Oecologia (2020) 193:619–630
https://doi.org/10.1007/s00442-020-04701-0

PLANT-MICROBE-ANIMAL INTERACTIONS – ORIGINAL RESEARCH

The role of toxic nectar secondary compounds in driving differential bumble bee preferences for milkweed flowers

Eris Villalona[1] · Briana D. Ezray[2,3] · Erica Laveaga[1] · Anurag A. Agrawal[4] · Jared G. Ali[2] · Heather M. Hines[1,2]

Received: 24 January 2020 / Accepted: 30 June 2020 / Published online: 15 July 2020

Abstract

While morphological differences such as tongue length are often featured as drivers of pollinator floral preferences, differences in chemical detection and tolerance to secondary compounds may also play a role. We sought to better understand the role of secondary compounds in floral preference by examining visitation of milkweed flowers, which can contain toxic cardenolides in their nectar, by bumble bees (*Bombus* spp.), some of their most abundant and important pollinators. We examine bumble bee species visitation of common milkweed (*Asclepias syriaca*) compared to other flowers in the field and test whether observed preferences may be influenced by avoidance and tolerance of cardenolides, as measured by the cardenolide ouabain, in the lab. We reveal that common milkweed is visited predominantly by one bumble bee species, *Bombus griseocollis*, in a ratio much higher than the abundance of this species in the community. We confirmed the presence and toxicity of cardenolides in *A. syriaca* nectar. Lab experiments revealed that *B. griseocollis*, compared to the common bumble bees *B. impatiens* and *B. bimaculatus*, exhibit greater avoidance of cardenolides, but only at levels that start to induce illness, whereas the other species exhibit either no or reduced avoidance of cardenolides, resulting in illness and mortality in these bees. Toxicity experiments reveal that *B. griseocollis* also has a substantially higher tolerance for cardenolides than *B. impatiens.* Together, these results support a potential evolutionary association between *B. griseocollis* and milkweed that may involve increased ability to both detect and tolerate milkweed cardenolides.

Keywords Asclepias · Cardiac glycosides · Plant–pollinator interactions · Niche partitioning · Detoxification · *Bombus* · Specialization

Introduction

Bumble bees are abundant, temperate pollinators involved in numerous long-term co-evolutionary interactions with flowering plants. In highly populated bumble bee communities, which often peak around 12–20 species (Pyke 1982), competition for floral resources is thought to promote divergent traits that enable bumble bee species to coexist through resource partitioning (Inouye 1978, 1980; Pyke 1982; Pyke et al. 2012). Evolutionary synchronization of traits between bumble bees and many flowering species has led bumble bee species to develop preferences, reinforced by their own morphological and physiological constraints, for specific plant traits. Variation in tongue length, for example, has been established as a primary factor promoting different foraging patterns between bumble bees, as it restricts floral access and impacts handling efficiency depending on floral corolla length (Inouye 1980; Goulson 2009). Bumble bees' foraging patterns are also likely to be impacted by nutritional

---

Communicated by Carlos L. Ballare.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00442-020-04701-0) contains supplementary material, which is available to authorized users.

✉ Heather M. Hines
hmh19@psu.edu

[1] Department of Biology, The Pennsylvania State University, University Park, PA, USA

[2] Department of Entomology, The Pennsylvania State University, University Park, PA, USA

[3] Department of Research Informatics and Publishing, The Pennsylvania State University Libraries, University Park, PA, USA

[4] Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY, USA

preferences for certain pollen and lipid ratios (Vaudo et al. 2016) and sugar content (Willmer 2011).

Bumble bee visitation patterns could also be affected by their ability to tolerate certain secondary compounds found in nectar (Adler 2000); however, little is known about their effects on bumble bee foraging behavior. The presence of toxic compounds in nectar may simply be a consequence of systemic generation of these compounds by the plant to deter herbivory (Adler 2000). However, their presence could also be adaptive in serving to deter nectar robbers (Barlow et al. 2017), in medicating against pollinator pathogens (e.g., Manson et al. 2010; Richardson et al. 2015), and through ensuring pollinator fidelity and efficiency by toxin-tolerant specialists (Stevenson et al. 2017). Toxicity would add another level of complexity to factors that facilitate resource partitioning, as toxins may allow tolerant specialists to monopolize nectar rewards of specific plants. This would provide an additional mechanism of sustaining community diversity in bumble bees, considering that tongue length is often highly overlapping. Toxin-based partitioning and specialization has previously been observed with *B. consobrinus*, a European bumble bee that tolerates floral alkaloids, allowing it to specialize on monkshood (*Aconitum septentrionale*) (Gosselin et al. 2013).

A better understanding of the extent to which toxins impact pollinator visitation can be gained using milkweed (*Asclepias syriaca*). Milkweeds are known to produce cardenolides, which are found in many plant species, but especially in the Apocynaceae, rendering tissues of these plants toxic for both invertebrates and vertebrates (Agrawal et al. 2012). Cardenolides act by specifically inhibiting Na^+/K^+ ATPase pumps, which are important for maintaining cellular and neural membrane potentials. The bioactivity of these toxins in milkweed has led to the evolution of a community of milkweed-specialized herbivores capable of tolerating these toxins (Agrawal et al. 2012). Most famously, milkweed is known as the host plant for monarch butterflies (*Danaus plexippus*) who spend their caterpillar stage feeding on milkweed leaves, sequestering the toxins into their tissues as a defense against predation (Brower et al. 1968). Other aposematic specialists, including several species of milkweed bugs (Duffey et al. 1978) and beetles (Ali and Agrawal 2017), also sequester and are defended by these cardenolides.

Cardenolides have been found not only to occur in milkweed tissues, but also in the nectar, with concentrations depending on the species (Manson et al. 2012). As such, visitors to milkweed flowers may be impacted, depending on their degree of susceptibility to the toxins. Monarch butterflies (specialists, but ineffective pollinators) were shown not to be deterred by the presence of cardenolides in nectar, but laid fewer eggs on plants with cardenolide-laced flowers compared to controls (Jones and Agrawal 2016). Previous studies have examined pollinator visitors to various *Asclepias* species, revealing large bodied Hymenoptera (including bumble bees) to be the most abundant and effective pollinators (Robertson 1887; Kephart 1983; Jennersten and Morse 1991; Betz et al. 1994; Fishbein and Venable 1996; Kephart and Theiss 2004; Howard and Barrows 2014). Natural variation and adaptation to tolerate cardenolides may have promoted specialized interactions between milkweed and certain pollinators like bumble bees, thus enhancing resource utilization for bee specialists and potentially enabling more efficient milkweed pollinia transfer.

The potential effects of cardenolides in nectar have been previously noted, as some bees have been observed to appear "drunk" (Kevan et al. 1988) and show inhibition in feeding on cardenolide-laced nectar (Detzel and Wink 1993). Toxicity of cardenolides to honey bees supports that they are susceptible to these compounds. For example, Detzel and Wink (1993) found that LD_{50} for honey bees fed on commercially available cardenolides (convallatoxin, digoxin and ouabain) were within levels found in some milkweed species (as high as ~ 100 ng μL^{-1}, Manson et al. 2012). The bumble bee, *B. impatiens,* was found not to be impacted by digoxin-rich nectar (up to 50 ng μL^{-1}, Manson et al. 2012), but they avoided the cardenolide, ouabain, at potentially natural concentrations (100 ng μL^{-1}) after lengthy foraging experiences (Jones and Agrawal 2016). Our examination of records of bumble bee visits to milkweed species revealed unusually high visitation rates from *B. griseocollis* (Betz et al. 1994; Theiss et al. 2007; Baker and Potter 2018). For example, *B. griseocollis*' visitations account for ~ 50% of all pollinator visitors and 98% of all bumble bees visiting milkweed in a study by Theiss et al. (2007), a number that is particularly high given that this is not typically the most abundant species in eastern North American bumble bee communities. The unusual frequency of *B. griseocollis* visitors to this plant has previously been noted (Macior 1965; Kephart 1983) and could represent a case of specialization resulting from tolerance to milkweed cardenolides.

