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# Consequences of toxic secondary compounds in nectar for mutualist bees and antagonist butterflies

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**Abstract.** Attraction of mutualists and defense against antagonists are critical challenges for most organisms and can be especially acute for plants with pollinating and non-pollinating flower visitors. Secondary compounds in flowers have been hypothesized to adaptively mediate attraction of mutualists and defense against antagonists, but this hypothesis has rarely been tested. The tissues of milkweeds (*Asclepias* spp.) contain toxic cardenolides that have long been studied as chemical defenses against herbivores. Milkweed nectar also contains cardenolides, and we have examined the impact of manipulating cardenolides in nectar on the foraging choices of two flower visitors: generalist bumble bees, *Bombus impatiens*, which are mutualistic pollinators, and specialist monarch butterflies, *Danaus plexippus*, which are herbivores as larvae and ineffective pollinators as adults. Although individual bumble bees in single foraging bouts showed no avoidance of cardenolides at the highest natural concentrations reported for milkweeds, a pattern of deterrence did arise when entire colonies were allowed to forage for several days. Monarch butterflies were not deterred by the presence of cardenolides in nectar when foraging from flowers, but laid fewer eggs on plants paired with cardenolide-laced flowers compared to controls. Thus, although deterrence of bumble bees by cardenolides may only occur after extensive foraging, a primary effect of nectar cardenolides appears to be reduction of monarch butterfly oviposition.

**Key words:** cardiac glycoside; egg laying; flower constancy; learning; nectar foraging; plant–insect interactions; toxic nectar.

## INTRODUCTION

Flower nectar is generally considered to function as a reward to attract and retain pollinators. However, secondary metabolites with toxic effects have been found in the floral nectar of a broad range of plant species (Baker 1977). The presence of these compounds in a food reward is counterintuitive. Some empirical research has shown reduced pollinator visitation to flowers containing these secondary metabolites (Adler and Irwin 2005, Gegeer et al. 2007), which could impose fitness costs for the plant. In many cases, the compounds present in nectar are the same ones that function to defend leaves against herbivores, and their presence in nectar may be a pleiotropic effect, or consequence of circulation between tissues (Adler et al. 2006, Kessler and Halitschke 2009).

In contrast, a number of studies have proposed that rather than simply byproducts of leaf defense, secondary compounds may have benefits in nectar (Janzen 1977, Rhoades and Bergdahl 1981, Adler 2000, Manson et al. 2012, Irwin et al. 2014). Proposed hypotheses suggest that nectar compounds may (1) “filter” flower visitors, encouraging more effective pollinators over less effective ones, (2) manipulate pollinator behavior to increase

visitation rates (or decrease selfing), (3) deter nectar robbers, and (4) act as anti-microbial nectar preservatives (Adler 2000). While in many cases natural concentrations of secondary metabolites in nectar appear to have no effects on pollinators (Tiedeken et al. 2014), there is some empirical data to support each of these hypotheses. Secondary metabolites have been shown to filter flower visitors, deterring inferior pollinators and nectar thieves (Stephenson 1981, Johnson et al. 2006), to increase the number of pollinator visits to flowers by enforcing modest nectar consumption (Kessler and Baldwin 2006), to increase pollinator memory for flower odors (Wright et al. 2013), to deter nectar-robbing ants (Adler and Irwin 2005), and to have anti-microbial properties (Lokvam and Braddock 1999, González-Teuber et al. 2009). Additionally, some nectar secondary metabolites appear to have medicinal benefits for pollinators, such as reducing parasite loads in bumble bees (Manson et al. 2010, Baracchi et al. 2015, Richardson et al. 2015). The current literature therefore does not support a single adaptive explanation for the presence of secondary compounds in nectar, but instead suggests that such compounds may have different effects in different plant species and ecological communities.

In this study, we examined the effects of nectar secondary metabolites on pollinators and herbivores of milkweed plants. Milkweed plants in the genus *Asclepias*

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contain cardenolides, or cardiac glycosides, toxic steroids that bind to and inhibit the function of animal sodium–potassium ATPases. Cardenolides in leaves reduce herbivore damage by a wide range of insects (Agrawal et al. 2012), and are also present in nectar. Milkweed plants have been shown to have mixtures of cardenolides in nectar that range from none in *Asclepias texana* to >100 ng/ $\mu$ L in the closely related *A. pumila* (Manson et al. 2012). Although there is a positive correlation between leaf and nectar concentrations, there are also both qualitative and quantitative differences in the cardenolides present in nectar and leaves of milkweeds. An average of 68% of the cardenolide compounds present in a milkweed species' nectar are also present in its leaves. This suggests that the presence of cardenolides in nectar is not simply a byproduct of circulating levels for leaf defense, but could have benefits for the plant (Manson et al. 2012).

