly along Gradients of Plant Toxicity and Larval Ontogeny

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Abstract

Monarch butterflies, *Danaus plexippus*, migrate long distances over which they encounter host plants that vary broadly in toxic cardenolides. Remarkably little is understood about the mechanisms of sequestration in Lepidoptera that lay eggs on host plants ranging in such toxins. Using closely-related milkweed host plants that differ more than ten-fold in cardenolide concentrations, we mechanistically address the intake, sequestration, and excretion of cardenolides by monarchs. We show that on high cardenolide plant species, adult butterflies saturate in cardenolides, resulting in lower concentrations than in leaves, while on low cardenolide plants, butterflies concentrate toxins. Butterflies appear to focus their sequestration on particular compounds, as the diversity of cardenolides is highest in plant leaves, lower in frass, and least in adult butterflies. Among the variety of cardenolides produced by the plant, sequestered compounds may be less toxic to the butterflies themselves, as they are more polar on average than those in leaves. In accordance with this, results from an *in vitro* assay based on inhibition of Na⁺/K⁺ ATPase (the physiological target of cardenolides) showed that on two milkweed species, including the high cardenolide *A. perennis*, extracts from butterflies have lower inhibitory effects than leaves when standardized by cardenolide concentration, indicating selective sequestration of less toxic compounds from these host plants. To understand how ontogeny shapes sequestration, we examined cardenolide concentrations in caterpillar body tissues and hemolymph over the course of development. Caterpillars sequestered higher concentrations of cardenolides as early instars than as late instars, but within the fifth instar, concentration increased with body mass. Although it appears that large amounts of sequestration occurs in early instars, a host switching experiment revealed that caterpillars can compensate for feeding on low cardenolide host plants with substantial sequestration in the fifth instar. We highlight commonalities and striking differences in the mechanisms of sequestration depending on host plant chemistry and developmental stage, which have important implications for monarch defense.

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Introduction

Sequestration of plant chemicals into animal tissues for defense occurs in more than 250 insect species (Duffey 1980; Opitz and Müller 2009), and is known from diverse groups including beetles (Termonia et al. 2001), true bugs (Duffey and Scudder 1972; Scudder and Duffey 2011), and sawflies (Björkman and Larsson 1991). Sequestration is more common in specialist than generalist herbivores (Dyer 1995; Petschenka and Agrawal 2016), and can be effective as a defense against parasitoids (Sime 2002), and parasites (Sternberg et al. 2012) as well as invertebrate (Björkman and Larsson 1991; Dyer 1995) and vertebrate (Brower et al. 1967) predators. Sequestration is probably best known from the

Lepidoptera where it occurs in multiple families, from a broad range of host plants, and includes the storage of diverse compounds such as grayanoids, iridoid and cyanogenic glycosides, pyrrolizidine alkaloids, and cardiac glycosides (Nishida 2002).

Many sequestering species have adaptations to cope with plant defenses (Opitz and Müller 2009), but they are typically not entirely resistant to these toxins (Adler et al. 1995; Björkman and Larsson 1991; Dimarco and Fordyce 2017; Mason and Bowers 2017; Smilanich et al. 2009; Zalucki et al. 2001; but see Zvereva and Kozlov 2016; Zvereva et al. 2017). This pattern fits a coevolutionary interpretation because the arms race should not end with sequestering specialists if they still impose natural selection on the plant. Moreover, physiological mechanisms of insect resistance to toxins are not absolute, but only increase their level of tolerance. Consequently, a key question in the study of sequestration is whether herbivores selectively store particular compounds. Selectively sequestered compounds could result in a reduced burden on the insect compared to non-sequestered compounds, or non-exclusively, sequestered compounds could be especially toxic to maximize the protective effect against antagonists.

Monarch butterflies, *Danaus plexippus*, were the first system for which sequestration was definitively demonstrated and have been a model system to understand the process for over 50 years (Agrawal 2017; Brower et al. 1967; Reichstein et al. 1968). Monarchs sequester cardenolides, a subgroup of cardiac glycosides, from milkweed host plants into their body tissues as defenses against predators (Brower and Glazier 1975; Duffey and Scudder 1972), and with impacts on protozoan parasites (Sternberg et al. 2012). Cardenolides bind to and inhibit the ubiquitous Na^+/K^+ -ATPase in cell membranes (Palasis et al. 1996) and are therefore expected to be toxic to nearly all animals. Reciprocally, the monarchs' high level of resistance to cardenolides is determined by a few amino acid substitutions that influence the binding properties to their Na^+/K^+ -ATPase (Dobler et al. 2012; Holzinger et al. 1992; Holzinger and Wink 1996). Nonetheless, as with other sequestering specialists, this resistance is not absolute (Bramer et al. 2015; Moore and Scudder 1986; Petschenka et al. 2013; Vaughan and Jungreis 1977), and several studies report some negative effects of cardenolides on monarch performance both as larvae and as adults (Agrawal 2005; Malcolm 1995; Tao et al. 2016; Zalucki et al. 1990; Zalucki and Brower 1992; Zalucki et al. 2001).

Sequestration of cardenolides has been studied with regard to host plant chemistry (Brower and Moffitt 1974; Brower et al. 1982; Martin et al. 1992), compound polarity (Malcolm et al. 1989; Nelson 1993; Roeske et al. 1976), toxicity to vertebrate predators (Brower et al. 1967, 1972; Brower and Moffitt 1974; Brower et al. 1982, 1984; Martin et al. 1992; Roeske et al. 1976), and effects on protozoan parasites

(de Roode et al. 2008, Gowler et al. 2015; Sternberg et al. 2012). Nelson (1993) proposed a model for the processing and storage of cardenolides, which hypothesized that monarchs selectively store cardenolides of high and intermediate polarities and metabolize less polar compounds into compounds of intermediate polarity for storage. If this model is correct, we predict that the compounds which occur in butterfly tissue are predominantly of high or intermediate polarity, and apolar compounds that occur in leaves should not occur in butterflies. Frass might be expected to contain apolar compounds that are not metabolized by butterflies, or other compounds that are not stored. Accordingly, we propose that an input-output analysis of leaves and frass directly complement analyses of sequestered compounds in insect body tissues.

Given that sequestration inherently continues over development, and other major changes occur as larvae mature (including changes in feeding rate, tolerance to plant defenses, etc.) the study of sequestration would profit from linking larval ontogeny to final sequestration in the adult stage (Boege et al. 2018). Furthermore, although sequestration begins with plant feeding, it typically occurs via uptake into the caterpillar hemolymph and deposition into body tissue. Thus, in the current work, we examine sequestration in monarchs across ontogenetic stages. Additionally, by examining leaves, caterpillar hemolymph, body tissues, gut contents, frass, and adult butterflies, we comprehensively address the intake, sequestration, and excretion of cardenolides by monarchs.

We reared monarch butterflies on five closely-related *Asclepias* host plants in the well-resolved Incarnatae clade (Fishbein et al. 2011). A benefit of investigating sequestration among these close plant relatives is that many of the cardenolides are shared between species. Nonetheless, our host plants exhibit tremendous quantitative variation in cardenolides of more than ten-fold in total concentration (Züst et al. 2018). We estimate cardenolide concentrations and diversity using high performance liquid chromatography (HPLC), and additionally we assess inhibition of Na^+/K^+ -ATPase *in vitro* in order to quantify the inhibitory effects of leaf, frass, and adult butterfly extracts to address the relative toxicity of consumed *versus* sequestered cardenolides. Different cardenolide compounds have been shown to vary in their toxicity to monarchs themselves as well as vertebrates (Rasmann et al. 2009; Petschenka et al. 2018; Seiber et al. 1983), and potentially to parasites (Sternberg et al. 2012). We predict butterflies to sequester cardenolide mixtures with the most inhibitory effects on vertebrate enzymes to maximize defense against predators, as long as they are not directly toxic to monarchs themselves (Brower and Moffitt 1974; Tao et al. 2016).

