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Population-specific patterns of toxin sequestration in monarch butterflies from around the world

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Abstract

Monarch butterflies are one of the preeminent examples of a toxin-sequestering animal, gaining protection against predators via cardenolides obtained from their milkweed host plants. Although cardenolide sequestration by monarchs has been studied in ecological, physiological, and phylogenetic contexts, relatively little research has surveyed genetic variation in the ability to sequester, nor has monarch sequestration been studied in relation to divergent host plant assemblages or variation in exposure to predation. Here, we use the monarch's recent global range expansion to test hypotheses about how cardenolide sequestration evolves over relatively contemporary time scales. First, we test for whether sympatric monarch/milkweed combinations have a sequestration advantage by rearing six geographically disparate monarch populations on six associated milkweed host species and measuring levels of sequestered cardenolides in a set of 440 adult butterflies. Second, we use monarchs from Guam—an oceanic island where birds have been functionally extirpated for approximately 40 years—to test hypotheses about how exposure to avian predation affects cardenolide sequestration. We find little overall evidence for increased sequestration on sympatric hosts. However, one monarch population (Puerto Rico) shows strong support for cross-host tradeoffs in sequestration ability, primarily driven by limited sequestration of polar cardenolides from two temperate North American milkweeds (*Asclepias syriaca* and *A. speciosa*). Monarchs

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from Guam show some evidence for reduced cardenolide sequestration in both a cross-island comparison of wild-caught butterflies as well as population-level comparisons of greenhouse-reared butterflies. Our results suggest that there is substantial genetic variation in sequestration ability (both within and between monarch populations) and that evolutionary history and contemporary species interactions may influence patterns of cardenolide sequestration.

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Introduction

Plant-feeding insects comprise the vast majority of described terrestrial biodiversity (Futuyma and Agrawal 2009; Wiens et al. 2015), with most species showing a high degree of host plant specialization and feeding exclusively from single host plant families, genera, or species (Forister et al. 2015). Host plant specialization is often explained in terms of cross-host performance tradeoffs (Rausher 1984; Futuyma and Moreno 1988), though other explanations involving enemy-free space and selection by predators have also been invoked (Gilbert and Singer 1975, Bernays and Graham 1988, Petschenka and Agrawal 2016). One way in which herbivores may gain protection from their predators and other natural enemies is through sequestration, defined as the selective uptake, transport, modification, storage, and deployment of plant secondary compounds (Heckel 2014). Sequestration as a defense against higher trophic levels is common across the tree of life (Brodie 2009) and has been documented in taxa as diverse as snakes (Hutchinson et al. 2007), poison dart frogs (Santos et al. 2003), and African crested rats (Kingdon et al. 2012).

Monarch butterflies (*Danaus plexippus*) are perhaps the single best-studied example of a toxin-sequestering animal. Monarch larvae feed on milkweeds (Apocynaceae: Asclepiadoideae) and incorporate toxic cardiac glycosides (cardenolides) from these hosts that remain in their tissue throughout development (Brower et al. 1967, Reichstein et al. 1968, Roeske et al. 1976). The toxicity of monarchs and other Danaine butterflies has been the subject of intense speculation and research dating back to the late 1800s (Trimen 1887; Poulton 1914; Reichstein et al. 1968; Agrawal 2017), and the

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physiological and biochemical basis of this behavior has been studied in considerable detail (Duffey 1980; Seiber et al. 1980; Frick and Wink 1995; Agrawal et al. 2021). Cardenolide sequestration in monarchs is thought to reflect a combination of passive and active processes: nonpolar cardenolides passively diffuse across the membrane of the monarch midgut, while active transport is likely required for transmembrane movement of polar cardenolides and subsequent deposition in storage tissues (Frick and Wink 1995; Agrawal et al. 2012; Groen et al. 2017). Cardenolides sequestered by monarchs confer protection against bird predators, as demonstrated in the iconic series of experiments by Lincoln Brower and colleagues (Brower et al. 1968; Brower et al. 1972; Brower and Moffitt 1974) and the associated image of a vomiting blue jay. Sequestered cardenolides also deter invertebrate predators (Rayor et al. 2004) and parasitoids (Stenoien et al. 2019), and high-cardenolide diets are likewise associated with resistance to the protozoan parasite *Ophryocystis elektroscirrha* (de Roode et al. 2008; Sternberg et al. 2012; Gowler et al. 2015).

Early studies of sequestration in monarchs focused on characterizing differences in the amount and composition of cardenolides across a variety of milkweed species (Brower et al. 1982; Brower et al. 1984; Malcolm and Brower 1989), partly with the goal of informing studies that used “cardenolide fingerprinting” to identify the natal origins of migratory monarchs (Seiber et al. 1986; Malcolm et al. 1989; Dockx et al. 2004). Results from these studies showed that sequestration is highly variable across milkweed species, with more than 20-fold variation in the amount of cardenolide sequestered (Malcolm and Brower 1989). Likewise, sequestration efficiency seems to be highest on milkweed species with low to intermediate levels of cardenolides, including common milkweed

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(*Asclepias syriaca*) (Malcolm and Brower 1989). More recently, phylogenetic comparative studies have placed monarchs' ability to sequester cardenolides into a broader evolutionary context (Aardema et al. 2012; Zhen et al. 2012; Petschenka et al. 2013; Karageorgi et al. 2019). Monarchs are part of a relatively small clade of milkweed butterflies (Nymphalidae: Danaini) that sequester cardenolides, and the stepwise evolution of cardenolide target site insensitivity in monarchs appears to be a byproduct of selection for sequestration ability, rather than dietary specialization (Petschenka and Agrawal 2015).