Large Hymenoptera such as bumble bees are the most common and effective pollinators of milkweeds, while, in contrast, lepidopterans such as monarch butterflies visit milkweed flowers less frequently and transfer pollinia less effectively (Jennersten and Morse 1991, Betz et al. 1994, Fishbein and Venable 1996, Kephart and Theiss 2004, Howard and Barrows 2014). Nectar chemistry that affects bumble bee foraging preferences or behavior is therefore likely to have important consequences for milkweed outcrossing. As ineffective pollinators, monarch butterflies are functionally “nectar thieves” from the plant's perspective, and any mechanism by which monarchs could be deterred from nectar feeding might be to the plant's advantage. Not only are monarchs ineffective pollinators, they are also detrimental herbivores on milkweed leaves. Monarchs are specialized on milkweeds, having evolved  $\text{Na}^+/\text{K}^+$  ATPases that are largely resistant to the toxic effects of cardenolides (Holzinger et al. 1992, Dobler et al. 2012), and allow them to sequester cardenolides for their own defense from birds (Brower et al. 1968) and likely also parasites (Lefèvre et al. 2012). Cardenolides can still be toxic to monarchs at high concentrations (Zalucki et al. 2001), and females therefore preferentially oviposit on milkweed plants with intermediate levels of cardenolides (Oyeyele and Zalucki 1990, Van Hook and Zalucki 1991). In other plant–visitor systems, nectar foraging has been proposed as a mechanism by which female herbivores make oviposition decisions (Adler et al. 2006, Sharp et al. 2009, Kessler 2012). Oviposition decisions may therefore be an additional agent of selection on nectar chemistry, potentially with very different effects from selection imposed by pollinators. Accordingly, in this study, we not only examined the effects of nectar chemistry on nectar foraging, but also on oviposition.

From the plant's perspective, the most beneficial scenario would be one in which nectar chemistry filters flower visitors by not deterring pollinating bumble bees, but deterring non-pollinating monarchs both from drinking nectar and ovipositing. Nonetheless, generalist bumble bees are expected to be deterred by the toxic effects of cardenolides, but monarch butterflies are not

expected to be deterred due to their specialized  $\text{Na}^+/\text{K}^+$  ATPases. To test these conflicting hypotheses, we assessed how bumble bees, important milkweed pollinators and generalist flower visitors, are influenced by the presence of cardenolides in nectar at the individual and colony level, and how monarch butterflies, poor pollinators and specialized herbivores, are affected by the presence of cardenolides in nectar when making nectar-foraging and oviposition decisions.

## METHODS

### *Nectar stimuli*

Milkweed nectar contains a mixture of cardenolides that range in polarity (Manson et al. 2012). To mimic these natural stimuli we used two commercially available cardenolides: ouabain, which is a highly polar cardenolide, and digitoxin, which is non-polar. Although these two cardenolides are not naturally found in *Asclepias* nectar (naturally occurring milkweed cardenolides are not commercially available), the mode of action of cardenolides is highly conserved (binding to the  $\text{Na}^+/\text{K}^+$  ATPase) and is expected to be consistent (Agrawal et al. 2012). We added 50 ng/ $\mu$ L of each cardenolide to artificial nectar, creating a total cardenolide concentration of 100 ng/ $\mu$ L, which was the highest natural concentration in nectar reported among the 12 milkweed species examined (Manson et al. 2012). Due to the hydrophobicity of digitoxin, dimethyl sulfoxide (DMSO) at 1% by volume was added to the solution to ensure full dissolution. We confirmed that the two cardenolides were fully dissolved in solution in equal amounts using high performance liquid chromatography. Bees and butterflies were always provided with a choice between two nectar stimuli: the cardenolide mixture in water with 1% DMSO and 20% sucrose by volume (sucrose + cardenolides) or water with 1% DMSO and 20% sucrose by volume (sucrose). The two nectar solutions were paired with different colored flowers to enhance learning of the nectar stimuli. New nectar solutions were made every week, stored at 4°C, and vortexed before each use.

For individual bumble bees and monarch nectar foraging, we also tested responses to two other stimuli, the cardenolide ouabain at 10 $\times$  natural concentrations (1,000 ng/ $\mu$ L) and quinine at 0.12% by volume (see Appendix S1). We included the unnaturally high cardenolide concentration so that we could determine if pollinator deterrence by high cardenolide concentrations is constraining milkweed nectar chemistry to the natural levels observed. If the insects, and particularly the bees, are not deterred at natural concentrations, but are deterred at unnaturally high concentrations, this might suggest that pollinator behavior constrains plants to the levels observed in nature. If bees are not deterred at high concentrations, this would indicate some other factor is limiting nectar cardenolide concentrations in nature. We used the cardenolide ouabain for the unnaturally high

