Effects of Simulated Herbivory on Cardenolide Levels in *Asclepias syriaca* and Consequences for Butterfly Pollinators

Mary Wald

Final Undergraduate Research Grants for the Environment Report

On Site Supervisor:

Dr. Ann Fraser
Biology Department, Kalamazoo College
Kalamazoo, MI

October 2012

Abstract

Secondary metabolites, produced by plants in response to damage or injury, serve as feeding deterrents and toxins, thereby reducing attack by herbivores. They have also been reported in floral nectar, however, raising interesting question about their function in nectar. In milkweed plants (*Asclepias* spp.), cardenolides are key secondary defense compounds that protect plants against all but specialized milkweed feeders. Their presence in milkweed nectar, however, may also adversely affect pollinators. In this study we attempted to manipulate cardenolide production the common milkweed (*A. syriaca*) by applying jasmonic acid to leaves to simulate herbivory. We collected leaf and nectar samples from control and experimental plants and analyzed them for cardenolide content. We also tested the effect of cardenolides in nectar on butterfly pollinator behavior using artificial flowers containing nectar composed of sucrose solution with different concentrations of the cardenolide digoxin added. Cardenolides were detected in leaf and nectar samples of *A. syriaca*. Application of JA did not increase cardenolide concentrations in experimental plants above those in control plants. The addition of different concentrations of cardenolides to nectar did not alter visitation and feeding patterns by any of the three butterfly species we tested (*Papilio polyxenes, Colias philodice, Danaus plexippus*), regardless of whether their larvae were specialized to feed on milkweeds or not. We conclude that although cardenolides are present in nectar of *A. syriaca*, their influence on pollinators and milkweed pollination is still unclear.

Introduction

To enhance biological fitness, many flowering plants entice pollinators to visit and feed from them by offering nectar and pollen rewards. As a byproduct of foraging at flowers, pollinators transport pollen between plants of the same species, allowing plants to achieve pollination necessary for reproduction. In addition to attracting pollinators, however, plants must also defend themselves against herbivores that consume tissues and fluids of plant roots and shoots. Defenses come in a variety of forms that include mechanical defenses such as hairs, thorns and tough leaves, and chemical defenses whose compounds reduce the digestibility of plant tissue or are deterrent or toxic to herbivores (Bernays and Chapman 1994; Karban and Baldwin 1997). Damage caused by herbivore feeding often triggers increased production of plants' characteristic defense mechanisms, as process known as induction (Karban and Baldwin 1997).

Deterrent and toxic compounds are also known as plant secondary metabolites because they are not necessary for the primary growth and development of plants (Rosenthal and Berenbaum 1991). Secondary metabolites are found not only in the leaves of plants, but also in the roots, stems and flowers (McCall and Irwin 2006). The concentrations of secondary compounds found in plant tissues vary with plant genotype, tissue type and plant condition, but are typically highest in new shoots and reproductive structures (flowers and seeds) (Bernays and Chapman 1994). Constitutive toxin levels are low in undamaged plants, but increase significantly within hours or days following herbivore attack (Bernays and Chapman 1994). Thus, herbivory leads to an increase in, or induction of, plant defense compounds. This effect may be simulated by application of jasmonic acid (JA), a compound that is naturally produced in plant cells after herbivore attack or wounding and that acts as a signaling molecule leading to increased production of many plant defense compounds (Thaler 1999). Consequently, JA is commonly used by researchers to experimentally induce defenses in a controlled fashion.

Milkweed plants, members of the genus *Asclepias,* are good examples of plants that are chemically defended against insect herbivores. Latex fluids are exuded by the leaves when tissue is cut by an insect, and this inhibits further feeding by the insect (Malcolm and Brower 1989; Malcolm 1995; Agrawal and Fishbein 2006). Secondary defense compounds known as cardiac glycosides or cardenolides are also produced within plant tissues, are bitter tasting and presumably distasteful to herbivores, both vertebrates and invertebrates alike (Dussourd and

Hoyle 2000; Rosenthal and Berenbaum 1991). Only insects that have evolved specialized counterdefenses to avoid latex ingestion or detoxify ingested cardenolides are able to feed on these plants (Brower et al. 1972; Malcolm and Zalucki 1996). The monarch butterfly, *Danaus plexippus*, is one such milkweed specialist. Larvae feed on milkweed leaves and sequester cardenolides for their own defense against predators (Malcolm and Brower 1989). The process of herbivory induces the production of jasmonic acid in milkweeds and causes the plant to produce a variety of responses, including increased cardenolide production, to protect itself from further herbivore damage (Rasmann et al. 2009; Van Zandt and Agrawal 2004a,b). Adult female and male monarchs often visit milkweed plants to consume nectar (although they also feed from a wide range of nectar sources), and females actively seek out milkweeds on which to lay their eggs (Agrawal and Fishbein 2006).