Specifically, in this study we address the following questions: 1) What are the patterns of cardenolide sequestration of monarchs (concentration, diversity, and polarity) on different

host plant species? 2) How does the concentration of sequestered cardenolides shift over the course of development? 3) Can caterpillars initially raised on a low cardenolide host plant compensate for little sequestration as early instars with sequestration on high cardenolide plants during the final instar? And 4) Does the relative compound diversity, polarity, and pharmacological potency (inhibition of Na^+/K^+ -ATPase) of cardenolides in leaves, butterflies, and frass correspond to the previously proposed hypotheses about selective sequestration?

Methods and Materials

Experiment 1: Sequestration of Cardenolides by Monarchs on Different Host Plants We examined cardenolide sequestration and excretion by monarch caterpillars on four milkweeds in the clade Incarnatae: *A. perennis*, *A. curassavica*, *A. incarnata* subspecies *incarnata* and *A. incarnata* ssp. *pulchra*. These milkweeds are closely related, have similar stem and leaf morphologies and all typically occur in mesic habitats, but vary broadly in cardenolide concentrations (Züst et al. 2018). The subspecies *Asclepias I. pulchra* and *A. i. incarnata* are distinct phylogenetically (Fishbein et al. 2011), and differ in a range of traits including leaf shape and trichomes (Agrawal et al. 2009). We surface-sterilized seeds with 5% bleach, scarified, and cold stratified them in damp petri dishes at 4 °C for 10–14 days after which we placed them in an incubator at 28 °C for 3–5 days. We planted seedlings in 10 cm diameter pots in potting soil and reared them in a growth chamber at 14 h day:light cycle, 28 °C during the day and 26 °C at night. We watered plants every other day and fertilized with dilute fertilizer (NPK 21–5–20, 150 ppm) approximately 5, 10 and 33 days after planting. Around 35 days after planting, we potted plants into 15 cm diameter pots and moved them into mesh cages in the field in Ithaca, NY, where we continued to fertilize them once a week. Experiments were conducted when plants were about 50 days from initial planting.

Monarchs for all experiments were a mixture of females (and lab-bred offspring of females) obtained from two commercial sources (MA and central FL), and wild females captured in Ithaca, NY. In this experiment caterpillars were from eggs laid by 12 monarch butterflies. Plants of the four *Asclepias* taxa were arranged in 2 × 2 × 3 m cages in the field, and we applied one freshly hatched caterpillar to each plant. On the seventh day after hatching all the caterpillars were moved to individual 9 × 12 × 6 cm transparent plastic cages in the lab where they were fed on cut leaves pooled from multiple individual plants of their assigned species for the remainder of their development. Frass was collected from fifth instar caterpillars, and caterpillars pupated in the lab.

We conducted high performance liquid chromatography (HPLC) on leaves ($N = 6$ individuals per species), caterpillar

frass ($N = 6$ per host plant species), and whole butterflies ($N = 6$ per host plant species, 3 males and 3 females). The three sample types were frozen at -80 °C and then freeze-dried at -40 °C. Each sample was then ground to a fine powder (Retsch Mixer Mill 300). To extract cardenolides we added 1.6 mL of MeOH to 50 mg aliquots of each of the powdered samples. We then added ~30 FastPrep beads to each sample and we agitated the samples twice for 45 s at a speed of 6.5 m/s on a FastPrep-24 homogenizer, followed by centrifugation at 12,000 rpm for 15 min. We pipetted 200 μL of each sample into separate tubes for the Na^+/K^+ -ATPase assay (see Experiment 4 below) and stored them in the -20 °C freezer. We added 1 mL of supernatant to the tubes in 96-well racks and added 20 μg of the internal standard cardenolide digitoxin (Sigma-Aldrich, MO, USA) to each tube. Samples were dried in a centrifugal concentrator at 35 °C. To resuspend we added 200 μL of MeOH to each sample and filtered with a MultiScreen Solvintert 0.45 μm low-binding hydrophobic PTFE 96-well plate. We analyzed samples using HPLC based on the method established by Rasmann et al. (2009). Briefly, 15 μL of the samples were injected into an Agilent 1100 series HPLC (Agilent, Waldbronn, Germany) equipped with a diode-array detector. A Gemini-NX C18 reversed-phase column 3 μm , 150 mm × 4.6 mm (Phenomenex, Torrance, CA, USA) was used for chromatographic separation. Cardenolides were eluted with a constant flow rate of 0.7 mL/min of a 0.25% phosphoric acid in water and acetonitrile gradient as follows: 0–2 min 16% acetonitrile, 24 min 70% acetonitrile, 24–25 min 70% acetonitrile, 30 min 95% acetonitrile, 30–35 min 95% acetonitrile. Absorbance spectra between 200 and 400 nm were recorded. We considered peaks with symmetrical absorption maxima between 216 and 222 nm to be cardenolides (Malcolm and Zalucki 1996). We calculated cardenolide concentrations based on integrated peak areas at 218 nm and standardized using the internal standard digitoxin. For estimates of peak diversity, we counted all peaks per sample showing a clear cardenolide absorbance spectrum.

We analyzed the estimated cardenolide concentrations across sample types within each plant species using one-way ANOVAs in R (version 3.4.4). To compare leaf to frass samples, and leaf to butterfly samples we conducted *Tukey multiple comparisons of means*. We calculated a polarity index score for each of our samples by weighting the retention time of each compound within a sample by the proportion of the total cardenolide concentration comprised of that compound, and then summed all of these weighted retention times within a sample to create a polarity score. Lower polarity scores indicate a larger proportion of more polar cardenolides and higher polarity scores indicate a larger proportion of less polar cardenolides (Rasmann and Agrawal 2011). We analyzed polarity scores in the same manner as total cardenolide estimates. We similarly compared the

cardenolide diversity, determined as the number of different cardenolide peaks in a chromatogram.

Experiment 2: Sequestration across Ontogeny To evaluate sequestration over the larval development of *D. plexippus*, we measured cardenolides in both hemolymph and body tissues (without the gut and its contents to include only cardenolides that had actually been sequestered into body tissues) from caterpillars between the second and fifth instar. Cardenolides dissolved in hemolymph are particularly relevant from a toxicological point of view, while the analysis of body tissues provides an estimate of the total amount of cardenolides sequestered.

A. curassavica seeds were prepared and grown the same as for Experiment 1 (above) except that they were planted in Metro-Mix 360 soil (Sun Gro Horticulture, Canada CM Ltd., Vancouver, Canada), and remained in the growth chamber for the entire experiment. We applied first instar caterpillars to five week old *A. curassavica* plants in a growth chamber. We enclosed plants with caterpillars in a mesh bag and we replaced plants if necessary. We collected hemolymph from each instar soon after molting but once the caterpillars had resumed feeding ($N=6$ caterpillars for instar 2–4, $N=24$ for instar 5, see below) by cutting off a caterpillars' tentacle. Exuded hemolymph was collected on a piece of parafilm (caterpillars recover from sampling readily and resume feeding) and samples of an exact volume were collected with a microliter pipette. Sample volumes depended on the overall amount of hemolymph obtained and ranged from 1 μl (2nd instar) to 120 μl (5th instar). To ensure exhaustive extraction of cardenolides, hemolymph samples $\geq 4 \mu\text{l}$ were extracted with a 10-fold volume of methanol. Volumes below 4 μl were extracted with 40 μl methanol. For estimating cardenolide quantifications on a mass per mass basis we converted 1 μl of hemolymph to 1 mg based on the density of water. We excluded first instar caterpillars from chemical analysis as they were too small to provide sufficient amounts of tissues for analysis.