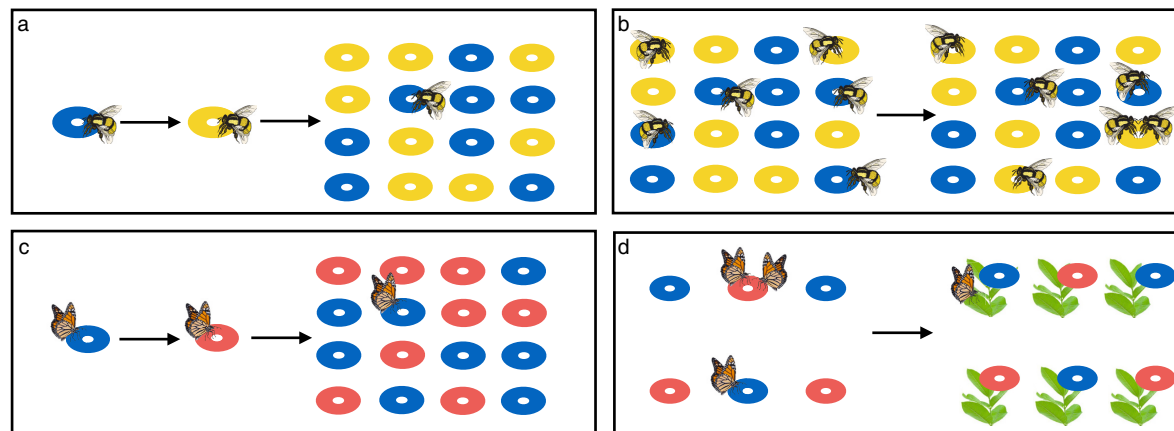


FIG. 1. The four panels represent the four experimental designs used in this study: (a) individual bumble bees were sequentially exposed to each flower type and then allowed to choose which flowers to visit during a 15-min period (bees not to scale); (b) an entire colony of bees foraged from an array of flowers for 5 d and then the nectar-stimulus-flower-color pairings were switched and the colony foraged for another 5 d; (c) individual monarchs were sequentially exposed to each flower type and then were allowed to choose which flowers to visit during a 15-min period; and (d) a group of monarchs foraged freely on a flower array for >5 d and then individual females from that group were exposed to the same flowers with added plants for 1 h for foraging and oviposition. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

concentration because its polarity permits full dissolution in water at high concentrations. Quinine solution is frequently used as an aversive stimulus in bee experiments (Whitney et al. 2009, Wang et al. 2013, Avarguès-Weber and Chittka 2014), and was used to ensure that the insects were capable of learning aversive nectar stimuli with this experimental design.

#### *Bumble bee individual foraging*

We examined whether cardenolides in nectar influenced foraging choices made by individual bumble bees in single foraging bouts. *Bombus impatiens* colonies were obtained from BioBest (Biobest Biological Systems, Leamington, Ontario, Canada). Colonies were provided with pollen ad libitum. Each colony was attached via a 4 cm diameter clear plastic tube to a  $117 \times 71 \times 32$  cm plywood arena with a clear acrylic top. The tube had sliding doors that allowed us to control which bees entered and exited the arena. On the bottom of the arena, we placed a white PVC  $56 \times 56$  cm sheet with 16 0.8 cm diameter holes spaced 11.1 cm apart in a grid. A QIAGEN Collection Microtube Cap (QIAGEN, Valencia, California, USA) was placed in each hole. Each cap could hold a maximum of 125  $\mu$ L of liquid. To habituate bees to collecting nectar in the arena, the entire colony was allowed free access to the arena for multiple days. For this habituation phase, the microtube caps were filled with 20% v/v sucrose solution and placed in the hole in the center of a 4 cm diameter white paper disk; no color stimuli were used during this habituation phase.

We began testing individual bees after 3–5 d of habituation. We first returned all the bees to the colony and cleaned the arena with 50% ethanol to remove any scent marks. We then allowed one bee to enter through the

sliding doors into the arena. The individual bee first received a no-choice experience with each flower type (Fig. 1a). The bee was presented with one paper flower consisting of a 4 cm diameter Color-aid (Color-aid, Hudson Falls, New York, USA) paper circle with the microtube cap in the center (Fig. 2). This flower was either blue (B-Hue) or yellow (Yw-Hue). The microtube cap of the flower was filled with 20  $\mu$ L of one of the two nectar stimuli (sucrose or sucrose + cardenolides). After the bee had tasted (extended proboscis into the solution) or consumed (drunk all 20  $\mu$ L) the first nectar stimulus/color pairing, we removed that paper flower and presented the bee with another flower of the alternative color (blue if the first had been yellow or vice versa) paired with the other nectar stimulus. We controlled the number of bees that received each flower color-nectar stimulus pairing, so that half of the bees received the cardenolide solution with yellow and the other half received the cardenolide solution with blue. We also controlled the order in which bees were exposed to the nectar stimuli such that half of the bees received the cardenolide solution first and the other half received the cardenolide solution second. This resulted in four blocks of bees with different flower-color-nectar-stimuli pairings and orders received. When the bee had tasted or consumed the second stimulus, we again removed the paper flower and set up the arena with 16 paper flowers, eight blue and eight yellow with the appropriate stimulus pairings, each containing 5  $\mu$ L of solution. Bees were allowed to forage for 15 min or until they attempted to return to the colony. We recorded the number of flowers visited by the bee in these 15 min (visits were only counted if the bee extended its proboscis into the solution). When a bee drained a flower, we refilled it with 5  $\mu$ L. Bees that made no visits in 15 min were excluded from the experiment. When the foraging period ended, the bee was removed and euthanized by freezing. Bees were thereby

