**Introduction**

Plant-feeding insects comprise the vast majority of 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). 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). Though perhaps best documented in insects, sequestration as a defense against higher trophic levels is common across the tree of life (Brodie 2009) and has been demonstrated 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 various species of milkweeds (Apocynaceae: Asclepiadoideae) and incorporate toxic cardiac glycosides (cardenolides) from these hosts that remain in their tissue throughout development (Brower and Corvino 1967, 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), and the physiological basis of this behavior has been studied in considerable detail (e.g. Duffey 1980, Frick and Wink 1995). Cardenolide sequestration in monarchs likely reflects a combination of passive and active processes: nonpolar cardenolides passively diffuse across the membrane of the monarch midgut, while active transport is required for the movement of polar cardenolides from the midgut and subsequent deposition in storage tissues (Frick and Wink 1995; Agrawal et al. 2012). Cardenolides sequestered by monarchs are thought to 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 “barfing blue jay.” Sequestered cardenolides also deter invertebrate predators (Rayor et al. 2004) and parasitoids (Stenoien et al. 2019), and dietary cardenolides are likewise associated with resistance to the obligate 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, Malcolm and Brower 1989, ref xx), 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 (1) sequestration is highly variable across milkweed species, with more than 100-fold variation in the amount of cardenolide sequestered (ref xx); (2) sequestration efficiency seems to be highest on milkweed species with low-intermediate levels of cardenolides, including common milkweed (*Asclepias syriaca*) (refs xx Malcolm and Brower 1989); (3) seasonal turnover in milkweed community composition results in seasonal turnover of monarch cardenolide fingerprints (ref xx). More recently, phylogenetic comparative studies have placed the monarch’s ability to sequester cardenolides into a broader evolutionary context (Aardema 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 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), throughout their development (Jones et al. 2019), across their migratory cycle (Malcolm and Brower 1989), and throughout the broader phylogeny of milkweed butterflies (Petschenka et al. 2013), little is known about how natural selection shapes sequestration strategies over more contemporary time scales. It is also unclear whether monarchs show genotypic tradeoffs in sequestration ability across host plants with divergent secondary chemistry: does the ability to sequester cardenolides on a focal host plant come at a cost to sequestering from an alternative host? 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 predator intensity to understand whether predators exert selection on cardenolide sequestration. For the first point, monarchs do show some evidence for local adaptation to host plants across their global range (Freedman et al. 2020a), as well as subtle variation in the terminal domain sequences of cardenolides’ target enzyme (Na+/K+-ATPase) (Pierce et al. 2016). It follows that sequestration ability might also vary across these monarch populations. For the second point, one might predict that sequestration should be disfavored in areas where predation of monarchs is minimal or absent, especially if there is an underlying physiological cost to sequestration or its underlying basis (e.g. Dalla and Dobler 2016, Karageorgi et al. 2019).

In this study, we report the results of two experiments aimed at understanding variation in sequestration ability across monarch butterflies. In the first experiment, 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 in sequestration ability (i.e. elevated sequestration on sympatric host plant species), as well as inherent variation in sequestration potential among monarch populations and host plants. In the second experiment, 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) with an intact bird assemblage. We also use the results from the first experiment to compare sequestration ability across host plants in monarchs from Guam versus other locations around the world with birds present.

**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 host plant species (Malcolm, Borders and Lee-Mäder 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 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 are themselves recent introductions from subtropical Africa, India, and the Neotropics. For a list of monarch populations and their associated host plants used in this study, see Figure 1.

*Experimental approach*

Over the course of two summers, we conducted a fully factorial rearing experiment using six populations of monarch butterfly from around the world and their associated host plants (Figure Sxx). Host plants were collected as seed and grown in 1 gallon pots in two greenhouses. For a full description of host plants used in this experiment, see Supplemental Table xx. Monarchs were collected in the field (typically as gravid adult females) and returned to UC Davis, 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 Sxxx). We then used mesh sleeves (Fiber-Aire xx) to enclose larvae onto live host plants. After pupation, monarchs were transferred into individually labeled plastic containers. Full rearing details are provided in Freedman et al. (2020a).

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 (Savidge 1988)—to the nearby island of Rota, which still has a mostly intact community of insectivorous birds. 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*. We also collected leaf tissue from *A. curassavica* in both locations and seed for use in greenhouse experiments (see above). Over the course of six days of observation on Rota and nine days on Guam, we did not record any predation attempts by birds on monarchs on either island. However, introduced black drongos (*Dicrurus macrocercus*) on Rota were observed pursuing and catching another aposematic species of Danaine butterfly (*Euploea eunice*) that sequesters pyrrolizidine alkaloids (Ackery and Vane-Wright 1984), and overall insectivorous bird densities on Rota are orders of magnitude higher than on Guam (ref).

*Tissue collection and processing*

For leaf tissue, we collected 12 leaf discs from the three pairs of opposite leaves using a ¼ inch diameter hole punch. Six discs were placed into 1 mL of 95% methanol and were held at 4°C until processing. The remaining six discs were placed into a coin envelope and were dried at 60°C, then weighed. For monarch wing tissue, butterflies were given ~6 hours for their wings to fully expand and dry post-eclosion. They were then placed into glassine envelopes and frozen at -20°C until processing. After approximately 2 months at -20°C, each of the four monarch wings was removed from the thorax, and the right hindwing was placed into a coin envelope and dried for ~48 hours at 60°C, then weighed. In total, we collected data from 183 leaf samples and 451 wing samples (Table Sxxx).