After hemolymph collection we immediately collected caterpillars ($N=6$) of the second, third and fourth instar each. As body mass increases most rapidly during the fifth instar and growth rate is likely to influence sequestration, the number of caterpillars sampled was increased to $N=24$ in the fifth instar. To prevent the presence of the food bolus in the gut from biasing cardenolide estimates in caterpillar body tissues, caterpillars chilled on ice were decapitated and the gut including its contents was separated from the body by gently squeezing towards the head opening and discarded. The remaining body tissues, i.e. the entire larval body without the head and the gut but including residual hemolymph, were freeze-dried, weighed, homogenized with a plastic pestle in 1 mL methanol including 0.1 $\mu\text{g}/\mu\text{L}$ digitoxin (internal standard), and treated in an ultrasound bath for 10 min. The next day, homogenates

were centrifuged (3 min at 20,000 $\times g$) and a 750 μL aliquot of the supernatant was filtered before analyses (KX Nylon Syringe Filter, 0.45 μm , Kinesis Scientific Experts, St. Neots, Cambridgeshire, UK). HPLC was performed using the same methods as in Experiment 1 (above). For quantification of cardenolide concentrations, only peaks were taken into account which had a clear cardenolide spectra in more than 50% of the samples. Given that cardenolide concentrations in hemolymph are on average 41 times lower than concentrations in body tissues (paired samples, $N=42$) we considered cardenolides removed with the hemolymph sample or contained in hemolymph adhering to the gut as having negligible effects on cardenolide estimates from body tissues.

As hemolymph cardenolides were estimated on a wet mass basis and body tissue cardenolides on a dry mass basis we separately analyzed the effect of larval instar on concentrations of cardenolides in hemolymph and in body tissues using one-way ANOVAs and checked for a linear trend over the course of development using polynomial contrasts (Wilsey and Potvin 2000). For each sample type we compared instars using Tukey-corrected post-hoc tests. Polarity scores and cardenolide diversity were compared between body and hemolymph samples, as these measures were not influenced by wet mass *versus* dry mass collections. We compared polarity scores and cardenolide diversity across sample types and instars using *two-way ANOVAs*. As we had a larger sample size for fifth instars, for the fifth instars we also used linear regression to examine the relationship between body mass and cardenolide concentrations.

Experiment 3: Sequestration in the Fifth Instar We grew plants of *A. barjoniifolia*, *A. curassavica*, *A. incarnata incarnata*, and *A. perennis* using the same methods as Experiment 2. *A. barjoniifolia* was included rather than *A. i. pulchra* because it is also from the same clade but has more intermediate cardenolide levels (Rasmann et al. 2009) between low cardenolide *A. i. incarnata* and high cardenolide *A. perennis* and *A. curassavica*. At four weeks, plants were repotted into bigger plastic pots (15 cm diameter) and moved from the growth chamber to an outdoor enclosure. At this point plants were fertilized one last time and subsequently watered as needed. We applied one caterpillar freshly molted into the fifth instar per plant when plants were 7–10 weeks old. The fifth instar caterpillars had been raised on *A. i. incarnata* to generate caterpillars with relatively low cardenolide concentrations. The fifth instar caterpillars were allowed to feed on the new host plants for one day and then we collected hemolymph samples from $N \geq 3$ different caterpillars per plant species, two to seven days later we collected a second hemolymph sample from the same caterpillars. After collecting the second hemolymph sample, we also collected several mature leaves from each plant ($N \geq 3$ individual plants per species) and caterpillar samples ($N=3$ per plant species) and froze them at

–80 °C. We added a 10-fold volume of MeOH to hemolymph samples, vortexed and treated them in an ultrasound bath for 10 min, then centrifuged for 3 min at 20,000 g and the supernatant was transferred into a fresh tube. For HPLC analysis 2 µL of a 1.5 µg/µL digitoxin (Sigma-Aldrich, MO, USA) solution were added to 28 µL hemolymph in MeOH sample as an internal standard. Leaves and whole caterpillars were freeze-dried and dissected (Petschenka and Agrawal 2015) subsequently into midgut (epithelium plus gut contents) and the corresponding body tissues surrounding the midgut portion. HPLC was performed using the same methods as Experiment 1.

As in Experiment 2, we analyzed cardenolide concentrations separately for samples that were estimated on a dry mass basis (leaves and body tissues with the addition of guts in this experiment) and for hemolymph samples that were estimated on a wet mass basis. We analyzed the concentrations of cardenolides across dry mass sample types separately for each different plant species using *one-way ANOVAs* and *Tukey-corrected post-hoc tests*. We compared cardenolide concentrations in hemolymph between the two subsequent sample collections for each caterpillar using a paired t-test. As they were not significantly different we averaged the two values and examined the effect of plant species on hemolymph estimates using a *one-way ANOVA* with *Tukey corrected post-hoc tests*. We averaged the polarity scores and number of cardenolide compounds across the two hemolymph samples to compare these values to body tissue polarity scores and cardenolides diversity. For polarity scores and number of compounds we conducted *linear mixed-effect models* using the *lme4* package in R (Bates et al. 2015) with host plant species, sample type (body *versus* hemolymph) and the interaction between plant species and sample type as fixed effects, and individual caterpillar as a random effect.

Experiment 4: *In Vitro* Inhibition of the Na⁺/K⁺-ATPase In order to determine the inhibitory effects of cardenolide extracts from butterflies, frass and leaves from Experiment 1 on the Na⁺/K⁺-ATPase we conducted an *in vitro* assay (Petschenka et al. 2013). We used Na⁺/K⁺-ATPase from the pig cerebral cortex (Sigma-Aldrich). For each extract we used three dilutions to generate the sigmoid enzyme inhibition function/a dose response curve. This allowed us to estimate cardenolide content relative to a reference curve obtained with the standard cardenolide ouabain (Sigma Aldrich). For all three sample types ($N = 3$ per sample type per plant species), we diluted extracts 1:5, 1:50, and 1:500 using 10% DMSO in water. We added 20 µL of the dilute extracts to 80 µL of a buffered reaction mix resulting in final concentrations of 2.5 mM ATP, 50 mM imidazole, 100 mM NaCl, 20 mM KCl, and 0.0015 units of pig Na⁺/K⁺-ATPase in a 96-well plate. On the same plate we replicated each reaction using a buffered background mix with identical composition