Because secondary compounds are usually associated with deterring insects and other herbivorous species from feeding on plant tissue, reports of toxins in nectar (Adler 2000), a food resource offered to attract and reward pollinators, raises interesting questions about their function with respect to pollination. Their presence in nectar may simply be a "consequence of defense," in which toxins produced in plant tissue leak into the nectar, but their presence does not serve an adaptive function (Manson et al. 2012). Adaptive hypotheses also have been proposed. These include increased probability of pollination success because the presence of toxins shortens the duration but increases the frequency of pollinator visits, and decreased probability of nectar robbing because ineffective non-specialist pollinators are deterred from consuming the nectar (Adler 2000; Adler and Irwin 2005).

Not much is known about the effects that milkweed defenses, specifically cardenolides in nectar, have on adult monarch behavior. Female butterflies are known to oviposit preferentially on milkweed plants with intermediate levels of cardenolides (Zalucki et al. 1990) and it is possible that feeding adults might be similarly influenced by cardenolide levels when nectaring at milkweed flowers if they can detect cardenolides with taste receptors located on the proboscis or tarsi (Bernays and Chapman 1994). This would be advantageous if adults could then sequester ingested cardenolides into their tissues for enhanced protection against predators, as occurs when larvae feed on leaf tissue (Brower et al. 1972).

The common milkweed, *Asclepias syriaca*, is an abundant milkweed species in the eastern United States. It grows in open fields and plants reproduce asexually by underground

runner, as well as sexually via seed production. Plants produce clusters of small pink flowers arranged in heads called umbels, with each flower containing five nectaries (Willson and Rathcke 1974). In the northern United States, *A. syriaca* flowers from June through mid-July and flowers produce measureable amounts of nectar (Southwick 1983; Southwick and Southwick 1983). The nectar serves as an important nutrient source for a wide range of insects, including bees, flies and butterflies (Southwick 1983; Agrawal 2005), although not all are equally effective as pollinators. (Wyatt and Broyles 1994; Kephart and Theiss 2004). Manson et al. (2012) recently reported cardenolides from nectar of a range of milkweed species but were unable to determine conclusively whether cardenolides served an adaptive function in nectar. *Asclepias syriaca* has low constitutive levels of cardenolides in its leaves (Malcolm and Brower 1989), but this species was not included in the analysis of nectar by Manson et al. (2012).

In this study, we sought to determine the effects of herbivory on cardenolide levels in leaves and nectar of the common milkweed, *Asclepias syriaca,* and determine whether cardenolides in nectar influence butterfly pollinator behavior. We hypothesized that plants treated with jasmonic acid, to simulate herbivory, would have higher levels of cardenolides in both leaves and nectar when compared to non-induced plants. With respect to pollinator behavior, we compared feeding behavior of monarch butterflies to that of two non-milkweed specialist butterfly species (one sulfur, family Pieridae, and one swallowtail, family Papilionidae) offered artificial flowers containing nectar with varying levels of the cardenolide digoxin. We predicted that monarch adults would make more visits and feed for longer at flowers containing cardenolides in nectar than would the non-specialist species. We also predicted that monarchs would prefer to feed from flowers with intermediate levels of cardenolides in the nectar over those with very high or very low cardenolides levels, similar to what has been documented for egg-laying behavior (Zalucki et al. 1990).

Materials and Methods

Study site

Fieldwork for this study was conducted from June through August, 2012 at the Pierce Cedar Creek Institute in Barry County, MI. Common milkweed (*Asclepias syriaca*) plants used in this study grew naturally in an open field east of the Education Building. Laboratory chemical

analysis of leaf and nectar samples collected from milkweeds were conducted by fellow undergraduate Conrad Liu at Kalamazoo College, Kalamazoo County, MI.

Plant manipulations

In early June, as some milkweed plants began to form buds, we selected twelve pairs of plants that did not appear to have been fed on by herbivores. Plants in each pair were separated by less than two meters, and were of similar size, developmental stage and condition. We enclosed each plant in a metal tomato hoop cage, and then covered the cage with a fine mesh bag to prevent herbivores from colonizing the plants. All plants were located within a 100 m radius. The appearance of each plant, such as number of leaf pairs and the presence of buds or flowers, was noted. One plant in each pair was assigned to a control treatment and the other to an experimental treatment for simulated herbivory manipulations. Additional milkweed plants that appeared to be free from herbivory were selected and labeled for use as needed as the summer progressed, but these plants were not enclosed in cages because herbivory at the site appeared to be low. As flowers neared opening, flower umbels of exposed plants were enclosed in fine mesh bags to prevent floral visitors from removing nectar before we could collect it.

As plants neared bud burst, plants were sprayed once daily for seven days with either a control solution containing water and acetone (12.5 µl acetone per 100 µl water), or a 0.5 mM solution of jasmonic acid (Sigma Aldrich, St. Louis, MI) dissolved in acetone and water (10.5 µl of JA dissolved in 12.5 µl of acetone, then added to 100 ml of distilled water). Control and JA solutions were applied in a fine mist using a spray bottle to the top two pairs of fully opened leaves until these leaves were shiny and wet, and the solution began to run off the leaves. In addition to spraying, control plants were handled to the same extent as experimental plants to control for any effects that touching or handling may have had on the plants.