To extract cardenolides, we added two stainless steel grinding beads to tubes containing leaf tissue and methanol, or to a 1.5 mL tube containing an oven-dried hindwing. Wing tissue was ground at 50 Hz for 2 minutes using a tissue lyser (Qiagen: 69980), and then 1 mL of 95% methanol was added to each tube. Leaf and wing tissue in methanol was then ground for an additional 2 minutes until thoroughly homogenized, after which samples were added to a sonicator (Thomas Scientific: 1207K36) for 1 hour. Next, samples were centrifuged at 14,000 RPM for 5 minutes, and 800 µL of the supernatant was pipetted into a new tube. These samples were then vacuum-evaporated for 3 hours or until dry and stored at -20°C until analysis. Samples were reconstituted in 500 µL of a 0.15 mg/mL solution of digitoxin (Sigma-Aldrich) in methanol, and 200 µL of each sample was added to a 96-well filter plate (AcroPrep™ Advance, 0.2 μm PFTE membrane), which was centrifuged at 1500 RPM for 1 minute.

Samples were injected at a volume of 2 μL onto Accucore™ C18 column (2.6 μm x 2.1 mm x 30 mm) (ThermoFisher Scientific) and processed using high performance liquid chromatography on a Thermo (Dionex) UHPLC 3000 instrument. We used the following solvent gradient of acetonitrile (ACN) and methanol that separated cardenolides according to their polarity, with the most polar compounds eluting first: 15% ACN for 5 minutes, ramping linearly to 30% for 3.5 min, then to 55% for 3 min, then ramping to 100% for 1 minute with a hold at 100% ACN for 10 min, then equilibration at initial conditions (15% ACN) for 5 minutes. Peaks were quantified using a diode array detector (DAD).

For wild-caught butterflies from Guam and Rota, cardenolide extraction and quantification were conducted using the methods described in Zehnder and Hunter (2007) and were similar to the methods described above.

*Cardenolide quantification*

A library of cardenolide peaks was established using ChromeleonTM 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). 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 lastly dividing by the corresponding dry tissue mass in grams. With the exception of the internal standard, we do not have definitive identities for any of the cardenolide peaks, although some qualitatively match descriptions given in other papers for calotropin, calactin, and asclepiocide (ref, Züst et al. 2018). For an example of chromatograms from wing tissue, see Figure 2A.

*Analysis*

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 across patterns of sequestration across host plants and monarch species. For each 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 (Xi = RTiAi) and then took the sum of these values (Pi…n = for each wing sample. The resulting values (P1…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.

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). Next, we used PERMANOVA within each milkweed species to test for whether leaf and corresponding wing samples had significantly different cardenolide profiles.

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 for two species (*A. fascicularis* and *A. incarnata*) (Figure xx), we restricted these analyses to only wing tissue from monarchs reared on the other 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, mon.pop is the monarch source population, and with random intercepts for plant ID nested within plant population of origin as well as monarch maternal family. 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 necessarily 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-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:

1. **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. 2020).

For 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 covariate. Next, we tested a second model that used wing concentrations adjusted for the average cardenolide concentrations in respective *A. curassavica* leaf samples from each location. Variation in *A. curassavica* cardenolides between Guam and Rota was further assessed using comparisons of greenhouse-grown plants. Finally, we used pairwise comparisons of greenhouse-reared 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 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). Sequestration efficiency differed strongly based on 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 differed 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 nonpolar (Figure 2C). Across all milkweed species, the composition of cardenolides available in leaves was significantly different from the composition of sequestered cardenolides (Table Sxxx).

We found strong support for a GxE interaction in sequestration ability, with monarch populations varying substantially in their ability to sequester across milkweed species (χ2 = 77.6, d.f. = 15, p < 0.001) (Table Sxx). In particular, monarchs from Puerto Rico showed a strong tradeoff in sequestration ability across milkweed species: 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 3). Despite the strong GxE pattern in our data, there was no support for local adaptation in sequestration ability. In fact, on average, monarchs sequestered slightly less when reared on their sympatric hosts (Table Sxx).

Papers to cite:

Malcom and Brower (1989 – Experientia): assayed sequestration across 12 species. Find asymptotic relationship where sequestration plateuas in medium cardenolide plants. Sequestration most efficient from A. syriaca (4.68 ratio), followed by A. californica (3.55), A. cordifolia (2.49), and A. speciosa (1.99). Also discusses seasonal turnover and fingerprints overwintering Mexican monarchs, matching them to A. syriaca.

Roeske et al. (1976 – Book chapter): compared sequestration across A. curassavica and Gomphocarpus. Found greater sequestration efficiency on Asclepias, and greater fraction of nonpolar sequestration on Gomphocarpus. Reported sequestration ratio for Gomphocarpus is very low (about 0.2).

Malcolm (1994 – Chemoecology): good review of studies up until that point

Decker et al. (2018 – Functional Ecology): measured sequestration across 4 species. Amounts sequestered in our study were marginally higher. Noteworthy because we used the same genotypes of A. syriaca and also found lower-than-expected leaf concentrations (~0.2 mg/g dry mass).

Seiber et al. 1986 – cardenolide profiles of 394 monarchs sampled at overwintering sites, with 85% having asclepioside that is characteristic of A. syriaca and A. speciosa