Despite research into variation in sequestration across monarch tissues (Brower and Glazier 1975; Frick and Wink 1995), across their ontogeny (Jones et al. 2019), and throughout their migratory cycle (Malcolm and Brower 1989), little is known about how natural selection shapes sequestration strategies over contemporary time scales. Two approaches that could improve our understanding of selective forces operating on sequestration in monarchs involve (1) using geographically disparate populations of monarch butterflies with divergent host plant assemblages to test for local adaptation in sequestration ability and (2) using naturally occurring gradients of predation intensity to understand whether predators exert selection on cardenolide sequestration. For the first point, monarch populations around the world show some evidence for local adaptation to their available host plants based on larval growth rate (Freedman et al. 2020a), as well as subtle variation in the terminal domain sequences of cardenolides' target enzyme (the sodium-potassium pump, Na^+/K^+-ATPase) (Pierce et al. 2016). It seems reasonable to expect that sequestration ability might also vary across these monarch populations. For the second point, sequestration could conceivably be disfavored in areas where predation

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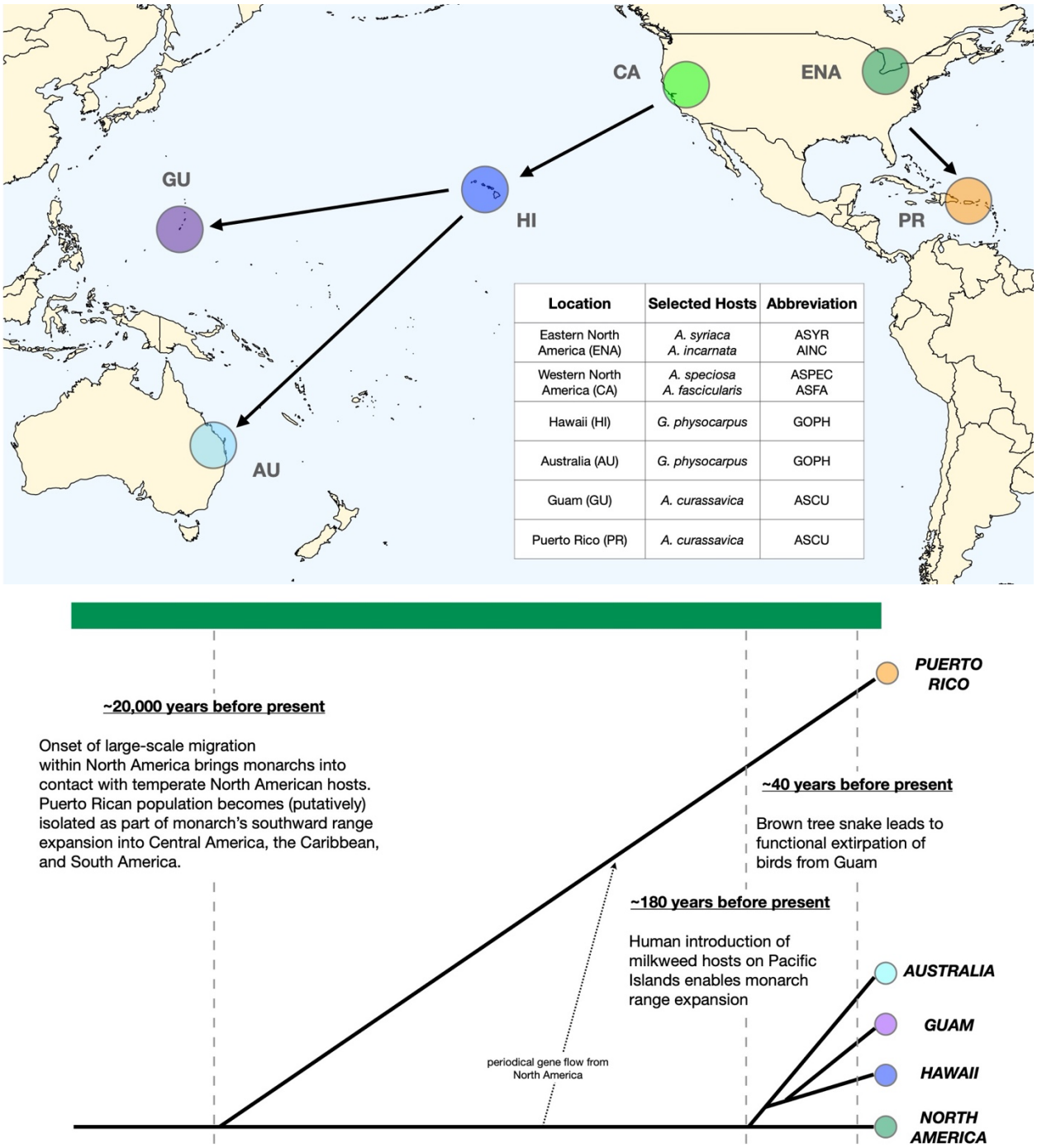

Location	Selected Hosts	Abbreviation
Eastern North America (ENA)	*A. syriaca* *A. incarnata*	ASYR AINC
Western North America (CA)	*A. speciosa* *A. fascicularis*	ASPEC ASFA
Hawaii (HI)	*G. physocarpus*	GOPH
Australia (AU)	*G. physocarpus*	GOPH
Guam (GU)	*A. curassavica*	ASCU
Puerto Rico (PR)	*A. curassavica*	ASCU

Figure 1 – (**Top**) Monarch populations and their associated host plants. Arrows indicate the direction of expansion out of the ancestral North American range. Pacific Island populations are part of a single westward expansion event, while Puerto Rican monarchs are part of an independent southward expansion event. Selected hosts for each population are considered sympatric in all analyses. (**Bottom**) Phylogram showing relatedness among populations and approximate timing of major events discussed throughout the manuscript. Note that timeline is not calibrated and that ENA and CA samples are treated as part of a single North American population.

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physiological cost to target site insensitivity (Dalla et al. 2017) or if sequestered cardenolides inhibit even the highly insensitive Na^+/K^+-ATPase of monarchs (Petschenka et al. 2018; Züst et al. 2019; Agrawal et al. 2021).

Here, we conduct a fully reciprocal rearing experiment using six monarch populations and six associated host plant species from around the world and measure cardenolide sequestration in a set of 440 butterflies. We test for local adaptation and tradeoffs in sequestration ability across hosts, as well as inherent variation in sequestration among monarch populations and host plants. In a second comparison, we focus on a monarch population from an oceanic island (Guam) that has lost its bird predators. We compare sequestration in wild-caught butterflies from Guam and a nearby island (Rota) that has an intact bird assemblage. We also use the results from the first experiment to compare sequestration ability across host plants in reared monarchs from Guam versus other locations around the world.