Sample Collection and Preparation

After the final day of the spray application, nectar and leaf samples were collected for chemical analysis. Nectar, when available, was always collected prior to removing leaves. In some cases only leaves were collected because the plants did not produce measureable quantities of nectar. Nectar was obtained from 3 control and 3 experimental plants but none of these were

from members of a pair. Leaves were collected from 8 control and 6 experimental plants, of which four from each treatment came from paired plants.

Nectar samples were collected using either 1 or 5 µl glass micro-pipettes (Drummond Scientific Co., Broomal, PA) by inserting the micro-pipette into the nectaries, being careful not to damage the flowers in the process. An aspirator tube attached to the micro-pipette was used if necessary to recover as much nectar as possible. Most nectar collections were performed at 0800 h, a peak nectar production time (Southwick 1983), but attempts were made at other times of day as time allowed. The amount of nectar collected per flower varied widely among plants but was generally 1 µl or less per flower. Nectar from multiple flowers of the same plant was pooled into a single plant sample to obtain approximately 20 µl for cardenolide analysis. For each micro-pipette, nectar volume (in µl) was determined by measuring the length (in mm) of micro-pipette occupied by nectar, multiplying this by total volume of the micro-pipette (1 or 5 µl) and dividing this by total micro-pipette length (32 mm). Total nectar volume collected per plant was calculated by summing nectar volumes across the multiple micro-pipettes filled from a given plant. Samples were stored in sealed glass vials at -80 °C for later chemical analysis. We prepared nectar and leaf samples using extraction procedures modified slightly from the protocol outline in Manson et al. (2012).

To prepare nectar samples for cardenolide analysis, we first soaked the nectar-containing micro-pipettes in a vial containing 500 µL of 95% ethanol (Sigma-Aldrich). Eluted samples were then dried down in a rotary evaporator. We added 1 mL of HPLC-grade methanol (Sigma-Aldrich) to the residuum and spiked the sample with 10 µL of a 200 ng/µL solution of digitoxin (92% purity, Sigma-Aldrich) as an internal standard. We then filtered each sample through a 0.22 µm-sized syringe filter into a 1.5 mL centrifuge tube and stored capped tubes in a -20 °C freezer for later analysis by HPLC.

Leaf samples were collected by removing the top two leaves from the plants at the stem. These leaves were placed in paper envelopes, transported in a chilled cooler to Kalamazoo College where they were placed in a -80 °C freezer for 1 day to kill leaves and cease plant metabolism. Freeze-killed leaves were then dried in an oven at 50 °C for three days before grinding them to a fine powder using a mortar and pestle. Leaf powder was stored in sealed glass vials for later chemical analysis.

To extract cardenolides from leaf samples, we weighed out 50 mg of dry leaf material per sample and added 1.5 mL of HPLC grade methanol (Sigma-Aldrich) to this. After spiking samples with 50 µL of a 200 ng/µL solution of the cardenolide digitoxin as an internal standard, we sonicated samples in a 55^{o}C water bath for 50 minutes before centrifuging them for five minutes at 12000 rpm. We removed the supernatant and used the rotary evaporator to dry down each sample and then resuspended the residuum in 1 mL of methanol. We then filtered each sample through a 0.22 µm-sized syringe filter into a 1.5 mL centrifuge tube and stored capped tubes in a -20 °C freezer for later analysis by HPLC.

Cardenolide Analysis by HPLC

We analyzed leaf and nectar samples for cardenolides by HPLC using a protocol adapted from Wiegrebe and Wichtl (1993). We injected 20 µL of each processed sample into a Waters 2487 Dual λ Absorbance Detector instrument that was equipped with a Nova-Pak reversed-phase 4-µm particle size C18 column (3.9 x 150mm). The solvent gradient was as follows (solvent A: milliQwater; solvent B: acetonitrile): initial = 20% acetonitrile (B), 35 min = 32% B, 45 min = 40% B, 55 min = 50% B, 59 min = 55% B, 61 min = 20% B. These changes occurred linearly at a flow rate of 0.5 mL/min. Following previously established protocols (Wiegrebe and Wichtl 1993; Manson et al. 2012) we set the HPLC to detect compounds at 225 and 218 nm because it has been shown that cardenolides can be identified from other compounds by their symmetrical peak shape at these wavelengths.

For each symmetrical peak contained within a chromatogram, including the internal standard, we used the system software to integrate area under the peak. Because our instrument was not equipped with an autosampler, we did not produce a standard curve for the internal standard on a routine basis. Therefore, we quantified cardenolide content by first dividing individual sample peak areas by area under the digitoxin (internal standard) peak for that sample, then by digitoxin dose contained in the sample (10 µg for leaf samples, 2 µg for nectar samples), to get area per µg digitoxin. This normalized areas and helped control for variability in manual injection volume among samples and day-to-day performance of the machine. We divided this normalized area by the amount of dry leaf mass or nectar volume processed in a sample to yield cardenolide content/mg or µL of sample, with cardenolide content measured as area in detector

absorbance units per μg digitoxin. Total cardenolide content for a given sample was then calculated by summing corrected cardenolide content for all detected peaks. We used an unpaired t-test assuming equal variances to compare mean total cardenolide content between control and JA treated plants, separately for leaf and nectar samples.