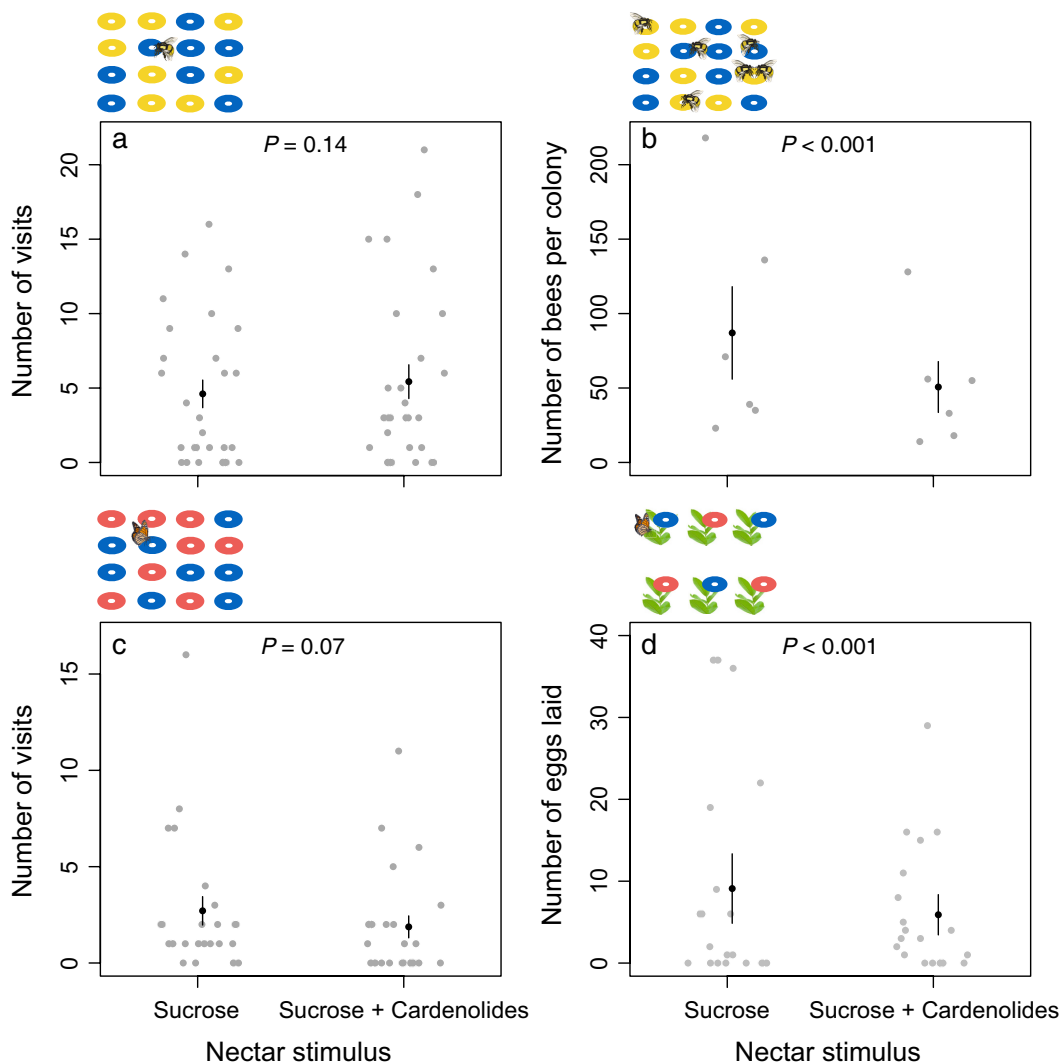


FIG. 2. Results from each of the four experiments. Gray dots indicate jittered raw data points for individuals or colonies, and black dots are mean  $\pm$  SE.  $P$  values are based on the effect of nectar stimulus on visits or number of eggs laid in generalized linear mixed-effects models. (a) The number of visits that individual bees made to each of the two nectar stimuli; (b) the number of bees recorded on each flower type summed across all the data collection intervals for each colony; (c) the number of visits that individual monarchs made to each of the nectar stimuli; and (d) the number of eggs laid by female monarchs on plants paired with each of the nectar stimuli. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

prevented from bringing cardenolide-laced solution back into the colony and potentially influencing the decisions of later foragers. The arena and flowers were cleaned with 50% ethanol between testing individual bees.

We tested 28 individual bees from five colonies (three colonies contributed four bees each and two colonies contributed eight bees each). We analyzed whether total number of visits to each flower type was affected by nectar stimulus or by flower color with a generalized linear mixed-effects model with a Poisson distribution using the `glmer` function in the `lme4` package in R (version 3.2.1). The model with the lowest Akaike information criterion (AIC) value included nectar stimulus, flower color, and the order in which each flower type was received as fixed effects, and colony and individual bee

were included as random effects. We determined whether each of the fixed effects had a significant effect on the number of visits to each flower type using the Anova function in the `car` package (Fox and Weisberg 2011).

#### *Bumble bee colony foraging*

Bumble bees are eusocial insects, and live in mature colonies of hundreds of workers. Individual worker bees will make multiple foraging trips per day (Heinrich 1976), and the foraging choices of other workers may be influenced by social factors including floral scents brought back to the nest by other workers (Molet et al. 2009), and the presence of other individuals on flowers (Jones et al. 2015). We wished to investigate how nectar chemistry

influenced foraging choices when individuals had the opportunity to make multiple foraging trips under social conditions. Therefore, we next conducted long-term foraging experiments with entire bumble bee colonies. As in the previous experiment, all of the bees from the colony were allowed to forage freely in the arena from white paper flowers with 20% v/v sucrose solution until multiple bees were making foraging trips to and from the colony. The arena was then set up with eight yellow and eight blue paper flowers with microtube caps (Fig. 1b). We filled each flower to the top of the microtube cap (approximately 125  $\mu$ L), with each of two nectar stimuli (sucrose vs. sucrose + cardenolides), corresponding to flower color. The doors to the colony were opened and bees were allowed to forage freely in the arena. When bees drained a flower, we refilled it. Using a timer, we photographed the arena every 30 min and recorded the number of individual bees on each flower color. This foraging period continued for 2.5 h, such that we collected five time point surveys per day. This design was repeated each day for 5 d, and was the only nectar that bees had access to during the experiment. After the 5 d, bees received 2 d of ad libitum foraging in the arena from feeders filled with 20% v/v sucrose solution. After these 2 d, we repeated the test for five more days with the same colony but with the color-stimulus pairing reversed.