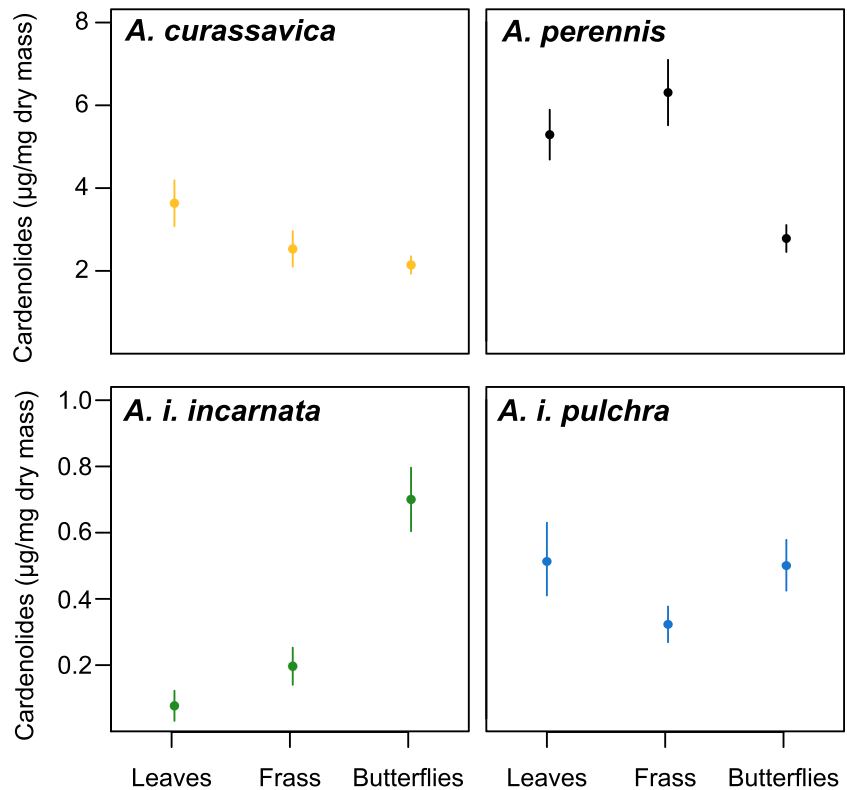
as the reaction mix but without KCl (Na⁺/K⁺-ATPase inactive) to control for the extract color and for any non-specific ATPase reactions as well as contaminating inorganic phosphate. We incubated plates at 37 °C for 20 min, and then stopped enzymatic activity by adding 100 µL of 10% sodium dodecyl sulfate containing 0.05% antifoam A to each well. We stained the inorganic phosphate released from enzymatically hydrolyzed ATP using 100 µL of a color reagent (Tausky and Shorr 1953). To quantify the amount of stained phosphate we measured absorbance of each well in the plate at 700 nm. We corrected the absorbance values using the absorbance of their respective backgrounds, and calculated percent residual activity using reference points of an uninhibited enzyme reaction as 100% activity and of a reaction inhibited by 2×10^{-3} M ouabain as 0% residual activity. Using the *gnls* function in the *nlme* R package (Pinheiro et al. 2018), we estimated the sigmoid dose-response curve using a logistic function with the upper and lower asymptotes set to 100% and 0% residual activity based on the residual enzymatic activity across the three sample dilutions. For each sample, we calculated the dilution at the inflection point (i.e., residual enzymatic activity of 50%). We estimated the concentration of cardenolides in our samples in ouabain equivalents by using the ouabain calibration curve that ranged from 10^{-3} to 10^{-8} M and was included in each plate.

For each sample we calculated the ratio of the Na⁺/K⁺-ATPase assay cardenolide estimate to the HPLC cardenolide estimate (enzyme assay estimate/HPLC estimate). The ratio gives us a measure of the toxicity (from the amount of enzyme inhibition) per concentration (from the HPLC estimate) for each sample extract. We analyzed differences in these ratios across sample types within host plant species using an ANOVA. We only wished to compare leaf ratio to butterfly ratios to determine if monarchs were sequestering a more toxic suite of cardenolides than occur in host plant leaves so we compared leaf ratios to butterfly ratios within each host plant using contrasts from the *lsmeans* package in R (Lenth 2016).

Results

Experiment 1: Sequestration of Cardenolides by Monarchs on Different Host Plants We found differences in total cardenolide estimates by HPLC across sample types (leaves, frass, and adult butterflies) for *A. perennis* ($F_{2,15} = 9.07$, $P < 0.01$), and *A. i. incarnata* ($F_{2,15} = 22.83$, $P < 0.001$), a marginal effect for *A. curassavica* ($F_{2,16} = 3.64$, $P = 0.050$), and no effect for *A. i. pulchra* ($F_{2,15} = 2.12$, $P = 0.16$; Fig. 1). For *A. curassavica* and *A. perennis*, the high cardenolide plant species, leaves contained 70–90% higher cardenolide concentrations than butterflies (*A. curassavica*: $P = 0.045$, *A. perennis*: $P = 0.026$), and there was no difference between leaves and frass for either

Fig. 1 Cardenolide concentrations measured by HPLC in plant leaves, caterpillar frass, and adult butterflies on a dry mass basis for each host plant species. Note the difference in scale between the high cardenolide plant species (*A. curassavica* and *A. perennis*) and the low cardenolide plant species (*A. i. incarnata* and *A. i. pulchra*). Shown are means \pm SE

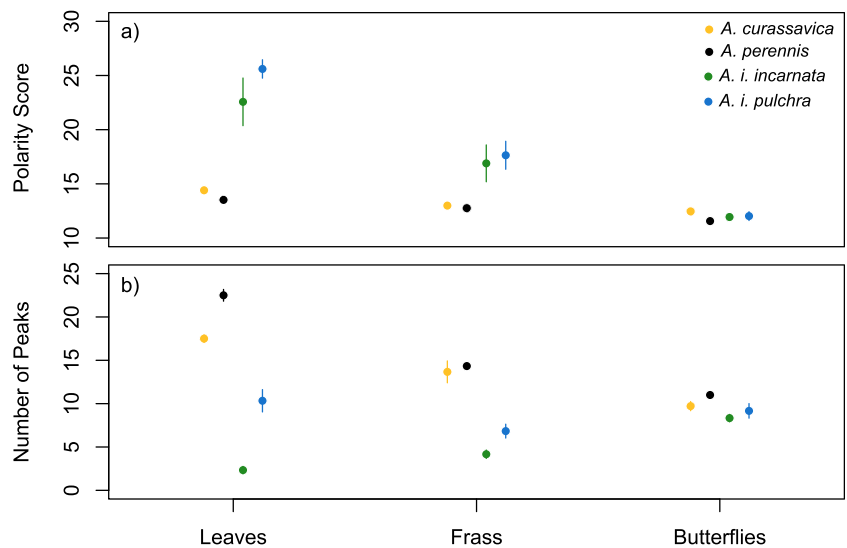


species (*A. curassavica*: $P = 0.18$, *A. perennis*: $P = 0.47$). For *A. i. incarnata*, in contrast, butterflies contained 800% higher cardenolides than leaves ($P < 0.001$), and there was again no difference between leaves and frass ($P = 0.46$).

Sample type had an effect on concentration-weighted polarity scores for all host plant species (*A. curassavica*: $F_{2,16} = 27.53$, $P < 0.001$, *A. perennis*: $F_{2,15} = 21.82$, $P < 0.001$, *A. i. incarnata*: $F_{2,15} = 11.0$, $P = 0.001$, *A. i. pulchra*: $F_{2,15} = 55.27$, $P < 0.001$; Fig. 2a). For all four host plant species, polarity scores decreased from leaves to frass to butterflies, with the

polarity scores showing that the polarity of cardenolides in frass and butterflies was higher than that of leaves, indicating that butterflies were sequestering (and excreting) more polar compounds than were in leaves. Polarity scores ranged from 90 and 100% higher in *A. i. incarnata* and *A. i. pulchra* leaves than those of butterflies reared on those leaves, to 16 and 17% higher in *A. curassavica* and *A. perennis* ($P < 0.001$ for all species), and leaves had average polarity scores around 6% higher than frass polarity scores ($P \leq 0.059$ for all species). Across the four plant species, the polarity scores for butterflies

Fig. 2 a Polarity scores for samples from each of the four host plant species as determined by concentration-weighted HPLC retention times. Lower values indicate a higher proportion of polar compounds (earlier retention times) and higher values indicate a higher proportion of apolar compounds. **b** Diversity of cardenolide compounds for samples from each of the four host plant species. Shown are means \pm SE



were equivalent, indicating that monarchs sequester only cardenolides with polarities within a certain range regardless of leaf chemistry.