Methods

Study system

Monarch butterflies are best-known from their ancestral range in North America, where they migrate seasonally and feed on more than 40 milkweed host species (Malcolm and Brower 1986; Xerces Society 2018). Over recent evolutionary history, monarchs have greatly expanded their geographic range and are now established in locations throughout Central and South America, the Caribbean, the Pacific, and the Atlantic (Vane-Wright et

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al. 1993, Pierce et al. 2014; Zhan et al. 2014), with Pacific and Atlantic populations likely becoming established in the last ~180 years (Zalucki and Clarke 2004; Freedman et al. 2020b). Nearly all recently-established monarch populations are non-migratory and breed year-round on restricted assemblages of host plants (Pierce et al. 2016, Freedman et al. 2020a). Monarchs have little coevolutionary history with many of their host plants in their introduced range, and host plant species available to monarch in locations throughout the Pacific and Atlantic—primarily *A. curassavica*, but also *Gomphocarpus spp.* and *Calotropis spp.*—are themselves recent introductions from subtropical Africa, India, and the Neotropics.

Experimental approach

Over the course of two years, we conducted a fully factorial rearing experiment using six populations of monarchs from around the world and their associated host plants (Figure 1A). We used the following six host plant species: *A. curassavica* (ASCU), *A. incarnata* (AINC), *A. fascicularis* (ASFA), *A. speciosa* (ASPEC), *A. syriaca* (ASYR), and *G. physocarpus* (GOPH). Host plants were grown from seed in 1-gallon pots in two greenhouses. Monarchs were collected in the field from six global sites as gravid adult females and returned live to [*name redacted for double blind review*] in glassine envelopes, where females laid eggs on cut stems of *A. curassavica*. Within 12 hours of hatching, we transferred neonate larvae onto a randomly assigned host plant using a paintbrush, typically adding 5 larvae per plant. When possible, we used a balanced design that assigned larvae from a single maternal family to all possible host plants (Table S1).

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We then used mesh sleeves to restrict larvae to a single live host plant. For each caterpillar, we recorded its mass after 8 days, the number of days until pupation, and the number of days until eclosion. After pupation, monarchs were transferred into individually labeled plastic containers. Full rearing details, including host plant provenance, are provided in Freedman et al. (2020a) and Supplementary Appendix 1.

To study patterns of sequestration in relation to bird predation, we compared wild-caught monarchs from Guam—an oceanic island where birds have been functionally extirpated since the 1980s due to the introduction of the brown tree snake (Savidge 1987)—to the nearby island of Rota, which still has a mostly intact community of insectivorous birds and bird densities that are orders of magnitude higher than on Guam (Camp et al. 2015). Brown tree snakes also prey on rodents—which can be major monarch predators (e.g. Glendinning and Brower 1990)—and other insectivorous vertebrates on Guam (Savidge 1987). Monarchs from these two islands are genetically distinct (Hemstrom et al., *in revision*), although divergence times between Guam and Rota monarchs are uncertain. We generated sequestration data for 54 wild-caught monarchs from Guam and 27 wild-caught monarchs from Rota (collected in 2015), all of which had cardenolide fingerprints consistent with feeding on *A. curassavica* (Figure S1). We also collected leaf tissue from *A. curassavica* in both locations and seed for use in greenhouse experiments (see above).

Tissue collection and processing

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We extracted cardenolides from milkweed leaf discs and entire butterfly hindwings. Adult monarchs store cardenolides primarily in their wings and integument, with wing cardenolides thought to be an adaptation for deterring bird predation (Brower and Glazier 1975). For a full description of cardenolide extraction methods, see Supplementary Appendix 2. In total, we collected data from 183 leaf samples and 451 wing samples (Table S2).

Cardenolide quantification

A library of cardenolide peaks was established using Chromeleon™ software (Thermo-Fisher) by manually scanning chromatograms of each species and tissue type and selecting all peaks with absorbance spectra between 216-222 nm. This resulted in a library of 70 peaks, including the internal standard (digitoxin). We note that some of these peaks are likely constituent fragments of larger, more intact cardenolides; for example, the widespread cardenolides calotropin, calotoxin, calactin, and uscharin share a common aglycone precursor (calotropogenin). Total cardenolide concentrations (expressed in mg of cardenolide per g of dry tissue) were calculated by summing across all peak areas, dividing by the peak area for digitoxin (0.15 mg/mL), dividing by 0.8 to account for the fraction of cardenolide extract saved after centrifugation, and dividing by the corresponding dry tissue mass in grams. We ran authentic standards for the compounds calactin, calotropin, and frugoside—reported to be the three major compounds sequestered from *A. curassavica* (Agrawal et al. 2021)—as well as aspecioside, reported to be a major sequestered compound from *A. syriaca* (Seiber et al.

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1986; Malcolm et al. 1989) (Table S3). For an example of chromatograms generated from wing tissue, see Figure 2A. We also note that we did not record voruscharin from leaf tissue of either *A. curassavica* or *G. physocarpus* (see Agrawal et al. 2021) because of its very late retention time and potential degradation under our storage conditions, although we did record its sequestered derivatives calactin and calotropin in monarch wings.

Analysis

For each leaf and wing sample, we generated a polarity index based on the retention times of cardenolide peaks (Rasmann and Agrawal 2011). We multiplied each retention time by its corresponding relative peak area ($X_i = RT_iA_i$) and then took the sum of these values ($P_{1...n} = \sum_{i=1}^{i=n} X_i$). The resulting values ($P_{1...n}$) were then scaled between 0 and 1 and subtracted from 1 so that higher polarity indices correspond to samples with higher relative proportions of polar cardenolides.

For each of the six milkweed species sampled, we calculated a sequestration ratio that corresponds to the average cardenolide concentration in monarch wings divided by the average cardenolide concentration from corresponding leaf tissue. We also plotted raw data to explore variation in patterns of sequestration across host plants and monarch species. We visualized multivariate disparity in cardenolide profiles of wings and leaf tissue using non-metric multidimensional scaling implemented in the package 'vegan' (v2.5-7) (Oksanen et al. 2020). We used PERMANOVA (implemented using the adonis2 function, a matrix of Bray-Curtis dissimilarities, and with 1000 permutations) within each

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milkweed species to test whether leaf and corresponding wing samples had significantly different cardenolide profiles. We also analyzed multivariate disparity in sequestered cardenolides using PERMANOVA and a model that considered milkweed species, monarch population, and their interaction as predictors.