We tested six different colonies of bumble bees. Three of these colonies had previously contributed bees to the individual foraging experiment (but all single foragers used in the individual foraging experiment were sacrificed, and did not return to the colony after testing). We analyzed the numbers of bees recorded on flowers at each time point with different nectar stimuli with a generalized linear mixed-effects model with a Poisson distribution. The model with the lowest AIC value included as fixed effects: nectar stimulus (sucrose vs. sucrose + cardenolides), flower color, whether it was the first or second week of testing for that colony, and the interaction between the nectar stimulus and the week of testing. Day of testing and colony were included as random effects.

#### *Monarch foraging*

We also examined how the presence of cardenolides in nectar affected foraging choices by adult monarch butterflies from our lab colony (see Appendix S1). Adult monarchs were tested between 24 and 48 h after emergence, during which time they were housed in single-sex cages (38  $\times$  36  $\times$  60 cm) and provided no access to nectar. The testing arena was a columnar mesh cage (58  $\times$  58  $\times$  158 cm) with a PVC sheet on the bottom containing 16 0.8 cm diameter holes at 11.1 cm spacing (same as used for the bees). We first exposed monarchs to a single flower color made from Color-aid paper, which was either red (Rw-Hue) or blue (B-Hue; Fig. 1c). We used red and blue colored paper flowers for the monarchs because previous research showed these colors (using the same Color-aid papers) fall distinctly in monarch color vision space and

are equally preferred (unlike yellow, for which there was a strong innate preference; Blackiston et al. 2011). The flower had a microtube cap in the center with 20  $\mu$ L of a nectar stimulus. To ensure the butterfly sampled the nectar stimulus, we unrolled the butterfly's proboscis into the solution using an entomology pin. The butterfly was then provided with the other flower color (blue if the first was red, or vice versa) and 20  $\mu$ L of the other nectar stimulus. As with the bees, monarchs were divided into four blocks with all the combinations of nectar-stimulus-color pairings and order stimuli received. After sampling both nectar stimuli, the butterfly was released into the arena with the 16 flowers, eight of each color with the assigned nectar stimulus pairings (5  $\mu$ L each). Over a 15-min period, we recorded the number of flowers the butterflies visited (extended proboscis into solution), and we refilled empty flowers with 5  $\mu$ L of the stimulus solution.

The butterfly experiment differed from that of the bees in that, after the 15 min test, we again allowed them to sample 20  $\mu$ L of each nectar stimulus paired with each color, and then the entire sequence of tests was repeated the following day. We added this experience because 42% of monarchs made no choices on the first day. Choices were then pooled from the 2 d of testing. We tested 24 adult monarchs in this experiment (nine females and 15 males). We analyzed whether the number of visits that monarchs made to each flower type was affected by nectar stimulus or by flower color with a Poisson distributed generalized linear mixed-effects model. The model with the lowest AIC was chosen; nectar stimulus, flower color, the order in which each flower type was received, and the interaction between nectar stimulus and order were included as fixed effects, and individual butterfly was included as a random effect. Sex had no significant effect on foraging choices and was not included in the model.

#### *Monarch oviposition*

We examined whether the presence of cardenolides in nectar could be a source of information influencing oviposition choices made by female monarch butterflies. Oviposition trials were conducted in two 1.8-m<sup>3</sup> cages. In each cage, we placed six 10 cm diameter pots filled with potting soil. In the soil of each pot was a 15 cm wooden stick attached to a 5 cm diameter Color-aid paper disk (Fig. 1d). Three of the disks in the cage were red (Rw-Hue) and three were blue (B-Hue), arranged in alternating order. These flowers supported 2-mL Eppendorf tubes filled with nectar stimulus solutions. The two cages differed in the nectar-stimulus-color pairings, i.e., in one cage the cardenolide nectar was paired with red flowers and in the other cage the cardenolide nectar was paired with blue flowers. Pots were spaced in two columns of three plants 60 cm apart. The columns were 110 cm apart, and 40 cm from the edges of the cage. Freshly emerged male and female monarchs were individually labeled by

writing numbers on their wings with permanent marker. The butterflies were allowed to forage and mate in the cage for at least 5 d (range 5–10 d). Each day we replaced the nectar solution, and switched the spatial arrangements of the pots within a cage but maintained the color–nectar–stimulus pairings.

After all the butterflies had been in the cage for at least 5 d, all of the butterflies were removed and placed in a 38 × 36 × 60 cm cage for 24 h with no access to nectar. We then tested individual females one at a time for oviposition and foraging preferences in the large (1.8-m<sup>3</sup>) cage. Approximately one-month-old milkweed plants (see Appendix S1) were placed in the cage and paired with a stimulus flower. Each of the 20 tested butterflies received a set of six full-sibling plants from the same genetic family (seeds collected from a single pod). We used 18 different families total. Using full-sibling plants allowed us to manipulate nectar (i.e., gustatory cues) independent of leaf traits, as females are known to sample leaf chemistry when ovipositing (Baur et al. 1998, Haribal and Renwick 1998).