Pollinator Behavior toward Nectar Containing Cardenolides

To determine whether cardenolides in nectar influenced pollinator feeding behavior, we quantified responses of three butterfly species to artificial nectar solutions containing different amounts of the cardenolide digoxin (Sigma Aldrich). Three butterfly species were collected. These included the monarch butterfly, *Danaus plexippus* (Nymphalidae), whose larvae are specialist feeders on milkweed foliage, the black swallowtail, *Papilio polyxenes* (Papilionidae) and the clouded sulphur, *Colias philodice* (Pieridae). The latter two species were chosen because of their abundance at the time the study was conducted, and because larvae of these species do not feed on milkweeds (family Asclepiadaceae) but are specialist feeders on other plant families (Apiaceae and Brassicaceae, respectively). Adults of all three species have been observed nectaring at milkweed flowers, however (M. Wald, personal observation). Butterflies were obtained through netting in fields and forests around the PCCI property and Grass Lake, Jackson County, MI. Following capture, butterflies were held indoors in an insect cage (temperature ca. 22 °C, florescent lighting during daylight hours) until used in experimental trials. All specimens were handled with bare hands and butterfly age was not known. A container of water stuffed with cloth was placed in the holding cage to increase humidity within the cage and to provide a source of water from which butterflies could drink. The cage was also misted with water at least once per day.

Behavioral trials were conducted in an outdoor net enclosure (42 x 42 x 76 cm) using artificial flowers equipped with nectaries. Artificial flowers were constructed based on details outlined in Rodrigues et al. (2010). Each flower contained one nectary constructed using a 10 μL plastic pipette tip (0.5 cm in diameter across top and 3 cm deep) that was flame sealed at the tip and inserted through a hole made in the plastic lid of a 50 ml collecting vial. Prior to inserting the pipette tip, the lid was covered by a 4 cm circle of magenta-colored origami paper (Aitoh Co., San Francisco, CA) that was folded crosswise several times to provide butterflies with tactile guides toward the nectary and thereby facilitate nectar feeding. Preliminary research

showed that this color of paper was most attractive to the butterflies. Although our artificial flowers were considerably larger, and nectaries significantly deeper, than those of individual milkweed flowers, the purpose of this experiment was to test butterfly feeding preferences on flowers containing different levels of cardenolides, not on the suitability of these flowers to mimic milkweed flowers. Because the butterfly species used in this study have been observed feeding from *Phlox* species that closely resemble our artificial flowers (A. Fraser, personal communication) we believe that results obtained from this study are applicable to natural systems.

The nectary of each flower was filled to within 1 mm of the top with one of four solutions: a 30% w/w sucrose in water solution, or a 30% sucrose solution containing digoxin at a final concentration of 100, 250 or 1000 ng/µL. Digoxin and the concentrations used here were chosen to mirror a feeding preference study by Manson et al. (2012) conducted with bumblebees. Four artificial flowers, one for each cardenolide concentration treatment, were placed in a haphazard pattern on the floor of the enclosure to control for position effects on behavior.

Behavioral trials were conducted under sunny or partly sunny skies in both morning and afternoon. After a butterfly was released into the testing enclosure, it was observed until it interacted in some way with one of the flowers. This was deemed the start of a behavioral trial. Its actions, including the flower treatments visited and amount of time spent with each, as well as its behavior while on the flowers, were recorded using the program JWatcher. Behaviors coded in JWatcher included *landing* on flowers, *probing* flowers with the proboscis, and *feeding* from the nectary through the proboscis. Each butterfly was given 5 minutes to interact with the different flower treatments once a trial began. At the end of the trial a mark was made on the wing of the butterfly with a felt tip marker to avoid using it in subsequent trials.

If a butterfly did not interact with the flowers within twenty minutes of introduction, it was removed from the enclosure and kept for a later trial. If a butterfly began, but did not complete, a behavior (such as landing on a flower but performing no further behaviors the entire trial period) the behavior recorded was considered unusable. After three behavioral trials, the nectaries were replaced with new ones, freshly filled. This kept the sucrose solution from becoming too viscous, which might have deterred feeding.

We used chi-square tests of independence to determine whether behavior of each butterfly species was influenced by cardenolide concentration in the nectar. Data were analyzed

in a 4 x 3 matrix with cardenolide concentration and behavior as the two variables, respectively, and number of individuals responding as the dependent variable. Because the number of individuals successfully tested per species differed, we plotted results as the proportion of responders per treatment and behavior so that we could qualitatively compare behavioral patterns among butterfly species.

Results

Effects of Tissue Type and Simulated Herbivory on Cardenolide Content

A total of seven symmetrical peaks, presumed to be cardenolides, were detected in both leaf and nectar samples. Mean cardenolide content was not significantly different between control and JA treated plants for either leaves ($t_{12} = 0.18$, $p = 0.86$; Fig. 1a) or nectar ($t_4 = 0.23$, $p = 0.83$; Fig. 1b).