To test for quantitative variation in cardenolide sequestration across host species and monarch populations, we used linear mixed models implemented in the lme4 package (Bates et al. 2015) in R version 4.0.3 (R Development Team). Since sequestration amounts were consistently low across all populations for two species (*A. fascicularis* and *A. incarnata*) (Figure 3A) (also see Malcolm 1994), we restricted these analyses to only wing tissue from monarchs reared on the remaining four milkweed species (n = 327). We first tested a model of the form:

(1) conc ~ species*mon.pop + sex + (1|plant.pop/plant.ID) + (1|maternal.family)

where conc is the cumulative total concentration of wing cardenolides, species is the natal milkweed species, and mon.pop is the monarch source population, with random intercepts for plant ID nested within plant population of origin and for monarch maternal family. Sex was included as a categorical factor to account for potential differences between males and females in sequestration (Brower and Glazier 1975). Here, the primary effect of interest is the interaction between milkweed species and monarch population, which reflects GxE interactions in sequestration ability but does not by itself imply local adaptation for sequestration. Model results were summarized using Type III ANOVAs implemented in the ‘car’ package (Fox and Weisberg 2019). We assessed post-

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hoc pairwise differences between monarch populations and milkweed species using TukeyHSD tests implemented in the 'multcomp package' (Hothorn et al. 2008). Next, to explicitly test for local adaptation, we also fit the model:

(2) conc ~ sym.allo + species + mon.pop + sex + (1|plant.pop/plant.ID) + (1|maternal.family)

where the sym.allo term corresponds to whether a given monarch was reared on a sympatric or allopatric milkweed host. Here, a significant positive intercept for sympatric combinations is diagnostic of local adaptation (Blanquart et al. 2013, Freedman et al. 2020a). We note that using total cardenolide concentrations as our response variable when testing for local adaptation presumes that natural selection favors higher cardenolide concentrations in sympatric combinations. However, a more appropriate metric might be sequestration efficiency, which takes into account both the concentration of sequestered cardenolide as well as developmental rate (see Tao and Hunter 2015). Hence, we also analyzed our data using total cardenolide concentration divided by the number of days from egg to eclosion to get a measure of sequestration efficiency.

For monarch samples collected in the field from Guam and Rota, we calculated total cardenolide concentrations for both wing and leaf samples. We then fit a basic linear model comparing wing concentrations between Guam and Rota, with sex as a categorical factor. Next, we tested a second model that used wing cardenolide concentrations adjusted for the average cardenolide concentrations in natural *A. curassavica* leaf samples from each location. Finally, we used pairwise comparisons of greenhouse-reared

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Guam butterflies and all other populations to provide broader context for the sequestration abilities of this population.

Results

Milkweed species varied greatly in their cardenolide composition (Figure 2A, 2B) as well as their average cardenolide concentration, ranging from as low as 0.11 ± 0.03 mg/g (*A. incarnata*) to as high as 7.86 ± 0.66 mg/g (*A. curassavica*). Monarchs, regardless of population of origin, had the highest levels of sequestered cardenolides on *A. curassavica* (12.11 ± 0.53 mg/g) and the lowest on *A. fascicularis* (0.31 ± 0.03 mg/g) (Figure 3). Sequestration efficiency varied strongly across milkweed species: the sequestration ratio was highest in *A. syriaca* (12.78) and lowest in *G. physocarpus* (0.74) (Figure 3). The polarity index of sequestered cardenolides also varied strongly across species: in general, monarchs reared on *A. syriaca* and *A. speciosa* sequestered a high proportion of polar cardenolides, while the subset of sequestered cardenolides on other species was predominantly compounds with low to intermediate polarity (Figure 2A, 2C). Across all milkweed species, the composition of cardenolides present in leaves was significantly different from the composition of sequestered cardenolides (Table S4). Calactin, calotropin, and frugoside were present in monarchs reared on *A. curassavica* and *G. physocarpus*, and together comprised approximately 50% of the total amount sequestered for both species (Table S5). Aspecioside was the predominant compound sequestered from both *A. syriaca* and *A. speciosa* (Table S5). Within milkweed species, concentrations of individual sequestered cardenolides were generally positively

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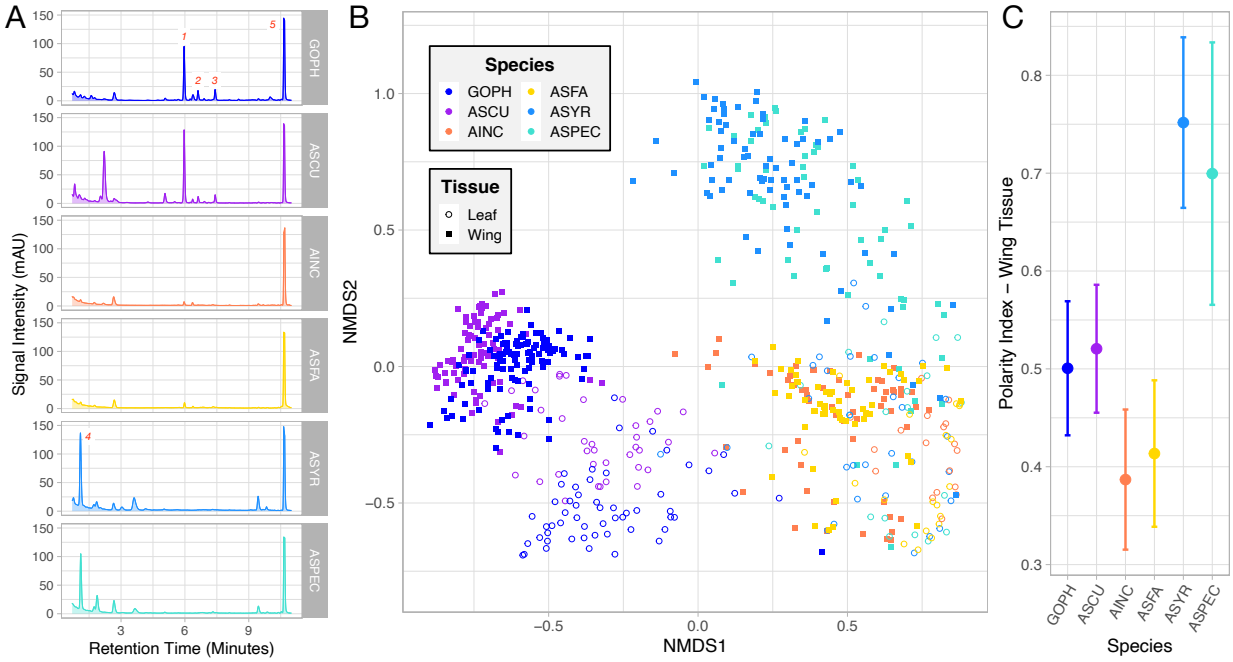