In this study, we test whether bumble bee species exhibit differential preferences for milkweed and the mechanisms that may explain those differences using field observations and lab experiments. We first determine whether visitation patterns of bumble bee species are different on common milkweed (*A. syriaca*) compared to other members of the floral community by observing visitation patterns at several sites in central Pennsylvania. These data are enhanced by examining the proportion of bumble bee species visiting common milkweed compared to the proportion present in records in the eastern United States, inferred using photos in the public repository, *iNaturalist*. Common milkweed is recognized as one of the less toxic milkweeds (Züst et al. 2019), thus to assess whether nectar of common milkweed contains cardenolides, we inferred cardenolide levels and enzymatic toxicity of this plant using in vitro assays. To examine whether field visitation may be influenced by tolerance to cardenolides, we reared the three most common eastern bumble bee species in the lab (*B. griseocollis*, *B. impatiens*, *B. bimaculatus*) and tested whether (i) species are deterred by cardenolides using multi-day feeding-choice experiments with varying cardenolide doses, and (ii) species differ in their innate tolerance to cardenolides by assessing mortality to varying cardenolide doses.

Methods

Bumble bee visitation to milkweed

Field observations

To assess whether there is a difference in relative bumble bee species visitation on common milkweed (*A. syriaca*) compared to other plants present at the same sites as milkweed plants, field observations of bumble bee visitors to flowers were conducted over the course of two consecutive summers from late June to early July at four sites in State College, Pennsylvania, USA: The PSU Arboretum (1 ha, Site 1) and public city parks Bernel Park (0.5 ha, Site 2) and Tudek Park (0.5 ha, Site 3) sampled in years 1 and 2 and Roundhay Court (0.1 ha, a private garden, Site 4) sampled in year 2 only. These sites were selected because they contained abundant milkweed plants as well as an abundance of other plant species. Each location was sampled once in the morning, 9–12 a.m., and once in the afternoon, 1–5 p.m., for a total of two sampling events per site. During each sampling event, bumble bees visiting common milkweed were collected for 45 min using vials and nets. Bumble bee visitors were also collected from three other flower species at each site for 15 min each. These plant species were selected based on them being the most abundant plants in flower that were visited by bumble bees. The number of plants of these species were similar to the number of milkweed plants at the site. After field identification to species, sex and caste, bees were released. Local species have reliable field diagnostic traits, except for two species that are difficult to field ID, *B. perplexus* and *B. vagans*, which were lumped into one group. Caste was determined based on relative bee size. Multiple plants of each species were sampled from across these sites.

For analysis, bee visitation was classified on milkweed vs. non-milkweed plants given lack of power to examine effects by individual plant species for non-milkweed. To analyze whether individual bumble bee species preferred milkweed vs. non-milkweed plants (i.e., the bumble bee species' "preferred" plants), we utilized a Generalized Linear Model with a Poisson distribution and log link that tested the effects of Bee Species, Plant type and their interaction on abundance of bumble bee visitors. We also included Time and Site as fixed effects in this model. This was followed by Tukey-HSD tests to assess how abundance of each bee species differed on milkweed vs. non-milkweed. To assess whether each type of plant received a different composition of bumble bee visitors (i.e., the milkweed's "preferred" visitors), we tested the difference in proportional visitation by species to milkweed vs. non-milkweed plants at a site using a Chi-square test of independence. We applied this test for each site separately. All statistics were run in JMP Pro version 14.0.0 (SAS Institute).

iNaturalist data

As an additional source of data on bumble bee species visitation to milkweed that broadens the range of observations beyond central Pennsylvania, we utilized publicly accessible photograph records of common milkweed (*A. syriaca*) available in *iNaturalist*, an online platform where observations of natural objects can be submitted, identified using computer automated approaches and identifications confirmed by users. In total, 13,756 common milkweed records, submitted by 6211 different observers and confirmed by 1431 users, were scanned to look for bumble bee visitors on the flowers (accessed July 30, 2019). Bumble bee species were determined using photo identification (by H. Hines), only considering those that could be identified with confidence. Common milkweed identification was also confirmed. Most of the photos with bumble bees could be reliably identified, resulting in 60 bumble bee identifications. Bumble bee visitation frequencies on milkweed were then compared to the proportion of each bumble bee species observed within the region where common milkweed is mostly distributed. For this, the *iNaturalist* search was narrowed to Eastern United States and the number of identified records of each bumble bee species in this region were recorded. This region includes the areas from which the 60 observations were drawn (Table S1), which include records from 16 U.S. states spanning this region and the southernmost extent of Ontario and Quebec and represents the bounds of a major zone of endemicity in bumble bees. Identifications for bumble bees are often confirmed/checked by professional bee taxonomists and in our perusal of these were correctly identified for > 90% of the specimens. Although there are other sources of data that could provide relative metrics of species distribution, this is the best control for bias, as some species are more easily identified from photographs than others. The resulting data were analyzed using a Chi-Square goodness of fit test treating milkweed species records as observations and the community as the expectation.

Cardenolide analysis from A. syriaca nectar

To demonstrate whether *A. syriaca* nectar contains cardenolides, *A. syriaca* nectar was collected and analyzed from flowers using methods developed in Manson et al. (2012). We used nectar from the following samples collected in Ithaca, NY: 1 flower each from two separate plants extracted by vortexing the flowers upside-down in a 2 mL tube, two samples of 100 μL drawn via capillary tubes from multiple flowers of these same two plants, and total nectar from groups of four and eight flowers, extracted by vortexing as above. Nectar was added to 500 μL of 70% ethanol (following Blüthgen et al. 2004) and stored at − 80 °C prior to analysis.

High-performance liquid chromatography (HPLC) was used to quantify cardenolides in nectar following the protocol in Züst et al. (2019). Briefly, samples were prepared by drying down all water and ethanol from the stored samples using a rotary evaporator (Labconco, Kansas City, MO, USA). Residuum was extracted with 600 μL of 100% methanol of which 200 μL (1/3 of original sample) was run for HPLC with 0·2 g/L solution of the cardenolide digitoxin added as an internal standard. Cardenolides were analyzed on an Agilent 1100 series HPLC using a Gemini C18 reversed-phase column (3 mm, 150 × 4·6 mm; Phenomenex, Torrance, CA, USA). Cardenolides were quantified by integrating the peak area at 218 nm. Cardenolides were calculated relative to the peak area of the digitoxin internal standard.

To quantify the biological activity of cardiac glycosides, nectar extracts were tested for toxicity following the methods of Züst et al. (2019). This method involves in vitro activity of Na^+/K^+-ATPase from the porcine cerebral cortex (Sigma Aldrich, MO, USA) and measures inorganic phosphate (Pi) enzymatically released from ATP. Dried supernatants from 200 μL of the methanol-dissolved nectar extract samples described above (not containing digitoxin internal standard) were resuspended in 50 μL 10% DMSO with 20 μL run in each sample well (13.2% of the original sample). Five dilutions were prepared from the working stock solution in 10% DMSO and all dilutions were tested to produce an inhibition curve for each sample. Inorganic phosphate released from enzymatically hydrolyzed ATP was stained by adding 100 μL Taussky–Shor reagent and photometrically quantified by measuring absorbance 700 nm. To account for coloration of plant extracts, we included a background well for each reaction with identical content but lacking KCl. On each microplate we also included wells with an uninhibited control and a calibration curve made with ouabain ranging from 10^{-3} to 10^{-8} M. Based on the residual enzymatic activity inhibited by a biological sample, we estimated the sigmoid dose–response curve using a logistic function with the upper and lower asymptotes fixed to 100 and 0% residual activity (Züst et al. 2019). We estimated the cardiac glycoside concentration of the undiluted samples in ouabain equivalents based on the calibration curve.