Each butterfly was tested for 1 h in the arena. During this hour, we recorded all of the times that the butterfly landed on plants (termed in this study an oviposition visit) or on flowers (foraging visit). After 1 h, we removed the butterfly and counted the number of eggs on all of the plants. Each butterfly was retested for 1 h the next day, and we replaced any plant on which the female had laid eggs with a new plant from the same genetic family. The numbers of foraging visits, oviposition visits, and eggs laid on each flower type were pooled from the two testing days. We conducted separate analyses of foraging visits, oviposition visits, and eggs laid using Poisson-distributed generalized linear mixed-effects models. Nectar stimulus and flower color were fixed effects and individual butterfly was included as a random effect.

## RESULTS

### *Bumble bee individual foraging*

Across the 28 bees tested, there was no significant effect of cardenolides on bee visits ( $\chi^2 = 2.15$ ,  $df = 1$ ,  $P = 0.14$ ; Fig. 2a). In fact, bees made 18% more visits to flowers with cardenolides than to flowers with only sucrose. Nonetheless, bees showed a strong color preference ( $\chi^2 = 43.65$ ,  $df = 1$ ,  $P < 0.001$ ) with 123% more visits to blue flowers, and an effect of the order in which the flowers were received, with 51% more visits to the flower the bee received first ( $\chi^2 = 13.46$ ,  $df = 1$ ,  $P < 0.001$ ). In additional experiments using the same methodology, and different individual bees from the same five colonies, bumble bees were deterred by nectar solutions containing a much higher cardenolide concentration (ouabain at 10× natural concentrations) with 37% more visits to sucrose alone, and by quinine in nectar, with 388% more visits to sucrose flowers than quinine flowers (see Appendix S1).

### *Bumble bee colony foraging*

When colonies ( $n = 6$ ) were allowed to forage in the flower array for 10 d, 71% more bees were recorded on flowers with sucrose than flowers with sucrose and cardenolides (GLMM, Type II Wald chi-square tests:  $\chi^2 = 54.14$ ,  $df = 1$ ,  $P < 0.001$ ; Fig. 2b). We also found an effect of flower color (preference for blue,  $\chi^2 = 29.94$ ,  $df = 1$ ,  $P < 0.001$ ), no overall effect of week of testing ( $\chi^2 = 2.61$ ,  $df = 1$ ,  $P = 0.11$ ), but an interaction between stimulus and week of testing ( $\chi^2 = 19.60$ ,  $df = 1$ ,  $P < 0.001$ ). This latter interaction represents a stronger deterrence by cardenolides in the first week of testing (155% more bees on sucrose flowers) than in the second week of testing (27% more bees on sucrose flowers). The decrease in deterrence in the second week could be due to bees having habituated to the presence of cardenolides in nectar, or due to increased motivation in the second week; the latter hypothesis was supported by the observation that 20% more total bees were recorded on flowers in the second week.

### *Monarch foraging*

Among the 24 monarchs tested, we found a weak but nonsignificant effect of nectar stimulus ( $\chi^2 = 3.23$ ,  $df = 1$ ,  $P = 0.07$ ; Fig. 2c), with monarchs making 44% more visits to flowers with only sucrose. There was an effect of flower color ( $\chi^2 = 9.07$ ,  $df = 1$ ,  $P = 0.003$ ) with greater than two-fold visits to red over blue flowers, and an effect of flower order ( $\chi^2 = 8.55$ ,  $df = 1$ ,  $P = 0.003$ ), with 114% more visits to the first than the second flower type received. There was also an interaction between nectar stimulus and order ( $\chi^2 = 8.64$ ,  $df = 1$ ,  $P = 0.003$ ), with 31% more visits to cardenolides than sucrose when cardenolides were experienced first and 1,000% more visits to sucrose than cardenolides when sucrose was experienced first. In additional experiments, conducted with different individual monarchs at the same time, monarchs were not deterred by nectar solutions containing the cardenolide ouabain at 10× natural concentrations, but they were deterred by quinine solutions (see Appendix S1). We therefore showed that while there was a trend towards monarchs avoiding a natural concentration of mixed cardenolides in nectar, it was not statistically significant. Monarchs were also not deterred by an unnaturally high concentration of the cardenolide ouabain. Nonetheless, monarchs did show strong foraging preferences in the experimental design as demonstrated by the results for quinine and by the influences of flower color and order received.

### *Monarch oviposition*

Across the 20 adult female monarchs tested in this experiment, we again found no significant effect of nectar stimulus on foraging visits ( $\chi^2 = 1.75$ ,  $df = 1$ ,  $P = 0.19$ ; Fig. 2d), with the trend being 33% more visits to flowers

containing cardenolides than sucrose-only flowers. Nonetheless, there was again an effect of flower color on foraging visits ( $\chi^2 = 17.45$ ,  $df = 1$ ,  $P < 0.001$ ) with 180% more visits to blue than red flowers. Butterflies made 24% more landings on plants (oviposition visits) when the plants' flowers contained just sucrose compared to sucrose and cardenolides ( $\chi^2 = 4.89$ ,  $df = 1$ ,  $P = 0.02$ ). Nectar stimulus had an effect on numbers of eggs laid ( $\chi^2 = 13.87$ ,  $df = 1$ ,  $P < 0.001$ ), with an average of 61% more eggs laid per female on plants whose flowers contained only sucrose. There were no significant effects of flower color on oviposition visits ( $\chi^2 = 1.55$ ,  $df = 1$ ,  $P = 0.21$ ) or on the numbers of eggs laid ( $\chi^2 = 0.14$ ,  $df = 1$ ,  $P = 0.71$ ). Therefore, while flower color influences nectar foraging choices, it does not appear to impact oviposition decisions.