Comparisons of cardenolide diversity showed different patterns across sample types depending on host plant species. There were differences in the cardenolide diversity (number of different cardenolide compounds) across sample types for all species (*A. curassavica*: $F_{2,16} = 24.45$, $P < 0.001$, *A. perennis*: $F_{2,15} = 151.20$, $P < 0.001$, *A. i. incarnata*: $F_{2,15} = 48.62$, $P < 0.001$, and marginally for *A. i. pulchra*: $F_{2,15} = 3.21$, $P = 0.069$; Fig. 2b). Leaves contained, on average 38% higher cardenolide diversity than frass for three host plant species ($P \leq 0.01$) with the exception being *A. i. incarnata* in which frass contained ~ 2 more compounds than leaves ($P = 0.026$). Similarly, leaves contained more different compounds than butterflies for *A. curassavica* ($P < 0.001$) and *A. perennis* ($P < 0.001$), but there were more compounds in *A. i. incarnata* butterflies than leaves ($P < 0.001$).

Experiment 2: Cardenolide Sequestration over Larval Ontogeny For caterpillars reared on *A. curassavica*, there was an effect of larval instar on the cardenolide concentrations in hemolymph ($F_{3,40} = 3.11$, $P = 0.037$), however there was not a significant linear trend across all instars ($F_{1,40} = 0.061$, $P = 0.81$; Fig. 3a). Post-hoc tests revealed the only significant comparison to be between fourth and fifth instars (*Tukey HSD*: $P = 0.035$), with 270% higher cardenolide concentrations in fifth instars than fourth instars. When we examined the impact of caterpillar mass on cardenolide concentrations in the fifth instar hemolymph, there was a linear relationship with cardenolide

concentrations increasing with mass (*linear regression*; $P = 0.0012$, adjusted $R^2 = 0.33$; Fig. 3a).

Patterns of sequestration over development were quite different in caterpillar body tissues in comparison to hemolymph. There was an effect of instar on cardenolide concentrations ($F_{3,38} = 5.56$, $P = 0.0029$; Fig. 3b), with cardenolide concentrations declining over development in a significant linear trend (*polynomial contrast*, $F_{1,38} = 15.42$, $P < 0.001$). The only significant comparison was second instars having 160% more cardenolides in their body tissues than fifth instars ($P = 0.0029$). Although cardenolide concentrations generally decreased across developmental stages, the polarity scores of samples did not significantly change over ontogeny in caterpillar body tissues ($F_{3,38} = 2.14$, $P = 0.11$; Fig. 4a). Some second instars were missing a cardenolide with a retention time at 12.9 min that occurred in all later instars, resulting in an effect of instar on the diversity of cardenolide compounds that caterpillar body tissues contained ($F_{3,38} = 6.98$, $P = 0.0029$; Fig. 4b), with second instars being different from all other instars ($P < 0.01$). When we examined the impact of caterpillar mass on cardenolide concentrations in the fifth instar body tissues, there was positive relationship, with cardenolide concentration increasing with increasing caterpillar mass ($P = 0.044$, adjusted $R^2 = 0.13$; Fig. 3b).

While we cannot directly compare cardenolide concentrations between hemolymph and caterpillar body tissues as the former was calculated on a wet mass basis and the latter on a dry mass basis, we can compare polarity scores and cardenolide diversity. There was an effect of sample (hemolymph *versus* body tissues) on polarity scores ($F_{1,78} = 20.89$,

Fig. 3 **a** Concentration of cardenolides in hemolymph ($\mu\text{g}/\text{mg}$ wet mass) of caterpillars in comparison to caterpillar mass when reared on *A. curassavica*. Different colors indicate different instars. **b** Concentration of cardenolides in caterpillar body tissues ($\mu\text{g}/\text{mg}$ dry mass) over the course of development on *A. curassavica*

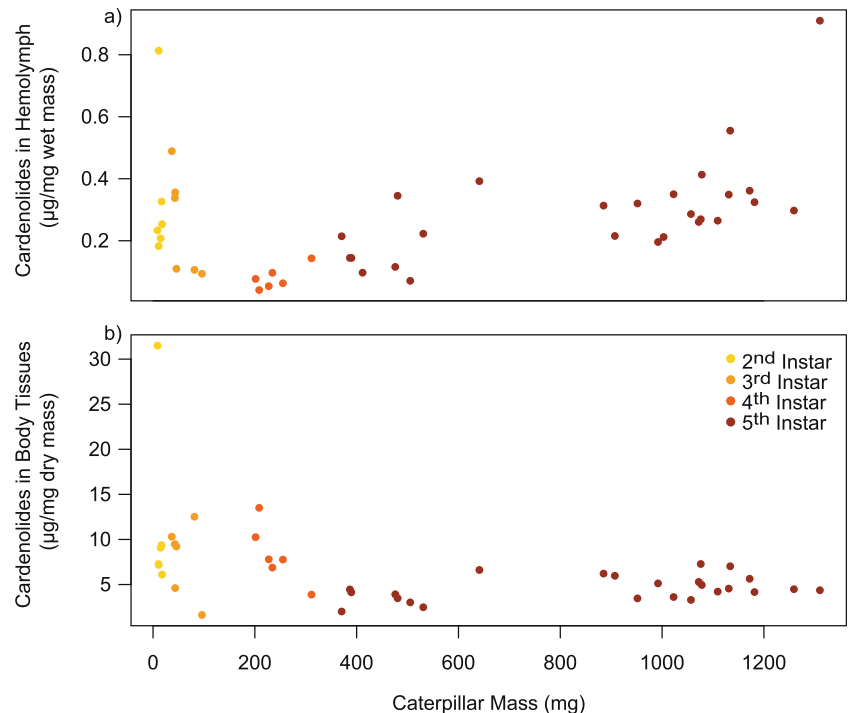
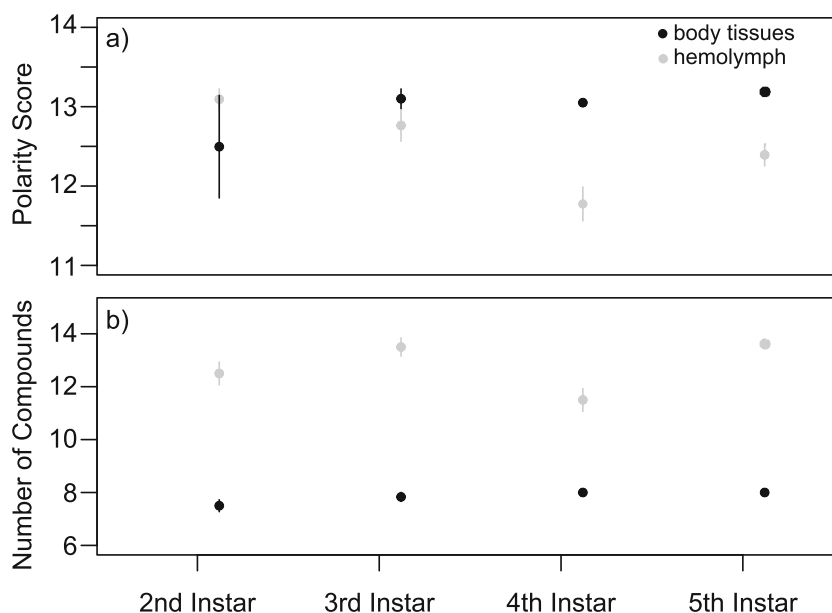