Figure 2 – (A) Example of chromatograms showing sequestered cardenolides in monarch wings. Each panel reflects a butterfly from one of the six milkweed species used during rearing. Retention times correspond to compound polarity, with more polar compound eluting first and less polar compounds eluting last. Compounds with red numbering were verified with authentic standards and are as follows: 1 = frugoside, 2 = calotropin, 3 = calactin, 4 = aspecioside, 5 = digitoxin (internal standard). Note that the y-axis is truncated and does not show the true values for the internal standard (digitoxin – 0.15 mg/mL), which elutes around 10.8 minutes and was generally the largest peak in each sample. **(B)** NMDS plot of leaf and wing tissue. Note the similarity in the profiles of sequestered compounds from *A. curassavica* and *G. physocarpus*, as well as *A. speciosa* and *A. syriaca*. **(C)** Polarity index of sequestered cardenolides in monarch wings. Here, values closer to 1 correspond to sequestration profiles biased towards polar compounds. Note the high polarity indices for monarchs reared on *A. syriaca* and *A. speciosa*, also visible in panel A.

correlated (Figure S2). The overall composition of sequestered cardenolides was most strongly determined by milkweed species identity ($F = 119.49$, $R^2 = 0.494$), followed by monarch population ($F = 4.77$, $R^2 = 0.033$), and finally the interaction between them ($F = 2.85$, $R^2 = 0.059$) (Figure 2B; Figure S3; Table S6).

We found strong support for GxE interactions in sequestration ability, with monarch populations varying substantially in their ability to sequester across milkweed species (χ^2

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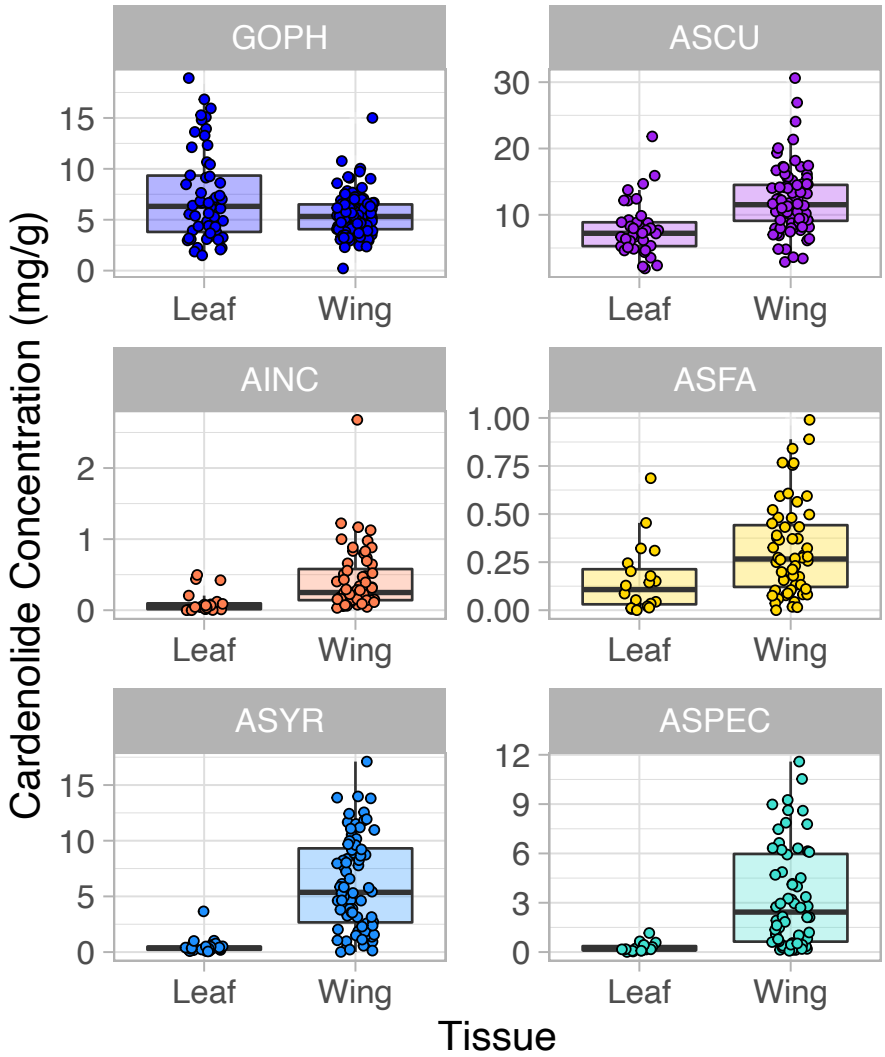
(A)



(B)

Species	Leaf Cardenolides	Wing Cardenolides	Ratio
GOPH	7.31 ± 0.61 (n = 54)	5.41 ± 0.19 (n = 112)	0.74
ASCU	7.86 ± 0.66 (n = 38)	12.11 ± 0.66 (n = 84)	1.54
AINC	0.11 ± 0.03 (n = 20)	0.42 ± 0.06 (n = 60)	3.73
ASFA	0.16 ± 0.04 (n = 20)	0.31 ± 0.03 (n = 59)	2.02
ASYR	0.48 ± 0.11 (n = 32)	6.16 ± 0.48 (n = 76)	12.78
ASPEC	0.28 ± 0.06 (n = 19)	3.34 ± 0.40 (n = 60)	12.09

Figure 3 – **(A)** Boxplots showing cardenolide concentrations (expressed as milligrams of cardenolide per gram of oven-dried leaf or wing tissue) for leaf and wing tissue of each milkweed species. Note that y-axes differ substantially between species. **(B)** Mean cardenolide concentrations (± MSE) and sample sizes for leaf and wing samples. The sequestration ratio reflects wing divided by leaf tissue.