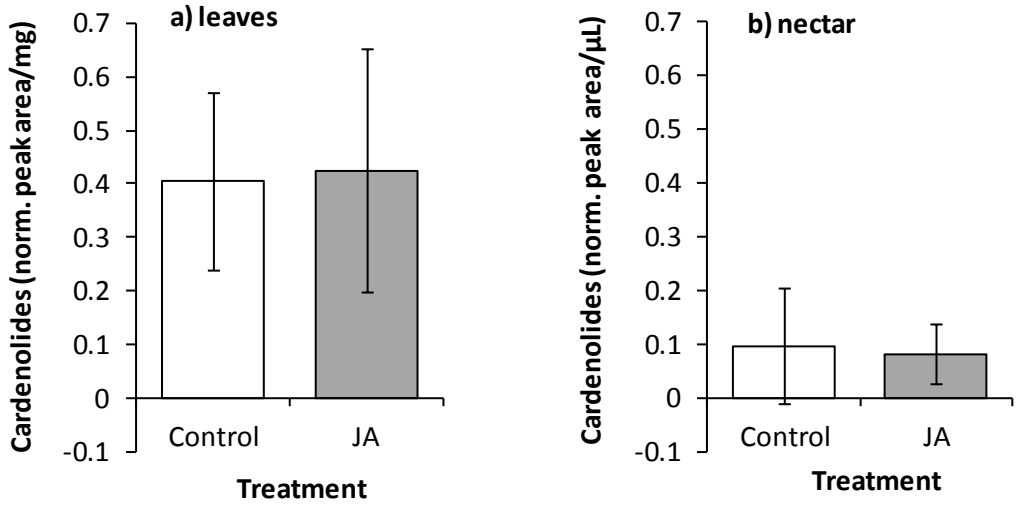

Figure 1. Mean (± 1 SD) total cardenolides detected in a) leaf tissue and b) nectar from milkweed *(Asclepias syriaca)* plants treated with control solution or with jasmonic acid to simulate herbivory. Cardenolide content is reported in detector absorbance units, normalized to peak area/µg digitoxin (internal standard) added to samples per mg dry weight of leaf tissue or per µL nectar. Sample sizes: leaves, n=8 control, 6 JA plants; nectar, n=3 control, 3 JA plants.

Effect of Cardenolides on Pollinator Behavior

Among all butterflies introduced into the outdoor testing enclosure, only half of the individuals from each species visited and interacted with the artificial flowers. A total of 7 monarchs, 6 black swallowtails and 4 clouded sulphurs interacted with artificial flowers, and at least some individuals of all species exhibited landing, probing or behavior (Fig. 2). Within each species, the number of individuals that engaged in each of three behaviors (land, probe, feed) was independent of digoxin concentration in nectar (monarch: $\chi^2 = 1.735$, p= 0.942; sulphur: $\chi^2 = 0.569$, p= 0.997; swallowtail: $\chi^2 = 3.145$, p= 0.790; Fig. 2). In other words, butterflies were not more likely to land, probe or feed on flowers containing any particular nectar cardenolide concentration.