Fig. 4 Mean (\pm SE) **a** polarity scores of hemolymph and body samples over the course of development and **b** cardenolide diversity in hemolymph and body samples over the course of development



$P < 0.001$; Fig. 4a), not of instar ($F_{3,78} = 1.66$, $P = 0.18$), and an interaction between sample and instar ($F_{3,78} = 5.71$, $P = 0.001$). We also compared the cardenolide diversity between hemolymph and body tissues over development: there was an effect of sample type ($F_{1,78} = 1244.32$, $P < 0.001$), instar ($F_{3,78} = 10.51$, $P < 0.001$), and an interaction between the two ($F_{3,78} = 8.13$, $P < 0.001$) (Fig. 4b). Hemolymph contained around four more different cardenolide compounds than body samples, with the number and identity of compounds in body tissues remaining steady over the course of development, but in hemolymph fourth instar caterpillars were missing cardenolides with retention times at one or more of 12.8, 13.1, 13.7, and 16.8 min, resulting in fewer total cardenolides in fourth instar caterpillars than in other instars.

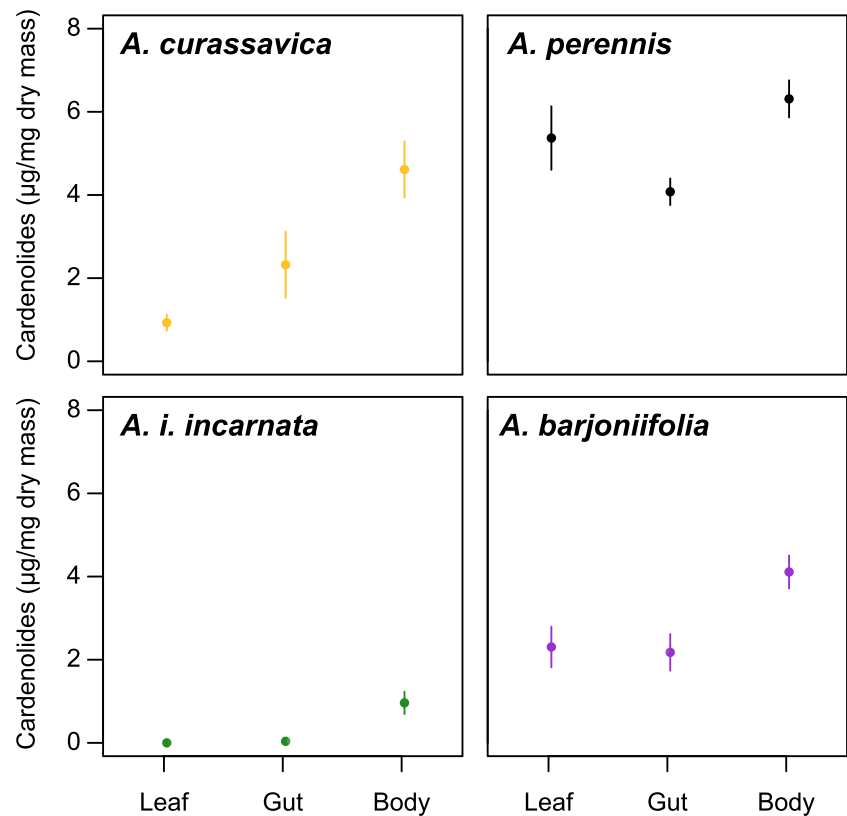
To summarize the effects of ontogeny on sequestration, it appears that the concentration of cardenolides circulating in hemolymph follows a U-shaped function with a reduction of cardenolides in the 4th instar and a subsequent recovery during the final stage. In contrast, cardenolide concentrations in caterpillar body tissues decrease over development (on a dry mass basis), indicating that sequestration may occur more intensively in body tissues of early instar caterpillars. In fourth and fifth instars, hemolymph contained a higher proportion of more polar compounds than caterpillar body tissues, and throughout development hemolymph had, on average, 66% percent more different cardenolide compounds than caterpillar body tissues.

Experiment 3: Sequestration in the Fifth Instar To determine the extent of sequestration in late instars, we reared caterpillars on low cardenolide *A. i. incarnata* until they molted into the fifth instar at which point they either continued on *A. i. incarnata* or were moved to one of three higher cardenolide plant species (*A. curassavica*, *A. perennis*, or *A. barjoniifolia*).

We then examined the levels of cardenolides in leaves, caterpillar guts (including the food bolus) and caterpillar body tissues on the four host plant species. Sample sizes were small ($N \geq 3$ per plant species). We found an effect of tissue type on cardenolide levels in *A. curassavica* ($F_{2,6} = 9.19$, $P = 0.015$), with the only significant comparison being body samples having 400% higher cardenolide levels than leaves ($P = 0.013$). In *A. perennis* there was no significant effect of tissue type on cardenolide levels ($F_{2,7} = 3.013$, $P = 0.11$). In *A. i. incarnata* and *A. barjoniifolia* there was an effect of tissue type (*A. i. incarnata*: $F_{2,6} = 12.31$, $P = 0.0075$; *A. barjoniifolia*: $F_{2,7} = 5.27$, $P = 0.040$), but only *A. i. incarnata* had significant post-hoc comparisons with body tissues having higher cardenolide levels than leaves ($P = 0.011$) and gut contents ($P = 0.013$). Leaves and guts (including their contents) were not different in cardenolide levels in any of the host plant species. It appears, therefore that fifth instar caterpillars (unlike caterpillars that are reared on high cardenolide host plants all through their development as in Experiment 1) are concentrating cardenolides in their body tissues on all host plants except *A. perennis*, on which body tissue concentrations are similar to the very high leaf concentrations (Fig. 5).

There were no significant differences in cardenolide concentrations in hemolymph collected on days one and three (seven) for any of the host plant species (*paired t-tests*; $P \geq 0.062$), so we used the average of the two for the analyses. There was a significant effect of plant species on hemolymph estimates ($F_{3,10} = 16.57$, $P < 0.001$). The pattern of concentrations in hemolymph was the same as leaf concentrations (*A. perennis* > *A. barjoniifolia* > *A. curassavica* > *A. i. incarnata*), but post-hoc comparisons indicate that the only significant differences ($P < 0.05$) were lower hemolymph cardenolide estimates for *A. i. incarnata* than any of the other three species (data not shown).

Fig. 5 HPLC estimates of cardenolide concentrations (in $\mu\text{g}/\text{mg}$ dry mass) in different tissue types across host plant species for fifth instar caterpillars that were initially reared on low cardenolide *A. i. incarnata* for the first four instars. Shown are means \pm SE



As before, we compared polarity scores and cardenolide diversity between hemolymph and body tissues. We found effects of host plant species ($\chi^2_3 = 263.59$, $P < 0.001$), sample type ($\chi^2_1 = 260.60$, $P < 0.001$), and their interaction ($\chi^2_1 = 545.49$, $P < 0.001$), on polarity scores. Hemolymph contained more polar compounds than body tissues, but only for *A. barjoniifolia* was the difference substantial with body samples having 49% higher polarity scores (more apolar compounds) than hemolymph samples. There were also effects of host plant species ($\chi^2_3 = 826.85$, $P < 0.001$), sample type ($\chi^2_1 = 1957.44$, $P < 0.001$), and their interaction ($\chi^2_1 = 189.49$, $P < 0.001$), on numbers of cardenolides in samples. Body tissue samples contained 40% more cardenolides than hemolymph for *A. curassavica*, 65% more for *A. perennis*, 81% more for *A. barjoniifolia*, and 100% more for *A. i. incarnata* (data not shown).