Cardenolide preference trials

Lab trials were performed on reared bumble bees to assess whether bumble bees sample cardenolide-laced sucrose solution differently from sucrose solution alone and whether patterns may differ by species. By assessing relative consumption of available solutions with different concentrations of cardenolide across 3 days, we were able to examine whether preferences developed over time (i.e., innate or learned) by contrasting patterns from day 1 to those from days 2 and 3. *Bombus impatiens* (5 colonies in 2018, 5 colonies in 2019), *Bombus griseocollis* (5 colonies in 2018, 5 colonies in 2019), and *Bombus bimaculatus* (4 colonies in 2018, 1 colony in 2019) colonies were reared from wild-caught queens in standard rearing conditions in laboratory incubators (26 °C, 60–80% humidity, dark) to obtain individuals used for laboratory trials. In the summer of 2018, worker bees were sampled evenly from across available colonies, with 10 bees per species placed individually into small plastic containers (Ziploc, 3.0 in (H) × 4.25 in (W)) drilled with holes for feeder insertion and air flow. Bees were each presented with four closed upside-down Eppendorf tubes with a 1-mm radius feeding hole at the base, that included 1 mL of 50% sucrose solutions (w/v, sucrose(g)/water(mL)) along with ouabain (Sigma: PHR1945, 300 mg) at concentrations of 0 ng μL^{-1}, 25 ng μL^{-1}, 100 ng μL^{-1} and 1000 ng μL^{-1}. Although ouabain is not one of the cardenolides found in *Asclepias*, those that are found are not commercially available. The mode of action among diverse cardenolides is similar (Agrawal et al. 2012) and ouabain is the standard cardiac glycoside used to test toxicity in other systems (e.g., Holzinger and Wink 1996; Jones and Agrawal 2016). Each vial was marked with a different similarly contrasting black and white symbol (vertical line, horizontal line, circle, or triangle) with a permanent marker to facilitate learning. The amount of consumption of each solution was determined 24, 48 and 72 h after trial initiation by weighing the Eppendorf tubes and making the following calculation $(W_{t(n+1)} - W_{tn}) \times$ (cardenolide solution density) where W represents tube weight at $t = 24$, 48, or 72 h, and $n = 0$, 1, or 2. To correct for evaporation loss, each value was normalized against the amount of volume loss in a control tube of each concentration that was not fed on by bees and kept in the same conditions. Before performing these trials, we confirmed that bees were able to learn and develop preferences with this experimental set-up by performing a trial with *B. impatiens* with four different concentrations of sucrose. In this experimental trial, after 24 h, which we considered a "learning period", the bees learned to avoid solutions with low sugar concentration and drink from the high-sugar solution. Throughout these experiments, individuals were kept at room temperature and under a white cloth, which diffused natural lighting and prevented bias from external landmarks.

In the 2018 experiments, we observed that *B. impatiens* and *B. bimaculatus* individuals had high levels of sickness and mortality, as the highest concentrations were at the observed lethal dose (see "Cardenolide tolerance trials"). As illness can affect learning, in 2019 we conducted the same experimental design as above (8 replicates each, using newly reared 2019 colonies), but removed the 1000 ng μL^{-1} ouabain solution. Thus, these bees were provided three vials: 0 ng μL^{-1}, 25 ng μL^{-1} and 100 ng μL^{-1} ouabain sugar–water solution. To assess whether observed patterns may be impacted by the number of choices, a final choice experiment was conducted (on bumble bees from 2019) where *B. impatiens* and *B. griseocollis* (10 bees/species) were only given two cardenolide-laced solutions, 0 ng μL^{-1} and 1000 ng μL^{-1} ouabain. To assess whether or not the bees sampled equally across all artificial nectar solutions, we performed a two-way factorial ANOVA, analyzing the concentration of Cardenolide, Time and their interaction, with bee individual ID as a random effect for each trial and species. As the above models did not directly compare between *B. impatiens* and *B. griseocollis*, to better understand the differences in effects between them for variables that came out significant in the above models, we also compared percent consumption of the highest dose between these two species using paired *t* tests for each time point for Trial 1 and 3. Data were sqrt ($N+0.5$) transformed where necessary to improve homogeneity of variances and normality of residuals; back-transformed means and standard errors (SEs) are reported in the figures. Tukey-HSD tests were used to compare means from each model (JMP Pro 14.0.0, SAS Institute).

Cardenolide tolerance trials

To assess if there are species-specific tolerances to cardenolides, toxicity trials were performed to determine relative mortality by dose concentration of ouabain for *B. impatiens* and *B. griseocollis*. In 2018, *B. impatiens* (10 individuals/condition) workers were evenly sampled from five colonies and were given an initial dose of 100 µL of sugar water with concentrations of 0, 25, 100, 500, 1000, or 2000 ng μL^{-1} ouabain. Similarly, *B. griseocollis* (10 individuals/condition) workers were evenly sampled from five colonies and were given an initial dose of 100 µL of sugar water with concentrations of 1000 or 2000 ng μL^{-1} ouabain. In 2019, the same procedure was repeated with *B. impatiens* (10 individuals/condition) and *B. griseocollis* (10 individuals for 0, 25, 100, 500, 1000 and 2000 ng μL^{-1} ouabain). Upon consumption of this solution, which happened within a 12-h window, *B. impatiens* and *B. griseocollis* were given sugar water ad libitum. All individuals in this sampling consumed the complete dose within 12 h. Sickness and mortality were recorded 24 and 48 h after being given the initial cardenolide dose. Bees were deemed to be sick if they exhibited reduced motor abilities (e.g., not standing upright, twitching) and a weak response to stimulation (did not move upon shaking or prodding). Throughout the experiment, individuals were kept in a room exposed to room temperature and natural lighting. To determine whether there were significant differences in survival for each treatment over time between the two species, Kaplan–Meier survival tests were performed comparing the two species by treatment using a log-rank test in R (Version 3.6.1) using the survival package (Therneau and Lumley 2015) and the survminer package (Kassambara et al. 2019). Data from both years were pooled for this analysis ($n=10$ for *B. griseocollis* 0, 25, 100 and 500 ng μL^{-1} ouabain; $n=20$ for *B. griseocollis* 1000 and 2000 ng μL^{-1} ouabain and all *B. impatiens* treatments). Alive and sick individuals were treated the same in this analysis as only mortality was assessed.

ATPase sequencing

Several milkweed specialist herbivores have become tolerant of cardenolides through shared, independently evolved amino acid mutations in a specific region of the sodium–potassium ATPase (Dobler et al. 2012; Karageorgi et al. 2019). To determine whether bumble bees might use a similar mechanism, we designed primers and sequenced the implicated protein-coding region of the ATPase in a single male of *B. griseocollis* from our reared colonies (queen collected from State College, PA). For this, the thoracic muscle of the bee was homogenized with metal beads and Trizol on an Omnibead ruptor. RNA was then extracted using a Zymo Direct-zol RNA Miniprep kit following standard protocols, followed by cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following recommended protocols. PCR was performed using custom-designed primers from this locus (5′-CAC AAA ATT ACA CCC GAG GA-3′, 5′-CAG CAA TCC TTC CCA TTA CA-3′), run at 95 °C (5 min(m)), 35 cycles of 94 °C (30 s), 52 °C (30 s), and 72 °C(1 m, 30 s) and 72 °C (1 min, 30 s). The amplified fragment was sequenced at the PSU Huck Genomics Core Facility, edited in Geneious 8.1.9 (https://www.geneious.com), and translated to examine amino acid sequence variation. Sequence and amino acid variation was compared to the genomic sequence for sodium/potassium-transporting ATPase subunit alpha in *B. impatiens* (BIMP_2.1 GCF_000188095.2; LOC100745576) and *B. terrestris* (Bter_1.0 GCF_000214255.1; LOC100647333) (Sadd et al. 2015), and to the typical vs. substituted alleles of milkweed specialists (Agrawal et al. 2012; Dobler et al. 2012; Karageorgi et al. 2019).

Results

Floral visitation patterns to milkweed

We found highly significant differences in proportional visitation patterns of bumble bee species to milkweed compared to the rest of the floral community for each site when considered separately ($P < 0.0001$ for each site and year, Fig. 1). This pattern was driven by markedly higher proportional visitation of *B. griseocollis* to milkweed and substantially lower visitation of *B. impatiens* and *B. bimaculatus* compared to visitation of other plant species, a pattern consistent across all sites. For example, *B. griseocollis* comprised 100%, 82%, and 91% of bumble bee visitors to milkweed compared to 14%, 23% and 25% of bumble bee visitors to other flowering plants at the respective sites in year 1 (Fig. 1a) and 100%, 59%, 89% and 85% of all visitation to milkweed compared to 11%, 11%, 0% and 13% of non-milkweed plants across sites in year 2. Overall, this amounted to *B. griseocollis* comprising 87% of bumble bee visitors to milkweed, but only 14% of non-milkweed visitors.