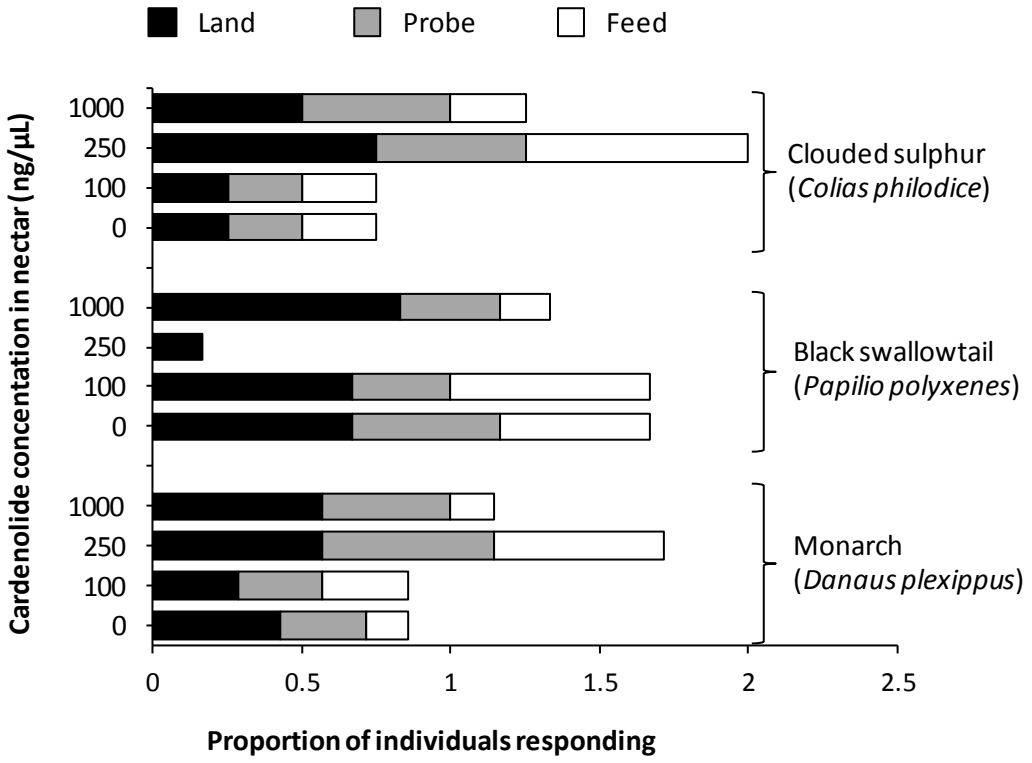

Figure 2. Comparison of behaviors exhibited by three butterfly species in response to artificial flowers offering nectar containing 30% sucrose and different levels of the cardenolide digoxin. Data were analyzed using number of individuals per species that responded, but are plotted as proportion of individuals responding here to allow for visual comparisons among species with unequal samples sizes (n = 7 monarchs, 6 swallowtails and 4 sulphurs).

Qualitatively, there were no obvious differences among butterfly species in their behavior toward the different nectar treatments. The only anomaly in pattern was that of black swallowtails, which were observed landing, but not feeding or probing, on the 250 ng/µL treatment. However, their behavior at the 1000 ng/µL treatment was similar to that of the other two species (Fig. 2).

Discussion

Cardenolides were present in both leaf and nectar samples of *A. syriaca*. Contrary to our prediction regarding effects of simulated herbivory on cardenolide levels, application of JA did not increase cardenolide concentrations in experimental plants above those in control plants. Contrary also to our prediction regarding the effects of nectar cardenolides on pollinator behavior, the addition of different concentrations of cardenolides to nectar did not alter behavioral patterns of any of the butterfly species we tested, regardless of whether their larvae were specialized to feed on milkweeds or not. Therefore, although we can conclude that cardenolides are present in nectar of *A. syriaca*, it remains to be determined whether cardenolide levels increase with herbivory and whether they serve an adaptive function to enhance pollination of milkweed plants.

Because leaves are solid tissue whereas nectar is a liquid, we could not compare cardenolide concentrations of leaves and nectar directly. A rough approximation of relative levels is possible, however, if we use the density of water (1 mg/µL) to represent that of nectar, and estimate that 30% of the weight of nectar is comprised of sucrose and other solutes, then the dry weight of a 1 µL sample of nectar would be approximately 0.3 mg. Cardenolide content of nectar averaged around 0.1 area units per µL nectar, which would equate to 0.3 area units per mg dry weight of nectar. Given that cardenolide content of leaves averaged around 0.4 area units per mg dry leaf tissue, then cardenolide content of nectar was only 1.3 times lower than that of leaf tissue. This is considerably higher than the 35-fold lower concentration of cardenolides in nectar compared to leaves reported by Manson et al. (2012) for their milkweed species. Their estimates were based on fresh, wet weights whereas ours are based on dry weights, which may account for at least some of the discrepancy. Furthermore, constitutive cardenolide levels in *A. syriaca* leaves are low compared with other *Asclepias* species (Malcolm and Zalucki 1996), so the possibility remains that nectar cardenolide levels in this species are high relative to leaves, especially if they serve some adaptive function.

We detected seven putative cardenolide peaks in our samples, and found all peaks in both nectar and leaf tissue. Without mass spectrometry or authentic standards, we cannot assign compound identities to our peaks, but future studies should try to do so. This result appears in line with data reported from Manson et al. (2012) in which they identified up to 11 cardenolide compounds from nectar, and up to 16 cardenolide compounds from leaves of a single *Asclepias* species. They also found a positive correlation between the number of cardenolide compounds in nectar and leaves, with nectar containing fewer, but in some cases unique, cardenolides compared to leaves.

Our simulation of herbivory through application of JA to plant leaves did not appear to increase cardenolide production. There are several possible explanations for why we did not see an induced defense response. The milkweed plants used this study endured a very low-resource environment due to the unusually hot, dry summer of 2012. Water stress was shown to result in an 86% reduction in leaf water potential, and a 20-30% decrease in cardenolide concentration in *Digitalis lanata* (Stuhlfauth et al. 1987). Our study plants grew naturally in an open field and drought-stress was evident throughout the region. We watered some plants for several days as flowers began to open, in an attempt to increase nectar production, but even with this scant watering it is quite likely that drought-induced stress adversely affected cardenolide production in our milkweed plants. Alternatively, both control and experimental plants used in this study may have been induced when JA was applied to experimental plant. This is because *Asclepias syriaca* reproduces asexually by underground stems, forming patches of clonal stems called ramets. This clonal growth habit, coupled with the fact that JA application in one location on a plant leads to systemic induction in more distant plant parts (Karban and Baldwin 1997; Thaler 1999), may lead to induction in attached ramets. The milkweed plants we sampled were in relatively close together and may well have been clones of the same plant. Consequently, if our application of JA led to an increase in cardenolide production in experimental plants, then it likely affected conjoined ramets, including our control plants. Martel and Malcolm (2004) documented such induction in two other milkweed species, although induction in unattacked ramets was lower than in the ramet being fed on by aphids. In future, we could avoid unintended induction by using potted plants. Thirdly, it is possible that jasmonic acid treatment was not effective at inducing plant defenses. Rasmann et al. (2009) found that jasmonic acid sprayed onto leaves at 0.5 mM, the same concentration and method used in our study, only partially