#### DISCUSSION

The presence of defensive secondary metabolites in flower nectar is a long-standing ecological mystery. These secondary metabolites may simply be byproducts of leaf defenses (Kessler and Halitschke 2009), or they could have an adaptive function in nectar (Adler 2000). One of the proposed adaptive explanations for the presence of secondary metabolites in nectar is that they "filter" flower visitors, selectively deterring ineffective pollinators or nectar robbers, but not effective pollinators (Adler 2000). A number of studies have shown that secondary metabolites in nectar can selectively deter ineffective pollinators or nectar thieves (Stephenson 1981, Johnson et al. 2006, Kessler and Baldwin 2006, Shuttlesworth and Johnson 2009). No previous study to our knowledge compared the effects of nectar secondary compounds on a mutualist pollinator vs. an antagonist herbivore. It could be that selection to deter herbivores through nectar chemistry is even stronger than selection to deter ineffective pollinators. We show that the addition of cardenolides to nectar deters bumble bees only after extended foraging periods, and, while cardenolides do not deter nectar foraging monarch butterflies, they do reduce oviposition rates on plants associated with cardenolide-laced flowers. We thus demonstrate that the presence of cardenolides could be costly to plants in deterrence of pollinating bees, but this cost may be countered by the benefit of deterring herbivore oviposition.

We conducted this study under controlled conditions in the laboratory. Some of the previous studies on filtering flower visitors have examined effects of secondary metabolites in nectar on flower visits in the field (Kessler and Baldwin 2006, Manson et al. 2013). While these field experiments have a number of advantages, particularly when it is possible to measure fitness effects (Adler and Irwin 2005), our controlled lab design allowed us to manipulate and control multiple factors simultaneously. In particular, we were able to examine not just nectar chemistry but how nectar chemistry interacts with other factors weighing into flower visitor decisions such as flower color and the order in which stimuli were experienced. Our results highlight that

these other factors have strong but divergent impacts on foraging decisions in different flower visitors, and also interact differently with nectar chemistry.

#### Bees

We tested individual bumble bees in single foraging bouts. While we found that bees were deterred by unnaturally high concentrations of cardenolides (see Appendix S1), we did not observe deterrence at natural cardenolide levels found in milkweed flowers. In contrast, we did observe deterrence in colony assays. We hypothesize that this difference could be due to individuals making multiple foraging trips, perhaps because bees take more than one foraging bout to learn the taste-color association, or have malaise in response to cardenolides that takes some period of time to develop. Alternatively, the accumulation of cardenolides in the nectar stores of the colonies might influence foraging choices. To our knowledge, no other study has examined how colonies of bees respond to secondary metabolites over extended time periods, although the same methodology has been used to study the effects of nectar secondary metabolites on ant foraging (Junker and Bluethgen 2008). Bumble bees forage under social conditions, and providing the opportunity not only for multiple foraging trips but also access to social information provides a different perspective on foraging behavior with regard to nectar chemistry.

Two previous studies have examined the effects of cardenolides on bee foraging behavior. One study with honey bees showed toxicity of ouabain ( $LD_{50}$  0.003% w/v) and digoxin (similar to the digitoxin used in this experiment but slightly more polar;  $LD_{50}$  0.5% w/v; Detzel and Wink 1993). The same study also tested for behavioral deterrence of honeybees by ouabain, digoxin, and digitoxin, and found no significant deterrence at concentrations up to 1% (w/v; Detzel and Wink 1993). A second study found that the addition of digoxin to nectar did not influence foraging choices of bumble bees at concentrations from 100 ng/ $\mu$ L up to 10 $\times$  natural concentrations at 1,000 ng/ $\mu$ L digoxin (Manson et al. 2012). Digoxin, however, while more polar than digitoxin, is still quite hydrophobic, with a water solubility of 64.8 ng/ $\mu$ L at 20°C, and neither of these studies added a solvent (such as DMSO used in our experiment) to ensure full dissolution of the non-polar cardenolide in water. It is unclear how well-dissolved the cardenolide was in the nectar solutions used in these previous experiments.

Our entire-colony results indicate that there could be costs to plants of cardenolides in nectar at high (but still natural) concentrations in terms of decreased numbers of visits by an important pollinator. In tobacco, nectar nicotine may be so costly to non-selfing plants that it drives an overall reduction in defensive compounds, highlighting the trade-offs produced by pollinator- and herbivore-mediated selection on plant chemistry (Adler et al. 2012). It is unclear whether we would see deterrence by cardenolides in a situation where bee colonies are foraging in a



mixed-plant community and exposed to a wide range of different nectar chemistries simultaneously. We might be less likely to see deterrence in the field, as different individuals tend to specialize on different flower types, diluting nectar cardenolides in colony honeypots (Heinrich 1976). Furthermore, given that the concentrations of cardenolides we used in this experiment are equivalent to the highest natural concentrations reported from the 12 species in which nectar cardenolides have been measured (Manson et al. 2012), nectar cardenolide concentrations may often be low enough to have no effect on bumble bee foraging preferences.