In comparing abundances of bumble bee species visits by plant type (milkweed vs. non-milkweed), we found an effect of time of day (bumble bees were most present in the morning, GLM: $\chi^2 = 7.338$, $df = 1$, $P = 0.0068$) and no effect by site (GLM: $\chi^2 = 5.236$, $df = 3$, $P = 0.1553$). Overall, species-specific bumble bee abundance was higher for *B. griseocollis* (GLM: $\chi^2 = 112.44$, $df = 3$, $P < 0.0001$, Table S2). There was a significant difference in the abundance of specific bumble bees species to milkweed vs. non-milkweed (bee species plant type, GLM: $\chi^2 = 170.95$, $df = 3$, $P < 0.0001$, Table S2). *B. griseocollis* was more abundant on milkweed than other plants ($P < 0.0001$), *B. impatiens* and *B. bimaculatus* were less abundant on milkweed than other plant species ($P < 0.0001$ for both species), and *B. perplexus/vagans* had similar abundance on both plants ($P = 0.9991$; Table S2). This suggests *B. griseocollis* has a preference for

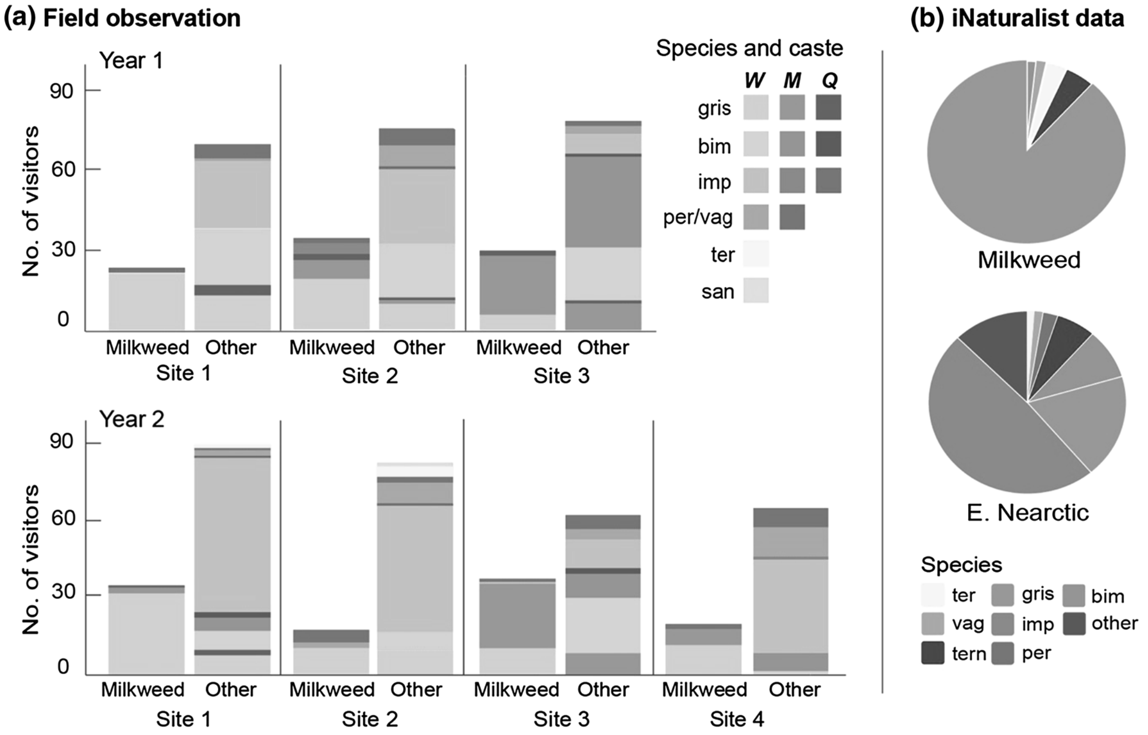

Fig. 1 Bumble bee species abundance on milkweed compared to the rest of the floral community: **a** Bumble bee abundance observed at four locations in central Pennsylvania, USA, separated by species, sex, and caste. Data are combined across two sampled time periods (AM and PM) for each year. Chi-square tests comparing visitation to milkweed vs. other plants were highly significant for all sites (Year 1 in order by site: ($\chi^2_{(3,92)} = 32.02$ $P = 5.2\text{E}^{-7}$), ($\chi^2_{(3,107)} = 56.80$, $P = 2.8\text{E}^{-12}$), ($\chi^2_{(3,116)} = 61.06$, $P = 3.4\text{E}^{-13}$); year 2: ($\chi^2_{(3,118)} = 100.5$, $P = 7.7\text{E}^{-21}$), ($\chi^2_{(3,96)} = 35.28$, $P = 1.3\text{E}^{-6}$, ($\chi^2_{(3,100)} = 55.80$, $P = 4.6\text{E}^{-12}$), ($\chi^2_{(3,81)} = 70.97$, $P = 2.6\text{E}^{-15}$). **b** Bumble bee milkweed visitation inferred from photo observation records on iNaturalist, including abundances of each bumble bee species visiting common milkweed (*Asclepias syriaca*) ($n = 60$ observations, top pie chart) and the abundance of each bumble bee species identified from photos submitted to *iNaturalist* in the Eastern United States ($n = 19{,}163$, bottom pie chart), the region where common milkweed is primarily located. Patterns are significantly different ($\chi^2_{(7,60)} = 203$, $P < 0.0005$). gris = *B. griseocollis*, bim = *B. bimaculatus*, imp = *B. impatiens*, per/vag = *B. perplexus/B. vagans*, san = *B. sandersoni*, ter = *B. terricola*. *Q* queen, *M* male, *W* worker

milkweed, while *B. impatiens* and *B. bimaculatus* exhibit avoidance of milkweed relative to other plant species.

Most non-*B. griseocollis* visitations to milkweed were from queens and males (only 8.3% were workers, $n = 24$ visitors), whereas workers of the other species were the most common visitors to non-milkweed plants (78.8% workers, $n = 443$ visitors; Fig. 1a), resulting in significant distributional differences by caste in non-*B. griseocollis* by plant ($\chi^2_{(1,467)} = 60.52$, $P < 0.0001$). *B. griseocollis* visitors to milkweed were from all three castes in a similar proportion to their frequencies at the rest of the flowering species (61.8% of 173 *B. griseocollis* on milkweed were workers, 56.5% of 69 *B. griseocollis* on other plants were workers: [$\chi^2_{(1,242)} = 0.58$, $P = 0.44$]). While *B. griseocollis* is observed more frequently on milkweed than other plants, one plant, *Echinacea,* also had disproportionally more *B. griseocollis* visitors, contributing substantially to non-milkweed visits by *B. griseocollis* at Site 3 (Tudek Park) in year 1 (Fig. S1).

iNaturalist

In iNaturalist photo records, *B. griseocollis* visitations to milkweed account for 88.3% of all bumble bee visitation while this species comprises only 18% of the Eastern bumble bee population (Fig. 1b, Table S1). *B. impatiens* comprises 49% of the bumble bee population, yet there were no photo observation records of *B. impatiens* on milkweed. Similarly, *B. bimaculatus* is the third most abundant Eastern species (9%), but it only made up 2% of the visitors to milkweed. A chi-square test revealed the bumble bee community on milkweed to be highly significantly different ($\chi^2(7,60) = 203$, $P < 0.0005$) than the composition of the eastern North American bumble bee community. This further confirms the biased behavior for milkweed observed in non-*B. griseocollis* bumble bee species and indicates that the visitation patterns observed in the field in State College, PA can be used to generalize visitations patterns to milkweed in the Northeastern United States. Of those non-*B. griseocollis* visiting milkweed, these were largely confined to southern Canada, where *B. griseocollis* reaches its northernmost range limit and is thus rare (Table S1).