mimicked the induced response observed in three other milkweed species fed on by monarch caterpillars. We originally intended to use caterpillars as our induction agent but were unable to do so because an unusually warm spring, followed by a summer drought, resulted in very few monarchs at our study site. Ideally, future induction studies should be done using herbivores. If a chemical elicitor is used, its effectiveness as an inducer may be improved by mechanically damaging leaves prior to JA application, applying a higher concentration of JA, or using methyl jasmonate as the elicitor instead of JA (Baldwin 1996).

All three butterfly species we tested exhibited similar behavioral activity toward nectar without cardenolides and with cardenolides at three different concentrations. Therefore, we conclude that none of these species are deterred from probing or feeding from nectar containing cardenolides at the concentrations we tested (0, 100, 250 and 1000 ng/μL). Manson et al. (2012) conducted a similar study using bumblebees and offered them the same concentrations of digoxin in artificial flowers. Although nectar cardenolides appeared to deter some bumblebees from feeding, no consistent pattern was observed. The lack of a clear deterrent effect is somewhat surprising, given that the concentrations used in both of our studies were 10 to 100-fold higher than those reported from milkweed nectar (Manson et al. 2012). Assays that look at feeding preference over a short time period are essentially tests of innate taste preference, however, and as Manson et al. (2012) note, it may be that aversion does not develop until insects have digested some the nectar and experienced ill effects. Future studies should therefore test pollinators on successive days to determine whether they must first learn to associate certain tastes with adverse physiological effects, and then avoid nectar with this taste.

We did notice, however, that when any of our butterfly species, either milkweed specialist or non-specialist, initially landed on the flower containing the highest level of toxins, they spent most of their time sitting or probing the flower and not much, if any, time feeding. Once they had tasted that nectar, they flew to a corner of the enclosure and did not return to any of the flowers for the remainder of the testing period. This suggests that encounters with high concentrations of cardenolides may deter further feeding on any of the flowers, and that there may be some innate taste aversion after all. Future tests should employ larger numbers of butterflies to help discern behavioral signal above the normal variation among individuals. This could require considerable time investment as we found behavioral tests with butterflies to be very time consuming, especially because only about half actually visited and interacted with

artificial flowers. Future studies could therefore try using actual milkweed plants and their flowers for behavioral tests as butterflies may be more likely to feed from these than from artificial flowers. This was our original intent but because of a hot, dry summer, the naturally growing milkweed plants were unavailable for behavioral testing. Finally, behavioral testing could be done to compare how butterflies respond to nectar cardenolides in intact flowers of uninduced, herbivore-induced and JA-induced plants. These tests should be accompanied by HPLC analysis of nectar cardenolides to correlate behavior responses with nectar chemistry.

In summary, evidence to date (Manson et al. 2012; this study) demonstrates that cardenolides are present in milkweed nectar, but there is no strong evidence that nectar cardenolides deter or otherwise affect pollinator behavior. Therefore, even if cardenolides levels in milkweed nectar increase in response to herbivory, as is known to occur in leaves, this may not reduce the frequency or duration of pollinator visits, and therefore should not impact pollination, either adversely or positively. Furthermore, milkweed herbivory may not affect physiological processes of pollinators, and therefore may not impact pollinator fitness. This may also explain why milkweed flowers are visited and fed upon by a wide range of insects (Southwick 1983; Wyatt and Broyles 1994).

Acknowledgements

I would like to thank Pierce Cedar Creek Institute in Barry County, MI. for funding this research and also for providing such amazing opportunities for research. I would also like to thank Dr. Hugh Brown and other staff at Pierce Cedar Creek Institute for all of their time, help and advice for me while I was working on this project. Thanks to Kalamazoo College, for providing me with the opportunity to participate in PCCI's undergraduate program and preparing me for my research work, and to my research mentor, Dr. Ann Fraser for all her time and assistance. I would also like to acknowledge my colleagues from Kalamazoo College, Nick Smith and Conrad Liu who were instrumental in helping me set up my project, and who offered so much helpful advice. Finally, I'd like to thank Dr. Steve Malcolm for all of his advice and for his knowledge of my research topic, and for letting me use his greenhouse plants whenever I needed them.

Literature Cited

Adler, L. S. (2000). The ecological significance of toxic nectar. *Oikos* 91: 409-420.

Adler, L.S., and R.E. Irwin. 2005. Ecological costs and benefits of defenses in nectar. *Ecology* 86: 2968-2978.

Agrawal, A. A. (2005). Natural selection on common milkweed (*Asclepias syriaca*) by a community of specialized insect herbivores. *Evolutionary Ecology Research* 7: 651-667.

Agrawal, A. A., and Fishbein, M. (2006). Plant defense syndromes. *Ecology* 87: S132-S149.