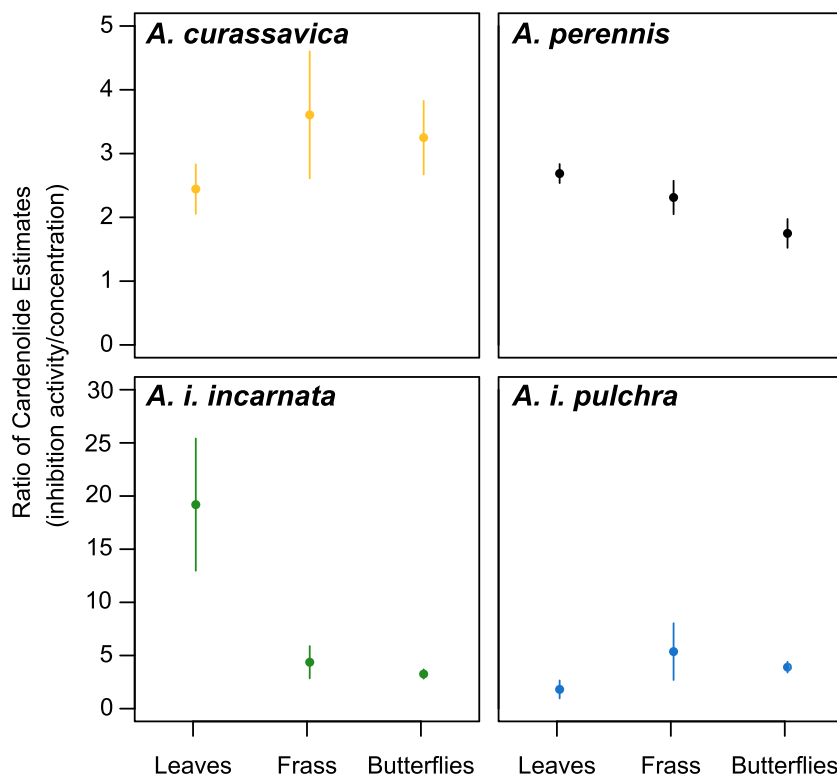
Experiment 4: *In Vitro* Inhibition of the Na^+/K^+ -ATPase We examined the ratio of the cardenolide estimates from the Na^+/K^+ -ATPase assay to the cardenolide estimate from HPLC in Experiment 1 to obtain a measure of the inhibitory strength/pharmacological potential of the cardenolides in different tissues for each host plant. Because we used the porcine enzyme, results from our Na^+/K^+ -ATPase assay can be interpreted as the pharmacological potential of sequestered cardenolides against a potential vertebrate predator. The plant species with effects of sample type on ratio were *A. i. incarnata* ($F_{2,6} =$

5.79 , $P = 0.04$) and a marginal effect in *A. perennis* ($F_{2,6} = 4.75$, $P = 0.058$). In *A. i. incarnata* and *A. perennis* leaves had higher ratios (i.e., more toxic than predicted by HPLC) than butterflies (contrasts *A. i. incarnata*: $P = 0.023$, *A. perennis*: $P = 0.022$). Therefore, in both a very low cardenolide plant species, *A. i. incarnata*, and a high cardenolide species, *A. perennis*, we found some evidence that the cardenolides sequestered by butterflies were less toxic to animal enzymes *in vitro* than the cardenolides present in leaves (Fig. 6). The patterns were reversed, although not significant, for the other two species.

Discussion

Monarch butterflies are a model system for studying sequestration of plant defensive compounds (Brower et al. 1967). At the same time, monarchs are long-distance migrants that encounter a wide range of host plants over their lifespans (Agrawal 2017). Variation in host plant chemistry likely has important consequences for caterpillar performance (Zalucki et al. 2001), as well as defense of both caterpillars and adults (Brower and Moffitt 1974), and adult life-span (Tao et al. 2016). Much remains unknown, however, about the mechanisms of sequestration by monarchs, particularly from different host plants and across development.

Fig. 6 Ratio of cardenolide estimates from the Na^+/K^+ -ATPase assay to the HPLC estimate from the same samples. Shown are means \pm SE



Experiment 1: Sequestration of Cardenolides by Monarchs on Different Host Plants

In our first experiment we reared monarchs for their entire development on one of four different closely related milkweed species that vary more than ten-fold in cardenolide concentrations. Our results confirm previous studies showing that the concentration of cardenolides in monarch butterflies varies depending on host plant chemistry (Brower and Moffitt 1974; Brower et al. 1982; Martin et al. 1992) and that on low cardenolide host plants (such as *A. i. incarnata* and *A. i. pulchra* in our study) monarchs concentrate cardenolides, resulting in higher concentrations in butterfly tissue (on a dry mass basis) than occurs in plant leaves (Brower et al. 1982; Malcolm et al. 1989). Our results also agree with previous research showing that on high cardenolide host plants, such as *A. curassavica* and *A. perennis*, monarchs only sequester cardenolides up to about 3 $\mu\text{g}/\text{mg}$ of dry mass (Malcolm et al. 1989), at which point sequestration saturates (Martin and Lynch 1988; Nishio 1980). These results are similar to those of another classic sequestering insect system: caterpillars of the buckeye butterfly, *Junonia coenia*, that sequester iridoid glycosides from host plants (Camara 1997a). As with monarchs, concentrations of iridoid glycosides in buckeye caterpillars are dependent on host plant concentrations (Lampert and Bowers 2010), and caterpillars reared on artificial diets high in iridoid glycosides do not achieve diet level concentrations, presumably because they metabolize the excess iridoids (Camara 1997b). Therefore, even in monarchs and buckeyes, both sequestering specialists,

there appear to be costs or constraints of sequestration such that insects do not achieve the concentrations of their most highly toxic host plants.

Previous research demonstrated that monarchs sequester a more polar suite of cardenolides than occurs in host plant leaves (Malcolm et al. 1989; Nelson 1993; Roeske et al. 1976). Our data supports these studies and shows that regardless of the wide variation in host plant leaf polarity, emergent butterflies converged in polarity, indicating that sequestered cardenolides are within a constrained range of polarities in comparison to host plant leaves. Part of this convergence in butterfly defensive chemistry occurs through a reduction in the diversity of compounds compared to leaves. For the two high cardenolide host plants, *A. perennis* and *A. curassavica*, leaves contained >80% more compounds than butterflies. Frass was intermediate between butterflies and leaves in both polarity and number of compounds, suggesting that caterpillars may be excreting some of the apolar compounds that occur in leaves but are not sequestered by butterflies, while other apolar leaf compounds are metabolized into polar metabolites lowering the polarity of frass (Seiber et al. 1983). In addition, in Experiment 2 we show that caterpillar body tissues contain slightly more apolar compounds than hemolymph. This result may be a product of apolar compounds being stored in fatty tissues, whereas circulating aqueous based hemolymph contains more polar compounds.