Nectar Cardenolides and Na^+/K^+-ATPase inhibition

HPLC analysis of cardenolides in *A. syriaca* nectar revealed three compounds (eluting at 11.3, 13.1 and 19.8 min), but they were only detectable in the samples of pooled nectar from four and eight flowers (yielding 0.110 and 0.139 µg total cardenolides, respectively). The in vitro sodium pump assay revealed cardenolide-mediated inhibition in all samples (Table S3). Confirming a dosage effect, inhibition of the Na^+/K^+ ATPase increased with extracts from 1, 4 and 8 flowers (Table S3, pearson $r = 0.964$, $P = 0.036$). The concentration of cardenolide in the sample was inferred to be ~ 1.4 ng μl^{-1} for both assays. This concentration is low compared to concentrations inferred in some other milkweed species (Manson et al. 2012) and less than the ouabain concentrations used in our experiments.

Cardenolide preference

The results of the feeding trials suggest that there are species-specific responses to the cardenolide-laced sugar solutions (Fig. 2, Table S4). In the first trial, *B. griseocollis* displayed significant differences in treatment consumption ($F_{(3,99)} = 62.196$, $P < 0.0001$) that was consistent across time points (no significant time or treatment*time effect), with similar consumption for 0, 25 and 100 ng μL^{-1} treatments, but reduced consumption for the highest (1000 ng μL^{-1}) treatment (Tukey test, $P < 0.0001$ for each pairwise comparison, Table S4). *B. impatiens* also had an effect of treatment ($F_{(3,99)} = 9.73$, $P < 0.0001$). The reduction in feeding was significant for 1000 ng μL^{-1} compared to the other ouabain treatments ($P = 0.0091$ from 0 ng μL^{-1}, and $P < 0.0001$ compared to 10 and 100 ng μL^{-1}). Nonetheless, this reduction in feeding at 1000 ng μL^{-1} was less than that observed for *B. griseocollis* (Fig. 2; *B. griseocollis* consumed proportionally less of this dose than *B. impatiens* at 24 ($P = 0.0028$) and 72 h ($P = 0.0407$), but not at 48 h ($P = 0.2071$)). Unlike *B. griseocollis*, *B. impatiens* also showed an effect of time ($F_{(2,99)} = 30.17$, $P < 0.0001$), with reduced overall consumption over time ($P < 0.0001$ between 24 h and 48 or 72 h, $P = 0.0135$ between 48 and 72 h). *B. bimaculatus* did not have a significant difference in consumption across treatments ($F_{(3,99)} = 0.89$, $P = 0.448$), and, like *B. impatiens*, showed a significant ($F_{(2,99)} = 55.91$, $P < 0.0001$) reduction over time, progressively reducing consumption from 0 to 48 to 72 h ($P < 0.0001$ between 24 h and 48 or 72 h, $P = 0.0008$ between 48 and 72 h). *B. impatiens* and *B. bimaculatus* showed signs of illness from consumption; thus reduced consumption was likely related to illness.

In Trial 2, when the more lethal 1000 ng μL^{-1} ouabain solution was removed from the choice trial, all individuals remained alive and healthy until the end of the experiment. For *B. griseocollis* and *B. bimaculatus*, there are no significant differences in consumption by time or treatment, while in *B. impatiens* there was a treatment effect ($F_{(2,56)} = 4.60$, $P = 0.0141$) due to somewhat increased consumption of the lowest (25 ng μL^{-1}) ouabain dose compared to the 0 ($P = 0.0648$) and 100 ($P = 0.0158$) ng μL^{-1} doses (Fig. 2, Trial 2; Table S4).

In Trial 3, where *B. griseocollis* and *B. impatiens* workers received only two choices, 0 ng μL^{-1} and 1000 ng μL^{-1} ouabain, both species demonstrated similar behavior as in the initial choice experiment (Fig. 2, Trial 3). For both species there were effects of cardenolide concentration

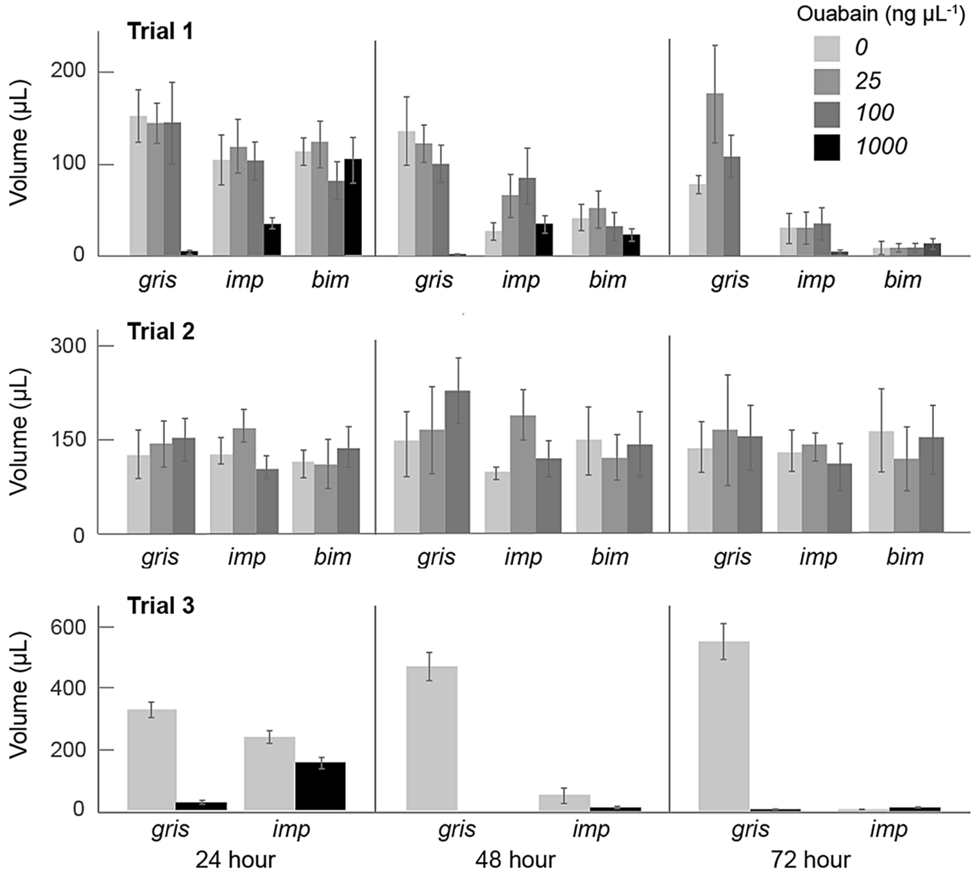

Fig. 2 Average consumption of cardenolide solution of varying ouabain concentrations in choice tests performed across three trials on bumble bee species *B. griseocollis* (*gris*), *B. impatiens* (*imp*), and *B. bimaculatus* (*bim*). For each species, $n = 10$ in Trials 1 and 3 and $n = 8$ in trial 2. Consumption levels were recorded for three 24-h increments including 0–24 h, 24–48 h, and 48–72 h. Error bars represent standard error

(*B. griseocollis* $F_{(1,45)} = 646.49$, $P < 0.0001$; *B impatiens* $F_{(1,45)} = 6.88$, $P = 0.0119$), although the difference in consumption was greater for *B. griseocollis* than *B. impatiens*, with *B. griseocollis* essentially avoiding the 1000-ng µL^{-1} ouabain solution (significantly different across all time points, Table S4), while *B. impatiens* maintained moderate, although reduced, levels of consumption of this dose (significant for 24 and 72 h). *B. griseocollis* consumed significantly less of the high dose in proportion to the lower dose than *B. impatiens* for 24 and 72 h ($P < 0.0001$), but not for 48 h ($P = 0.1479$). *B. impatiens* workers appeared sick by 48 h; thus their consumption strongly decreased at 48 ($P < 0.0001$) and 72 h ($P < 0.0001$) from levels at 24 h, consistent with previous trials (overall time effect, $F_{(2,45)} = 143.11$, $P < 0.0001$). In this trial, *B. griseocollis* demonstrated increased consumption over time ($F_{(2,45)} = 3.64$, $P = 0.0343$; significant between 48 and 72 h, $P = 0.0298$) and at the end of the trial, all *B. griseocollis* workers remained healthy and responsive, while 60% of *B. impatiens* workers died and 40% were sick after 72 h. For *B. griseocollis,* there was a significant or marginal decrease in 1000 ng µL^{-1} ouabain consumption after the first 24 h ($P = 0.0017$ from 24 to 48 h, $P = 0.0644$ from 24 to 72 h). This may be indicative of initial tasting of the 1000 ng µL^{-1} ouabain solution and avoidance thereafter (Fig. 2).