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by cross-host sequestration differences in monarchs from Puerto Rico. Puerto Rican monarchs sequestered 1.37 times more from *A. curassavica* and 1.46 times more from *G. physocarpus* than other populations, yet 4.96 times less from *A. speciosa* and 5.83 times less from *A. syriaca* (Figure 4A; Figure S4). The polarity index of cardenolides sequestered by Puerto Rican monarchs on *A. syriaca* and *A. speciosa* was significantly lower than for all other populations ($t = -6.86$, $p < 0.001$; Figure 4C), and the sequestration profile of Puerto Rican monarchs was distinct from other monarch populations on *A. syriaca* (Figure S3). Aspeciocide concentrations in Puerto Rican monarchs reared on *A. syriaca* were more than 23 times lower than for other populations, and 15 times lower on *A. speciosa* (Table S4). Despite the strong GxE pattern of sequestration in our data, there was no support for local adaptation in sequestration ability ($\chi^2 = 0.16$, d.f. = 1, $p = 0.687$), with roughly equivalent levels of sequestration in sympatric and allopatric *population x host* combinations (Figure 4A, Table S8). Accounting for development time did not meaningfully impact any of our inferences (Figure S5), and we did not find a strong correlation between development time and the total concentration of sequestered cardenolides (Figure S6). Across all species and populations, female monarchs sequestered slightly more than males, although this difference was not significant ($t = 1.688$, $p = 0.091$). Maternal families within populations varied substantially in their propensity to sequester cardenolides (Figure S7).

Wild-caught monarchs from Guam, an island with no birds, had modestly lower cardenolide concentrations (2.24 ± 0.25 mg/g) than wild-caught monarchs from Rota (2.66 ± 0.36 mg/g) ($t = -0.96$, $p = 0.340$). After accounting for average cardenolide concentrations of field-sampled *A. curassavica* on each island, which were 31% higher

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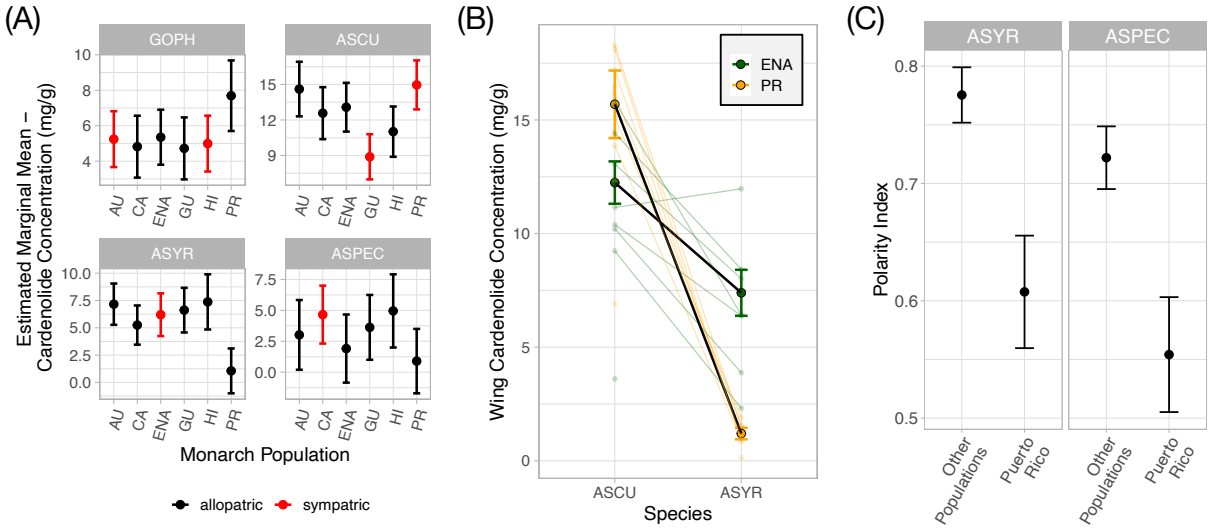

Figure 4 – **(A)** Model-averaged means showing wing cardenolide concentrations across the four primary milkweed species of interest, separated by monarch population of origin. Sympatric combinations of *monarch population x milkweed host* are shown in red; allopatric combinations are in black. Note the substantially lower concentrations of cardenolides in Puerto Rican monarchs from *A. speciosa* and especially from *A. syriaca*. **(B)** Reaction norm plot showing sequestration difference between Puerto Rican and Eastern North American monarchs on their respective host plants (*A. curassavica*, *A. syriaca*). Solid black lines connect mean values for each combination. Faint lines in the background correspond to individual monarch families reared on each host. **(C)** Polarity indices for Puerto Rican versus all other population when reared on *A. syriaca* and *A. speciosa*. Puerto Rican monarchs had significantly lower polarity scores on these species, suggesting comparatively poor sequestration of polar cardenolides (also see Table S5 and Figure S4).

from naturally occurring plants on Guam (3.96 ± 0.70 mg/g) than from Rota (2.72 ± 1.12 mg/g), monarchs from Guam sequestered significantly less than monarchs from Rota ($t = -3.23$, $p = 0.002$). Among all six monarch populations reared in the greenhouse, Guam had the lowest population-specific intercept for overall cardenolide sequestration; however, after correcting for multiple comparisons, no pairwise differences among populations were significant for overall cardenolide sequestration. The pattern of reduced sequestration by monarchs from Guam was most pronounced on *A. curassavica*, their