Nelson (1993) proposed a model for cardenolide sequestration and metabolism in which butterflies sequester mostly

polar or intermediate polarity compounds directly from leaves and metabolize apolar compounds into more polar compounds that are sequestered (Seiber et al. 1980, 1983). We therefore predicted that polar and intermediate polarity compounds that occur in leaves should also be found in butterflies, apolar leaf cardenolides should not occur in butterflies, and butterflies will contain some polar compounds missing from leaves (resulting from metabolism of apolar leaf compounds). Frass might therefore contain apolar leaf cardenolides that are not metabolized, as well as some of the polar metabolites that are not stored. Examination of individual peaks on chromatograms allowed us to investigate this hypothesis (Supplementary Fig. S1). A few compounds, that occur in host plant leaves with retention times of 11.9 (all host plants), 13.9 (*A. curassavica* and *A. perennis*), and 14.9 (*A. curassavica* and *A. perennis*) minutes (all reflecting intermediate polarity based on HPLC retention times) appear to be sequestered directly from host plant leaves. Some intermediate and many apolar compounds occur both in leaves and in frass but not in butterflies, indicating that they are passing through the caterpillar guts intact and are not sequestered by butterflies (although it is possible that some of them may be sequestered and metabolized to other compounds). Many compounds, often with retention times higher than 15 min, indicating apolarity, occur only in leaves, and do not occur in frass, potentially because they are being converted to more polar compounds by caterpillars. Compounds that occurred only in butterflies and were not found in leaves were generally polar (retention times less than 12 min). These results support the model of sequestration proposed by Nelson (1993) that monarchs generate polar metabolites, do not sequester apolar compounds that occur in leaves, and directly store cardenolides of intermediate polarity. We show in this study, however, that the compounds generated by monarchs through metabolism differ depending on host plant. These compounds could provide hints as to which host plants are less costly for sequestration, due to the potential for direct sequestration rather than metabolism.

Experiment 2: Cardenolide Sequestration over Larval

Ontogeny To test for sequestration across larval ontogeny, we examined cardenolides in monarch caterpillars from the 2nd to the 5th larval instar reared on *A. curassavica*. In caterpillar body tissues, the concentrations of sequestered cardenolides (on a per mass basis) steadily decreased over the course of development. We have two hypotheses for this decrease in sequestered compounds over ontogeny. One possibility is that there may be more active sequestration in early instars, potentially as a way to minimize predation risk to these vulnerable life stages. Another possibility is that young instars may be less able to actively regulate sequestration, and therefore on a high cardenolide host plant such as *A. curassavica*, they may passively absorb higher cardenolide loads. A

possible way to test these hypotheses would be to examine sequestration over development from low cardenolide host plants such as *A. i. incarnata*. If early instars on *A. i. incarnata* concentrate cardenolides this would indicate more active processes in early instars. Mechanistically, a decrease in cardenolide concentration in larval body tissues could be due to differential growth of the gut epithelium which is likely to mediate sequestration and the body tissues (Supplementary Fig. S2). In other words, if the ratio between gut epithelium surface area and volume of the body tissues shifts in favor of the body tissues, a dilution effect of cardenolides sequestered is to be expected. At the same time, in the fifth instar caterpillar, cardenolide concentrations increased with caterpillar mass, especially in the caterpillar hemolymph. This increase during the last instar could be related to the characteristics of caterpillar growth which are exponential (Supplementary Fig. S3). Consequently, while the growth rate across earlier instars remains relatively constant, it changes dramatically during the fifth instar. Thus, fifth instar caterpillars will have a substantially higher throughput of dietary cardenolides and sequestration is probably influenced by the interplay of physical parameters, such as allometric relationships between tissues, and phases of caterpillar growth.

Previous research with buckeyes has examined the pattern of sequestration of two different iridoid glycosides across the third, fourth, and fifth instars in *J. coenia* (Bowers and Collinge 1992). Concentrations of aucubin were similar between third and fifth instars but halved in fourth instars. Similarly, concentrations of catalpol were slightly lower in fourth than third instars but tripled in fifth instars. It is thus possible that the drop we saw in concentrations of hemolymph cardenolides in fourth instar monarchs was due to a metabolic process common to nymphalid butterflies, however we saw no corresponding drop in body concentrations of cardenolides. The different modes of sequestration of different iridoid glycosides over development in buckeyes indicates that future work may benefit from a focus on sequestration over larval ontogeny in a comparative framework, involving different species and classes of toxins.

Experiment 3: Sequestration in the Fifth Instar

Having shown that sequestration is prominent in the early instars, in Experiment 3 we tested how caterpillars reared initially on low cardenolide plants sequester cardenolides when moved to alternative host plants in the fifth instar. Caterpillars moved to high cardenolide plants such as *A. perennis* achieved high concentrations of sequestered cardenolides in their final instar. In fact, on all four host plants, caterpillars appeared to be concentrating cardenolides. These results highlight that caterpillars may be able to compensate for feeding on a low cardenolide host plants in early instars by sequestering large amounts of cardenolides in the fifth instar. This supports previous research indicating uptake of cardenolides in the final

instar (Frick and Wink 1995). In buckeyes, the host plant that the caterpillars consumed in the last instar is the most important determinant of caterpillar concentrations (Lampert and Bowers 2010). It appears therefore in monarchs that although early instars sequester high amounts of cardenolides relative to their body mass, the bulk of sequestration happens in the final instar.

Experiment 4: *In Vitro* Inhibition of the Na⁺/K⁺-ATPase

In Experiment 4 we used an enzyme assay to examine the inhibitory effects of the extracts of leaves, frass, and adult butterflies from Experiment 1. The same samples were partitioned for analysis in the Na⁺/K⁺ ATPase assay and HPLC, allowing direct comparison of estimates of cardenolide concentrations from their enzyme inhibition *versus* from peak area (Petschenka and Agrawal 2015; Züst et al. 2018; Züst et al. 2019). The enzyme assay offers the opportunity to examine biological activity, which can be more ecologically meaningful than HPLC-quantification. In addition, the Na⁺/K⁺ ATPase assay is more sensitive to low cardenolide concentrations than the HPLC, and generally gives estimates of cardenolide concentrations around three times those of HPLC (relative to the weakly inhibitive standard, ouabain (Züst et al. 2019)). We show that in both *A. perennis* and *A. i. incarnata*, the ratio of Na⁺/K⁺ ATPase to HPLC was significantly higher in leaves than in butterflies, indicating that leaf extracts are having a greater inhibitory effect per HPLC concentration than butterfly extracts. This result suggests that butterflies may have sequestered a less toxic cardenolide mixture than occurs in host plant leaves. This is in contrast to the oleander aphid, *Aphis nerii*, that sequesters a more toxic set of cardenolides than occur in host plant leaves (Züst et al. 2018). We found no differences in ratio, however, for the other two host plants *A. curassavica* and *A. i. pulchra*, indicating that this process is dependent on host plant chemistry. Further comparisons between milkweed host plants using both vertebrate enzymes and Na⁺/K⁺ ATPases extracted from monarchs (Petschenka and Agrawal 2015) could be informative for understanding the balance of toxicity to predators *versus* autotoxicity in monarch sequestration.

Conclusions Migrating monarchs encounter milkweeds that vary in many traits including plant chemistry (Agrawal 2017). Milkweed cardenolides are necessary for monarch defense from predation (Brower and Moffitt 1974), and parasites (Sternberg et al. 2012), but can be costly for monarch development at high concentrations (Zalucki et al. 2001). We have shown that monarch sequestration is highly dependent on host plant chemistry, but at the same time a highly selective process in which monarchs preferentially sequester particular compounds, metabolize some cardenolides into different forms, and shift their sequestration over the course of development. The degree of active regulation of cardenolide sequestration

may have evolved in monarchs as a means of coping with a wide diversity of milkweeds encountered by monarchs within and between generations.

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