Cardenolide toxicity trials

B. impatiens experienced higher levels of mortality and sickness than *B. griseocollis* (Fig. 3). For *B. impatiens*, negative impacts increased with cardenolide concentrations, reaching LD$_{50}$ at 1000 ng µL^{-1} and 90% mortality at 2000 ng µL^{-1} ouabain at 48 h. Compared to *B. impatiens*, *B. griseocollis* exhibited a lack of mortality at 1000 ng µL^{-1} ouabain and only 15% mortality at 2000 ng µL^{-1} ouabain at 48 h. Although mortality was higher for *B. impatiens* across treatments, survival analyses supported differences in mortality only for the two highest concentrations ($P = 0.00079$ at 1000 ng µL^{-1}, $P < 0.0001$ at 2000 ng µL^{-1}; Table S5). While our sample size does not allow for finer resolution, overall *B. griseocollis* appears to tolerate ~ 5–10X higher concentrations than *B. impatiens*. The level that starts to sicken *B. griseocollis* is the same as the dosage that they avoided in behavioral assays (Fig. 2).

ATPase amino acid variation

Monarch butterflies (*Danaus plexippus*) and other milkweed insects in different taxonomic orders have acquired resistance to milkweed through target site insensitivity to cardenolides as a result of specific amino acid substitutions

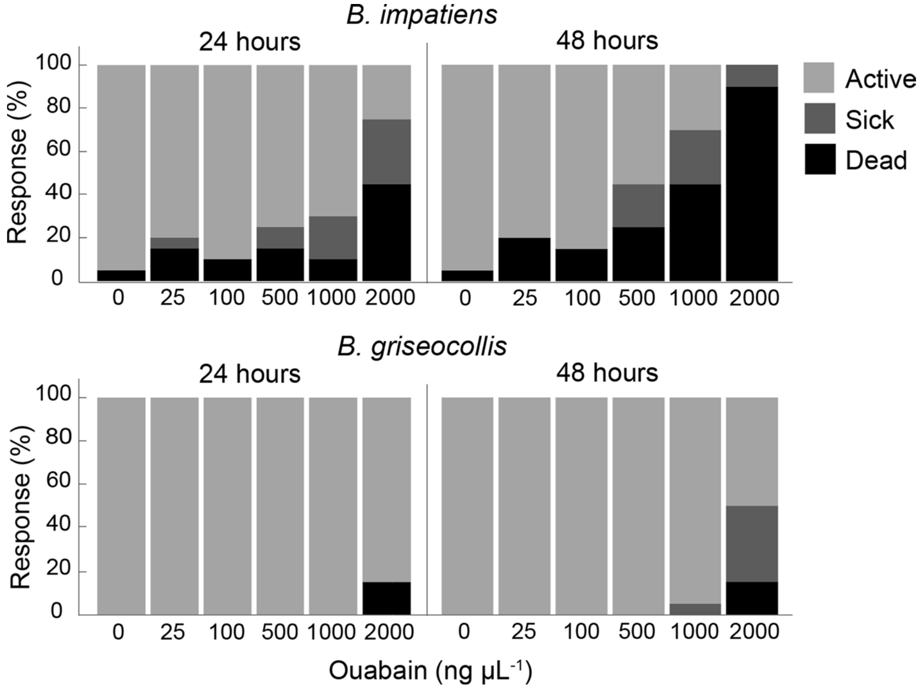

Fig. 3 Comparative mortality and sickness data in *B. impatiens* ($n = 20$/condition) and *B. griseocollis* ($n = 10$ for 0–500 ng μL^{-1} ouabain; $n = 20$ for 1000 and 2000 ng μL^{-1} ouabain) at specific time points post consumption of different concentrations of the cardenolide ouabain

(particularly at positions 111, 119 and 122) in the Na^+/K^+ ATPase (Karageorgi et al. 2019). Our sequence data from this gene (648 bp sequenced spanning this position, Genbank MT636066) reveal the same amino acid in all three of these sites for *B. griseocollis*, *B. impatiens* and *B. terrestris*, with a Q (Glutamine) at 111, N (Asparagine) at 119 and N at 122, suggesting these bases do not explain differences in tolerance between species. The amino acids present in bumble bees are similar to what is found across many non-cardenolide sequestering Hymenoptera and are not amino acids known to confer cardenolide resistance (Karageorgi et al. 2019).

Discussion

This study demonstrates a strong bias of bumble bees in their visitation to common milkweed. We have demonstrated that *B. griseocollis* is a disproportionate visitor to milkweed relative to its abundance in the field, with *B. griseocollis* dominating visitation at all four field sites across years (average 87%) at a ratio much higher than its visitation to other plants at the sites (average 14%). While our controlled design was better able to demonstrate this as a significant bias, similarly large proportions of *B. griseocollis* relative to other bumble bees were observed on milkweed in other studies (Theiss et al. 2007—98%; Betz et al. 1994—63%; Baker and Potter 2018—99%). A study by Macior (1965) collected counts of various bumble bee species on three species of milkweed vs. on other plants in the community. We performed a statistical analysis of these data similar to those performed on our own data (Fig. S2). This analysis supported highly significant differences ($\chi^2_{(6,278)} = 4764$, $P < 0.0005$) in visitation bias to milkweed, with milkweed again dominated by *B. griseocollis* (90% of common milkweed visitors, 4% of visitors on other plants). Similar proportional bias was also observed in iNaturalist data, where *B. griseocollis* occurs at a rate of 88% on milkweed in user-submitted photos, but only occurs at a frequency of 18% in eastern US bumble bee records.

In observing milkweed visitation, we noted that most non-*B. griseocollis* visitors were males and queens of other species and rarely workers. This observation may indicate that there is a learned avoidance by these species in the field, as queens and males are typically less experienced at foraging than workers. This could also be reflective of a differential tolerance to milkweed cardenolides between castes and sexes. Variation in the percentage of *B. griseocollis* is likely impacted by location of collection. For instance, a study in Maine, where *B. griseocollis* is uncommon, found alternative bumble bee species as dominant visitors (Jennersten and Morse 1991), and many of the non-*B. griseocollis* records from iNaturalist were recorded where *B. griseocolli* is rare at the northern edge of its distributional range. *B. pennsylvanicus* and *B. affinis* have been found previously in fairly high frequencies on this plant (Robertson 1886; Frost 1965; Betz et al. 1994); however, drastic declines in both of these species in the past 20 years (Cameron et al. 2011) would make more recent observations of these species unlikely.

Most notably absent on milkweed relative to their natural abundance is the most common eastern North American bumble bee, *B. impatiens*, thus drawing focus to understanding *B. griseocollis* vs. *B. impatiens* visitation biases to this plant. When dissecting the source of these visitation biases in the lab, we observed some avoidance of cardenolide (ouabain)-laced nectar by *B. impatiens* at the highest levels (1000 ng μL^{-1}) and none by another common species, *B. bimaculatus*, at any tested level. Therefore, these species did not avoid the inferred field-realistic levels of cardenolides (up to 100 ng μL^{-1}) from the literature (Manson et al. 2012). Previous research also found that *B. impatiens* individuals did not avoid field-realistic levels (100 ng μL^{-1}), but did show some avoidance at 10X this level (Jones and Agrawal 2016). This research found that whole colonies would show avoidance of cardenolides at field-realistic levels after 10 days of foraging, indicating more long-term learning was involved. Our *B. impatiens* data showed individual variance, with some individuals avoiding the highest dose and some not by 48 h, suggesting some learning may be taking place. Longer-term learning is one factor that could explain nearly complete avoidance by *B. impatiens* of milkweed in the field, but less so in the lab.