Baldwin, I.T. (1996) Methyl jasmonate-induced nicotine production in *Nicotiana attenuata*: inducing defenses in the field without wounding. *Entomologia Experimentalis et Applicata* 80: 213–220.

Bernays, E.A., and R.F. Chapman. 1994. *Host-Plant Selection by Phytophagous Insects*. Chapman & Hall, New York.

Brower L.P., McEvoy, P.B., Williamson, K.L., and Flannery, M.A. (1972). Variation in cardiac glycoside content of monarch butterflies from natural populations in eastern North America. *Science* 177: 426–429.

Dussourd, D. E., and Hoyle, A. M. (2000). Poisoned plusiines: toxicity of milkweed latex and cardenolides to some generalist caterpillars. *Chemoecology* 10: 11-16.

Karban, R., and Baldwin, I. T. (1997). Induced responses to herbivory. The University of Chicago Press, Chicago.

Kephart, S., and Theiss, K. (2004). Pollinator-mediated isolation in sympatric milkweeds (*Asclepias*): do floral morphology and insect behavior influence species boundaries? *New Phytologist* 161: 265-277.

Malcolm, S. B. (1995). Milkweeds, monarch butterflies, and the ecological significance of cardenolides. *Chemoecology* 5-6: 101-117.

Malcolm, S. B., and Brower, L. E. (1989). Evolutionary and ecological implications of cardenolide sequestration in the monarch butterfly. *Experientia* 45: 284-295.

Malcolm, S.B., and Zalucki, M. P. (1996). Milkweed latex and cardenolide induction may resolve the lethal plant defence paradox. *Entomologia Experimentalis et Applicata* 80: 193-196.

Manson, J. S., Rasmann, S., Halitschke, R., Thomson, J. D., and Agrawal, A. A. (2012). Cardenolides in nectar may be more than a consequence of allocation to other plant parts: a phylogenetic study of *Asclepias. Functional Ecology* 26: 1100-1110.

Martel, J.W., and Malcolm, S.B. (2004). Density-dependent reduction and induction of milkweed cardenolides by a sucking insect herbivore. *Journal of Chemical Ecology* 30: 545-561.

McCall, A. C., and Irwin, R. E. (2006). Florivory: the intersection of pollination and herbivory. *Ecology Letters* 9: 1351-1365.

Rasmann, S., Johnson, M. D., and Agrawal, A. A. (2009). Induced responses to herbivory and jasmonate in three milkweed species. *Journal of Chemical Ecology* 35: 1326-1334.

Rodrigues D., Goodner, B. W., and Weiss M. R. (2010). Reversal learning and risk-averse foraging behavior in the monarch butterfly, *Danaus plexippus* (Lepidoptera: Nymphalidae). *Ethology*. 116-3: 270-280.

Rosenthal, G. A., and Berenbaum, M. R., (eds.). (1991). Herbivores: Their interactions with secondary plant metabolites. Academic Press, New York, New York, USA.

Southwick, A. K., and Southwick, E. E. (1983). Aging effect on nectar production in two clones of *Asclepias syriaca*. *Oecologia* 56: 121-125.

Southwick, E. E. (1983). Nectar biology and nectar feeders of common milkweed, *Asclepias syriaca* L. *Bulletin of the Torrey Botanical Club* 110: 324-334.

Stuhlfauth, T., Klug, K., and Fock, H. P. (1987). The production of secondary metabolites by *Digitalis lanata* during CO2 enrichment and water stress. *Phytochemistry* 26: 2735-2739.

Thaler, J.S. (1999). Jasmonic acid mediated interactions between plants, herbivores, parasitoids, and pathogens: a review of field experiments in tomato. In: A.A. Agrawal, S. Tuzun and E. Bent (eds.) *Inducible Plant Defenses against Pathogens and Herbivores: Biochemistry, Ecology, and Agriculture*. A Press, St. Paul, MN. pp. 319-334.

Van Zandt, P. A., and Agrawal, A. A. (2004a). Community-wide impacts of herbivore-induced plant responses in milkweed (*Asclepias syriaca). Ecology* 85: 2616-2629.

Van Zandt P. A., and Agrawal, A. A. (2004b). Specificity of induced plant responses to specialist herbivores of the common milkweed, *Asclepias syriaca. Oikos* 104: 401-409.

Wiegrebe, H., and Wichtl, M. (1993). High-performance liquid chromatographic determination of cardenolides in Digitalis leaves after solid-phase extraction. *Journal of Chromatography* 630:402-407.

Willson, M. F., and Rathcke, B. J. (1974). Adaptive design of the floral display in *Asclepias syriaca* L. *American Midland Naturalist* 92: 47-57.

Wyatt, R., and Broyles, S. B. (1994). Ecology and evolution of reproduction in milkweeds. *Annual Review of Ecology and Systematics* 25: 423-441.

Zalucki, M. P., Brower L. P. and Malcolm, S. (1990). Oviposition by *Danaus plexippus* in relation to cardenolide content of 3 *Asclepias* species in the Southeastern USA. *Ecological Entomology.* 15: 231-240.