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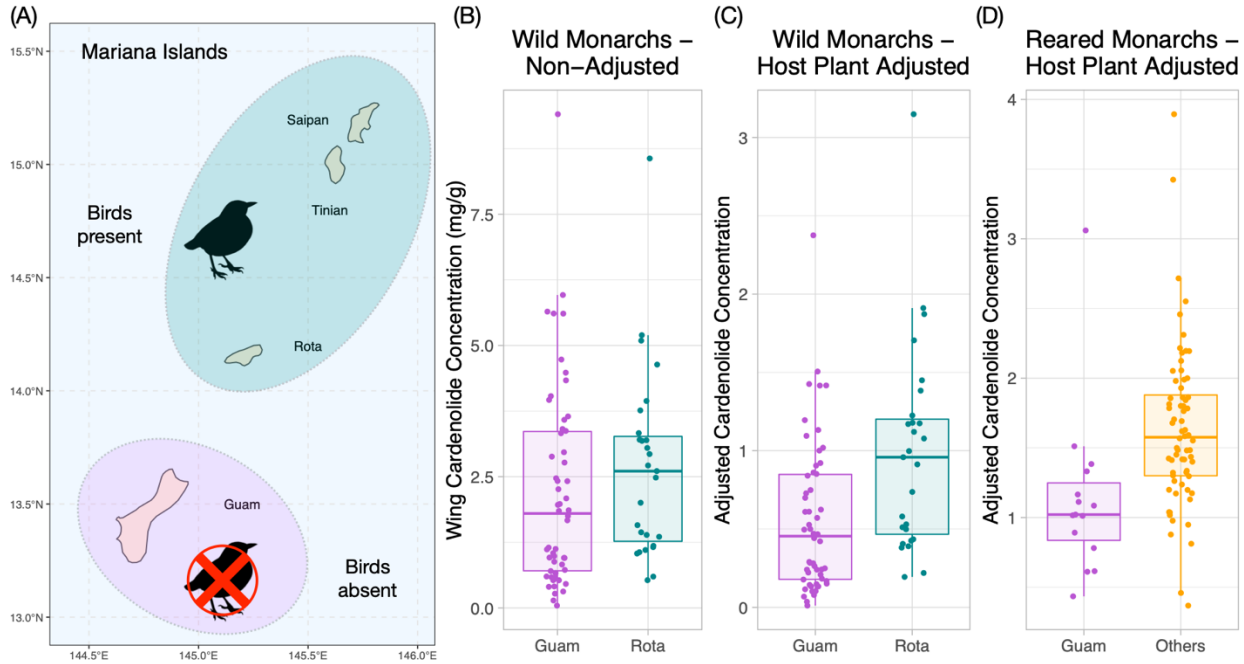

Figure 5 – **(A)** Map of the Mariana Islands, located in the western Pacific Ocean. Birds have been functionally extirpated from Guam for the last approximately 40 years, while the other islands in the archipelago still have largely intact bird assemblages. **(B)** Wing cardenolide concentrations of wild-caught monarchs from Guam (birds absent) and Rota (birds present) were not significantly different. **(C)** After accounting for the concentration of cardenolides in naturally occurring *A. curassavica* from each island, monarchs from Rota had significantly higher wing cardenolide concentrations. **(D)** When reared under controlled conditions on their sympatric host *A. curassavica*, monarchs from Guam sequestered lower concentrations of cardenolides than other monarch populations. In panels C and D, an adjusted cardenolide concentration of 1 means that wing and associated leaf tissue had equivalent concentrations.

sympatric host: Guam monarchs sequestered, on average, 33.1% fewer cardenolides on *A. curassavica* than other populations, and significantly less than populations from Australia, Eastern North America, and Puerto Rico on this host (Figure 5D; Figure S8; Table S10).

Discussion

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We found strong evidence for GxE interactions in sequestration ability, although this pattern was primarily driven by a single monarch population from Puerto Rico. Puerto Rican monarchs contained higher cardenolide concentrations than all other populations when reared on *A. curassavica* and *G. physocarpus*, but substantially lower concentrations when reared on *A. syriaca* and *A. speciosa* (Figure 4A; Table S10). One possible explanation for the inability of Puerto Rican monarchs to efficiently sequester from *A. syriaca* and *A. speciosa* is a lack of evolutionary history with these hosts. Divergence times between Puerto Rican monarchs and their migratory North American ancestors are uncertain but likely occurred within the last 20,000 years (Zhan et al. 2014), whereas other non-migratory populations included in this study likely diverged in the last 150-200 years (Zalucki and Clarke 2004; Freedman et al. 2020b). Unlike other non-migratory populations included in this study, Puerto Rican larvae and adults are phenotypically very distinct from North American migrants (see Freedman et al. 2020b). Thus, it is conceivable that the lineage of Caribbean and South American monarchs that includes Puerto Rico may have diverged prior to the onset of widespread adoption of *A. syriaca* and *A. speciosa* as hosts in North America, and this lineage may never have evolved the physiological capacity to efficiently sequester the primarily polar cardenolides from the temperate North American milkweed flora. Further research with additional monarch populations from the Caribbean and South America or additional North American milkweed species could help to resolve this question.

An alternative (but not mutually exclusive) explanation for the observed pattern of sequestration in Puerto Rican monarchs is a physiological tradeoff in sequestration efficiency, potentially driven by differences in the physical properties of cardenolides

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across milkweed host species. Puerto Rican monarchs sequestered high concentrations from *A. curassavica* and *G. physocarpus*, both of which are high cardenolide species and whose sequestration profiles are biased towards compounds with low to intermediate polarity (Roeske et al. 1976, Malcolm 1990). Interestingly, Puerto Rican monarchs sequestered higher cardenolide concentrations from *G. physocarpus* than any other population (Figure 4A), despite little apparent history of association with this species, suggesting that feeding on *A. curassavica* or other high cardenolide hosts may have pre-adapted them to sequestering from the chemically similar *G. physocarpus*. By contrast, Puerto Rican monarchs sequestered very low concentrations of polar cardenolides from *A. syriaca* and *A. speciosa* that were readily sequestered by all other monarch populations (also see Seiber et al. 1986, Malcolm et al. 1989), as indicated by the lower polarity index for Puerto Rican monarchs on these species (Figure 4C). More research into the biochemical basis of sequestration, as well as studies examining variation among monarch families in their ability to sequester, is needed to determine whether tradeoffs in sequestration ability across hosts are a viable explanation for the pattern observed here.