Learning is likely better in the field, given improved abilities for associative learning of toxic effects with floral cues (e.g., strong fragrance, large pinkish-white floral displays), as opposed to the more limited learning cues in our lab setting. *B. impatiens* may also be deterred innately by other milkweed cues, such as floral or plant odors or floral morphology. Tongue length in *B. griseocollis*, *B. impatiens* and *B. bimaculatus* are fairly similar, with *B. griseocollis* having lengths in between the other two species (av. worker glossa length of 4.91, 4.74 and 5.63 mm, respectively; Medler 1962); thus differences in corolla length is unlikely to explain observed field avoidance. Given that our analysis of common milkweed nectar found low cardenolide concentrations in nectar, factors in addition to cardenolides are likely to drive field bias. It will be important in future research to decouple the effects of floral morphology and floral toxins in understanding visitation bias in the field.

In contrast to what might be expected, we observed a stronger avoidance of the highest level of cardenolide by *B. griseocollis* than in the other species. *B. griseocollis* appears to be able to avoid cardenolides, but it only does so at levels for which they experience illness. At the 1000 ng μL^{-1} concentration, which they nearly completely avoided, a small percent of *B. griseocollis* showed signs of sickness, whereas at the lower levels, which they sampled equally and abundantly, there was no sickness or mortality. Interestingly, the highest cardenolide concentration was sampled in very small amounts during the first 24 h and was reduced to basically no sampling in the next two days. This suggests that *B. griseocollis* likely approaches and tastes the treatment and quickly learns to not feed thereafter, as the small amount sampled in the first day suggests limited sampling prior to avoidance. In contrast, *B. impatiens* consumed substantial amounts of cardenolides when exposed to concentrations where they experience moderate (~ 10–15% mortality, 100 ng μL^{-1}) or severe (50% mortality, 1000 ng μL^{-1}) morbidity, suggesting their ability to taste and/or remember the toxins may be reduced relative to *B. griseocollis*. Altogether, these data suggest *B. griseocollis* may have established a specialized interaction with milkweed that may be related to its ability to detect the milkweed toxins. The stronger avoidance of cardenolide by *B. griseocollis* compared to *B. impatiens* and the longer term avoidance observed in *B. impatiens* (Jones and Agrawal 2016) may indicate a difference in gustatory reception of cardenolides between the species, with *B. griseocollis* able to detect the cardenolides via taste to enable rapid learning and *B. impatiens* instead relying on longer-term associative learning with the negative consumptive effects (i.e., learning that milkweed makes them sick).

In further support of a potential adaptive association between milkweed and *B. griseocollis*, we observed *B. griseocollis* to have a markedly different ability to tolerate cardenolides compared to *B. impatiens*. *B. griseocollis* was able to handle ~ 5–10 times more concentrated cardenolides in nectar. The concentration of ouabain at which *B. griseocollis* gets sick is higher than the cardenolide levels typically found in nectar in nature ($\leq$ 110 ng μL^{-1}) in Manson et al. (2012) and much higher than the cardenolide concentrations we inferred for common milkweed (1.4 ng μL^{-1}). Levels of cardenolides in milkweed can vary substantially by plant, with stressed plants producing more cardenolides (Rasmann et al. 2009), and concentrations of cardenolide in nectar varying by species (Manson et al. 2012). The ability of *B. griseocollis* to avoid such high levels suggests that it may have an innate ability to avoid the most toxic exemplars of milkweed. Enhanced abilities to both detect and detoxify cardenolides would allow *B. griseocollis* to monopolize the abundant nectar of milkweed plants with little negative consequences. In contrast, *B. impatiens* becomes sick and dies at field realistic levels of cardenolide suggesting this can induce illness and result in toxicity-based preferences in the field. That levels which are toxic to bumble bees approach some of the highest levels found in nature may be relevant to the evolution of both the bumble bee and the plant, which may need to limit nectar toxicity to levels bumble bees can handle to ensure pollination by this important milkweed pollinator. Studying the effects of milkweed nectar itself and different types of cardenolides would help improve understanding of the extent to which these bumble bees are impacted by field concentrations, as milkweed nectar is comprised of different species–specific combinations of cardenolides (Manson et al. 2012), that each has different levels of toxicity on bees (Detzel and Wink 1993) and other insects (Petschenka et al. 2012). An examination of the visitation bias of bumble bees to different milkweed species and plants with varying concentration of cardenolides in their nectar would be valuable for analyzing the association between cardenolide content and field visitation patterns.

Resistance to toxic chemicals can be conferred by excretion, sequestration and degradation of the toxin, and/or target-site insensitivity (Després et al. 2007). Many of these methods may offer broad resistance to multiple toxins, a strategy that would be advantageous to generalist pollinators (Irwin et al. 2014). Sequestration into certain tissues using cellular transporters is common for most milkweed specialists, with an added benefit of making the insect toxic to predators (Després et al. 2007). Milkweed specialists have variably made use of changes in permeability of the perineurium around the nervous system (Petschenka et al. 2012), detoxification enzymes, such as cytochrome P450 monoxygenases (Marty and Krieger 1984) and improved metabolism (Scudder et al. 1986) to confer cardenolide resistance. For many milkweed specialists, mutations in the Na^+/K^+ ATPase pump leads to target-site insensitivity, preventing entry of cardenolides into the nervous system (Dobler et al. 2012). Our data do not support a role of protein-coding mutations in this gene in detoxification in *B. griseocollis*, at least not in the same amino acid mutations in which they have previously been detected. This would be a good system to study to improve understanding of mechanisms of detoxification in bees.

Our data support a potential role of secondary compound tolerance in driving field floral visitation preferences. Differential tolerance to these compounds can potentially serve as a means by which species can partition diverse floral resources in their environments, a form of cryptic specialization that helps to reinforce stable speciose communities. Further research should explore the role of secondary compounds, as opposed to other factors, such as tongue length, color and scent preferences, in floral resource visitation bias among bumble bee pollinators. Previous studies have shown that bumble bees are some of the most frequent and efficient pollinators of milkweeds (Jennersten and Morse 1991; Betz et al. 1994; Fishbein and Venable 1996; Kephart and Theiss 2004; Howard and Barrows 2014). As such, the common milkweed and other milkweeds may have an important coevolutionary connection with *B. griseocollis*. The monkshood plant *Aconitum* was found to have high concentrations of alkaloid toxins in pollen, thus encouraging higher fidelity pollen transfer by bumble bee specialists able to detoxify these compounds (Gosselin et al. 2013). By secreting cardenolides into their nectar, milkweed may reinforce a stronger mutualistic relationship with *B. griseocollis*, enabling higher fidelity pollination especially important in a plant species that places high-investment in pollinia for pollen transport. Furthermore, milkweeds, like *Aconitum*, produce large amounts of nectar, which can ensure that rewards outweigh the fitness cost of detoxification in specialists (Barlow et al. 2017). Frequency of pollinia captured in *B. griseocollis* compared to other bumble species did not suggest it was one of the more effective bumble bee pollinators (Macior 1965), so this interaction may not necessarily be fully mutualistic, although species-specific efficacy and the evolutionary trade-offs that govern them should be explored further. Together, these data enhance understanding of the extent to which adaptive coevolutionary interactions are involved between this toxic plant and its pollinators.

Acknowledgements Thanks to Alex Serpi, Ashley Heimann, and Swayamjit Ray for assistance in data collection and Amy Hastings for conducting the HPLC and sodium pump assays. We thank Christina Grozinger for intellectual discussion and guidance. This project was supported by an Apes Valentes Undergraduate Research Award (Center for Pollinator Research, Penn State) to EV and Penn State start-up funds for HMH.

Author contributions statement HMH, EV, and BDE conceived and designed the project. EV, EL, BDE, AA, and HMH performed experiments. JGA, BDE, EV, and HMH performed statistical analyses. EV, HMH, JGA and EL wrote the manuscript, with edits and intellectual contributions by AA and BDE. EV and HMH funded the research.

Data availability Data files from this study are available on ScholarSphere https://doi.org/10.26207/pgeq-he51.