### *Monarchs*

Nectar-foraging monarchs behaved very differently from bees. Nectar cardenolides did not affect monarch nectar foraging across three different experiments manipulating cardenolides in nectar. This is perhaps not surprising given that monarchs are largely resistant to the toxic effects of cardenolides (Holzinger et al. 1992). Nonetheless, the presence of cardenolides in flower nectar did affect oviposition visits (landings on leaves) and egg-laying by monarchs. These results support our hypothesis that nectar foraging could be a way for monarchs to acquire information about cardenolide content of plants. Field studies with monarchs have indicated that females preferentially oviposit on blossoming plants (Brower 1961, Zalucki et al. 1990, Knight et al. 1999). There is evidence from other lepidopterans that adults prefer to oviposit near to nectar sources (Murphy et al. 1984, Brommer and Fred 1999, Janz 2005, Fred et al. 2006), and in the tobacco hornworm moth, *Manduca sexta*, nectar traits may be used by adult females to acquire information used in oviposition decisions (Adler and Bronstein 2004, Sharp et al. 2009). From the monarch's perspective, it would only be an effective strategy to acquire information about leaf content from nectar as long as it is a good predictor of leaf chemistry. Previous research has shown that leaf and nectar cardenolide concentrations are positively correlated (Manson et al. 2012), and therefore it is possible that nectar could be such an honest predictor. Given that monarchs generally prefer to oviposit on milkweed species with intermediate cardenolide levels and avoid high leaf cardenolide concentrations (Oyeyele and Zalucki 1990), high nectar concentrations would therefore be expected to deter oviposition.

### *Color and order*

A foraging decision is a product of integrating multiple sources of information as well as biases in perceptual and cognitive processes. Flower visitors often have innate biases for particular flower colors (Leonard et al. 2011), and are influenced by previous experience (Nityananda and Patrick 2013, Muth et al. 2015) and social information (Worden and Papaj 2005). Our experiments allowed us to investigate the roles of flower color and experience order in foraging decisions. In all of our bumble bee experiments, we found a

significant preference for blue over yellow flowers. The trend was toward bumble bees being less deterred by cardenolides when they were paired with the preferred blue flower color, which highlights that flower color and nectar chemistry may have important interactive effects. Monarch butterflies showed less clear color preferences. In the quinine and ouabain experiments, we saw no color preference, supporting previous studies (Blackiston et al. 2011). For cardenolides at natural concentrations, we found a significant preference for red, whereas the foraging data in the oviposition experiment indicated a significant preference for blue. It is unclear why we found different color preferences in different experiments. The different backgrounds of the white PVC sheet of the foraging experiment vs. the plants and the black greenhouse cage in the oviposition experiment may have reversed color preferences, as background complexity has been shown to change color preferences in *B. impatiens* (Forrest and Thomson 2009).

One of the other factors influencing choices by flower visitors is experience. Many flower visitors continue to visit flowers of the same type as their first experience (Chittka et al. 1999). This phenomenon is called flower constancy, and has been noted since Aristotle (quoted in Grant [1950] and Darwin 1895]), but its mechanism and adaptive function is still a subject of debate (Chittka et al. 1999). In all of the monarch experiments and the majority of the bee experiments, individuals preferred the stimulus they experienced first. Preference for the first stimulus supports a model for flower constancy proposed by Nityananda and Patrick (2013) based on an inhibitory period after learning the first stimulus, although our research demonstrates that while there may be a reduction in learning, there is incomplete inhibition. Flower color and effects of experience are only some of the factors that influence foraging decisions by flower visitors. Our study highlights the importance of including these effects in examinations of foraging choices, as they can be influencing behavior in crucial, and not necessarily predictable, ways.

### CONCLUSIONS

We initiated these experiments to test two alternative hypotheses:

$H_1$ . Cardenolides in flower nectar filter flower visitors by selectively deterring poor pollinators (monarch butterflies) but not effective pollinators (bumble bees).

$H_2$ . Cardenolides in nectar do not deter specialist monarch butterflies, which have resistance mechanisms, but do deter generalist bumble bees which are sensitive to these secondary metabolites.

We found that nectar cardenolides decreased visits by bumble bees at the colony scale, but not individuals in single foraging bouts. In contrast, nectar-foraging monarch butterflies were not deterred. These two results support the second hypothesis, and indicate that the presence of cardenolides in nectar is potentially costly to the plant, raising the question of why they are there.

Levels of cardenolides in nectar may therefore be predominantly under selection from monarch oviposition. Increasing cardenolide concentration in nectar over evolutionary time might therefore decrease the likelihood of a plant being defoliated by herbivores.

The costs and benefits of cardenolides in nectar may be determined by the trade-off in herbivore and pollinator deterrence, as has been proposed for tobaccos (Adler et al. 2012). Given that bumble bee colonies are unlikely to forage exclusively on milkweed flowers for extended periods of time, deterrence of mutualist bees may not be common in the field. This inference is supported by findings that milkweed are not pollen limited (reviewed in Wyatt and Broyles 2002). Examining the effects of nectar chemistry on mutualistic and antagonistic flower visitors illuminates the costs and benefits of toxic nectar compounds from a community perspective. In the case of milkweeds, it appears that herbivores, more than pollinators, may be an important selective force on nectar chemistry.

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