Despite finding evidence for unique sequestration behavior in monarchs from Puerto Rico, we did not find general evidence for a pattern of local adaptation in sequestration ability, with no overall support for greater sequestration from sympatric host plants across monarch populations. This pattern remained even after accounting for development time, suggesting that butterflies with higher cardenolide concentrations do not have a longer development window in which to sequester. One possible reason for the lack of a sympatric sequestration advantage is that larval performance—including the process of sequestration—may be correlated across chemically similar host plants, even

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if they are geographically disparate and phylogenetically distant (e.g. Pearse and Hipp 2009). For example, the profile of cardenolides sequestered from *A. syriaca* and *A. speciosa* was nearly identical (Figure 2B; Table S5; Seiber et al. 1986), despite these two milkweed species having largely non-overlapping geographic ranges (Woodson 1954). Notably, we did not find evidence that monarchs from Hawaii or Australia had a sequestration advantage on *G. physocarpus*, despite apparently having >100 years of association with this host (Nelson 1993, Malcolm 1994). All derived Pacific Island populations (Hawaii, Australia, Guam) also retained their ability to sequester normally from ancestral North American hosts (*A. syriaca*, *A. speciosa*), even after spending as many as 1,500 generations isolated from these hosts. The intact sequestration ability of Pacific but not Puerto Rican monarchs supports the notion that Puerto Rican monarchs diverged from North American migratory monarchs substantially before other non-migratory populations (Figure 1B).

Our overall results generally corroborate earlier studies of monarch sequestration. For example, we found that sequestration ratios were high for monarchs reared on *A. syriaca* and *A. speciosa*, consistent with many other studies (Malcolm and Brower 1989, Malcolm 1994, Decker et al. 2019). Likewise, we found barely detectable levels of sequestered cardenolides from monarchs reared on *A. fascicularis*, as previously reported by Malcolm (1994), as well as relatively inefficient sequestration from *G. physocarpus* (Figure 3B), as previously reported by Roeske et al. (1976). Interestingly, variation in sequestration from *G. physocarpus* and *A. curassavica* was low relative to other species (Figure 4A; Table S9). The low level of variation in sequestration from *A. curassavica* likely stems from the fact that just three compounds—frugoside, as well as

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the stereoisomers calactin and calotropin—comprise >50% of the total cardenolides sequestered (Agrawal et al. 2021, Table S5). A qualitatively similar pattern was observed in *G. physocarpus*, suggesting a relatively simple and conserved biochemical process underlying sequestration from these species.

We found modest evidence for reduced cardenolide sequestration in monarchs from Guam, where birds have been functionally extirpated for the last ~40 years. Sequestration in wild-caught Guam monarchs was significantly lower than sequestration in Rota monarchs, but only after accounting for differences in *A. curassavica* cardenolide concentrations on each island. Despite their proximity, monarchs from Guam and Rota are genetically distinct (Hemstrom et al., *in revision*), indicating that adaptive divergence between these island populations is possible. As we were unable to rear monarchs from Guam and Rota side-by-side under controlled conditions, it is difficult to ascribe the differences in cardenolides between wild-caught butterflies to differences in bird predation. Furthermore, all comparisons using Guam necessarily involve a functional sample size of n=1, as it is the only location where it is tractable to assess the impact of long-term bird removal on monarch sequestration. The extirpation of bird predators from Guam has altered the island's trophic ecology (Rogers et al. 2012) and may be associated with a proliferation of mesopredators (Ritchie and Johnson 2009) such as wasps, which can be major predators of monarch larvae (Baker and Potter 2020), even when larvae are consuming high-cardenolide hosts (Rayor et al. 2004, McGruddy et al. 2021). Thus, in addition to direct effects of bird predation and its loss on monarch sequestration (Brower et al. 1967; Brower and Calvert 1985; Brower 1988), increased invertebrate

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predation on monarch larvae could indirectly favor faster development, which may be associated with reduced sequestration (Agrawal et al. 2021).

Notably, Guam monarchs sequestered significantly less from their sympatric host plant (*A. curassavica*) than three other monarch populations, whereas sequestration was comparable across other hosts. This pattern is consistent with selection against the specific processes involved with sequestration from *A. curassavica* (e.g. the conversion of voruscharin into calotropin [Agrawal et al. 2021]), but not against other processes involved in the broader context of sequestration (e.g. multidrug transporter activity [Groen et al. 2017]). The observation of reduced sequestration on a sympatric host, possibly only under altered predation regimes, highlights the importance of considering higher trophic levels when forming predictions about the outcomes of evolutionary interactions between plants and their specialized herbivores (Bernays and Graham 1988).

In conclusion, we have demonstrated that monarch butterflies show substantial genetic variation within and between populations for cardenolide sequestration. Cardenolide sequestration is likely shaped by both evolutionary history (including shifting host plant associations) and contemporary species interactions. Our research highlights the utility of “natural experiments”—both the monarch’s recent global range expansion and the recent extirpation of birds from Guam—for testing fundamental hypotheses in ecology and evolution.

Data Accessibility Statement

All raw data and code used in analysis are available through Github at this link:

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https://github.com/micahfreedman/manuscripts/tree/master/Cardenolide_Sequestration. Files will also be deposited on Dryad upon manuscript acceptance.

Acknowledgments and Author Contributions

We thank Asia Jones, Evan Jordan, and especially Christopher Jason for assistance with rearing monarchs used in this study. Hillary Streit assisted with generating chemical data for monarchs from Guam, and Ivan Munkres assisted with generating all other chemical data. Myron Zalucki provided Australian monarchs used in greenhouse rearing, and Louie Yang and Will Haines assisted with monarch collection from Hawaii. MGF, RLV, SYS, and SRR conceived of the study. MGF and SC performed caterpillar rearing. MGF, MDH, and RLV generated chromatograms used for chemical analysis. MGF performed all data analysis and wrote the first draft. Seeds for all milkweed plants grown during this experiment were collected and imported under USDA permit number P37-15-00580. Monarchs collected in the United States and U.S. territories were imported under USDA permit P526P-18-02061, and monarchs collected in Australia were imported under USDA permit P526P-17-02696. Funding to MGF was provided through the NSF Graduate Research Fellowship, National Geographic Society, and the Center for Population Biology. MDH was supported by NSF grant DEB-1256115. RLV was supported by USDA Multistate Award NE1501.

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