References

Adler LS (2000) The ecological significance of toxic nectar. Oikos 91:409–420

Ali JG, Agrawal AA (2017) Trade-offs and tritrophic consequences of host shifts in specialized root herbivores. Funct Ecol 31:153–160

Agrawal AA, Petschenka G, Bingham RA, Weber MG, Rasmann S (2012) Toxic cardenolides: chemical ecology and coevolution of specialized plant–herbivore interactions. New Phytol 194:28–45

Baker AM, Potter DA (2018) Colonization and usage of eight milkweed (*Asclepias*) species by monarch butterflies and bees in urban garden settings. J Insect Conserv 22:405–418

Barlow SE, Wright GA, Ma C, Barberis M, Farrell IW, Marr EC, Stevenson PC (2017) Distasteful nectar deters floral robbery. Curr Biol 27:2552–2558

Betz RF, Struven RD, Wall JE, Heitler FB (1994) Insect pollinators of 12 milkweed (Asclepias) species. In: Proceedings of the Thirteenth North American Prairie Conference, pp 45–60.

Blüthgen N, Gottsberger G, Fiedler K (2004) Sugar and amino acid composition of ant-attended nectar and honeydew sources from an Australian rainforest. Aust Ecol 29:418–429

Brower LP, Ryerson WN, Coppinger LL, Glazier SC (1968) Ecological chemistry and the palatability spectrum. Science 161:1349–1350

Cameron SA, Lozier JD, Strange JP, Koch JB, Cordes N, Solter LF, Griswold TL (2011) Patterns of widespread decline in North American bumble bees. Proc Natl Acad Sci 108:662–667

Després L, David JP, Gallet C (2007) The evolutionary ecology of insect resistance to plant chemicals. Trends Ecol Evol 22:298–307

Detzel A, Wink M (1993) Attraction, deterrence or intoxication of bees (*Apis mellifera*) by plant allelochemicals. Chemoecology 4:8–18

Dobler S, Dalla S, Wagschal V, Agrawal AA (2012) Community-wide convergent evolution in insect adaptation to toxic cardenolides by substitutions in the Na, K-ATPase. Proc Natl Acad Sci 109:13040–13045

Duffey SS, Blum MS, Isman MB, Scudder GGE (1978) Cardiac glycosides: a physical system for their sequestration by the milkweed bug. J Insect Physiol 24:639–643

Fishbein M, Venable DL (1996) Diversity and temporal change in the effective pollinators of Asclepias tuberosa. Ecology 77:1061–1073

Frost SW (1965) Insects and pollinia. Ecology 46:556–558

Gosselin M, Michez D, Vanderplanck M, Roelants D, Glauser G, Rasmont P (2013) Does Aconitum septentrionale chemically protect floral rewards to the advantage of specialist bumblebees? Ecol Entomol 38:400–407

Goulson D (2009) Bumble bees: behaviour, conservation, and conservation. Oxford University Press, Oxford

Howard AF, Barrows EM (2014) Self-pollination rate and floral-display size in *Asclepias syriaca* (common milkweed) with regard to floral-visitor taxa. BMC Evolut Biol 13:144

Holzinger F, Wink M (1996) Mediation of cardiac glycoside insensitivity in the monarch butterfly (*Danaus plexippus*): role of an amino acid substitution in the ouabain binding site of Na+, K+-ATPase. J Chem Ecol 22:1921–1937

Inouye DW (1978) Resource partitioning in bumblebees: experimental studies of foraging behavior. Ecology 59:672–678

Inouye DW (1980) The effect of proboscis and corolla tube lengths on patterns and rates of flower visitation by bumblebees. Oecologia 45:197–201

Irwin RE, Cook D, Richardson LL, Manson JS, Gardner DR (2014) Secondary compounds in floral rewards of toxic rangeland plants: impacts on pollinators. J Agric Food Chem 62:7335–7344

Jennersten O, Morse DH (1991) The quality of pollination by diurnal and nocturnal insects visiting common milkweed *Asclepias syriaca*. Am Midl Nat 125:18–28

Jones PL, Agrawal AA (2016) Consequences of toxic secondary compounds in nectar mutualist bees and antagonist butterflies. Ecology 97:2570–2579

Karageorgi M, Groen SC, Sumbul F, Pelaez JN, Verster KI, Aguilar JM, Hastings AP, Bernstein SL, Matsunaga T, Astourian M, Guerra G (2019) Genome editing retraces the evolution of toxin resistance in the monarch butterfly. Nature 574:409–412

Kassambara A, Kosinski M, Biecek P (2019) Survminer: drawing survival curves using ‘ggplot2’. R package version 0.4.6

Kephart SR (1983) The partitioning of pollinators among three species of *Asclepias*. Ecology 64:120–133

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

Kevan PG, Eisikowitch D, Fowle S, Thomas K (1988) Yeast-contaminated nectar and its effects on bee foraging. J Apic Res 27:26–29

Macior LW (1965) Insect adaptation and behavior in *Asclepias* pollination. Bull Torrey Bot Club 92:114–126

Manson JS, Otterstatter MC, Thomson JD (2010) Consumption of a nectar alkaloid reduces pathogen load in bumble bees. Oecologia 162:81–89

Manson JS, Rasmann S, Halitschke R, Thomson JD, Agrawal AA (2012) Cardenolides in nectar may be more than a consequence of allocation to other plant parts: a phylogenetic study of *Asclepias*. Funct Ecol 26:1100–1110

Marty MA, Krieger RI (1984) Metabolism of uscharidin, a milkweed cardenolide, by tissue homogenates of monarch butterfly larvae, *Danaus plexippus* L. J Chem Ecol 10:945–956

Medler JT (1962) Morphometrics studies on bumble bees. Ann Entomol Soc Am 55:212–218

Petschenka G, Offe JK, Dobler S (2012) Physiological screening for target site insensitivity and localization of Na+/K+-ATPase in cardenolide-adapted Lepidoptera. J Insect Physiol 58:607–612

Pyke GH (1982) Local geographic distributions of bumblebees near Crested Butte, Colorado: competition and community structure. Ecology 63:555–573

Pyke GH, Inouye DW, Thomson JD (2012) Local geographic distributions of bumble bees near Crested Butte, Colorado: competition and community structure revisited. Environ Entomol 41:1332–1349

Rasmann S, Johnson MD, Agrawal AA (2009) Induced responses to herbivory and jasmonate in three milkweed species. J Chem Ecol 35:1326–1334

Richardson LL, Adler LS, Leonard AS, Andicoechea J, Regan KH, Anthony WE, Manson JS, Irwin RE (2015) Secondary metabolites in floral nectar reduce parasite infections in bumblebees. Proc R Soc B 282:20142471

Robertson C (1886) Notes on the mode of pollination of Asclepias. Bot Gaz 11:262–269

Robertson C (1887) Insect relations of certain Asclepiads. I Botanical Gazette 12:207–216

Sadd BM, Barribeau SM, Bloch G, De Graaf DC, Dearden P, Elsik CG et al (2015) The genomes of two key bumblebee species with primitive eusocial organization. Genome Biol 16:76

Scudder GGE, Moore LV, Isman MB (1986) Sequestration of cardenolides in *Oncopeltus fasciatus*: morphological and physiological adaptations. J Chem Ecol 12:1171–1187

Stevenson PC, Nicolson SW, Wright GA (2017) Plant secondary metabolites in nectar: impacts on pollinators and ecological functions. Funct Ecol 31:65–75

Theiss K, Kephart S, Ivey CT (2007) Pollinator effectiveness on co-occurring milkweeds (*Asclepias*; Apocynaceae, Asclepiadoideae). Ann Mo Bot Gard 94:505–516

Therneau TM, Lumley T (2015) Package ‘survival’. R Top Doc 128.

Vaudo AD, Patch HM, Mortensen DA, Tooker JF, Grozinger CM (2016) Macronutrient ratios in pollen shape bumble bee (*Bombus impatiens*) foraging strategies and floral preferences. Proc Natl Acad Sci 113:4035–4042

Willmer P (2011) Pollination and floral ecology. Princeton University Press, Princeton

Züst T, Petschenka G, Hastings AP, Agrawal AA (2019) Toxicity of milkweed leaves and latex: chromatographic quantification versus biological activity of cardenolides in 16 *Asclepias* species. J Chem Ecol 45:50–60