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Cardenolides in nectar may be more than a consequence of allocation to other plant parts: a phylogenetic study of *Asclepias*

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Summary

1. The primary function of secondary plant metabolites is thought to be defence against herbivores. The frequent occurrence of these same noxious compounds in floral nectar, which functions primarily to attract pollinators, has been seen as paradoxical.

2. Although these compounds may have an adaptive purpose in nectar, they may also occur as a nonadaptive consequence of chemical defence in other plant parts. If nectar chemistry reflects physiological constraints or passive leakage from other tissues, we expect that the identity and relative concentration of nectar cardenolides to be correlated with those of other plant parts; in contrast, discordant distributions of compounds in nectar and other tissues may suggest adaptive roles in nectar.

3. We compared the concentrations and identities of cardenolides in the nectar, leaves and flowers of 12 species from a monophyletic clade of *Asclepias*. To measure putative toxicity of nectar cardenolides, we then examined the effects of a standard cardenolide (digoxin) on the behaviour of bumblebees, a common generalist pollinator of *Asclepias*.

4. We found that the average cardenolide concentrations in nectar, leaves and flowers of the 12 *Asclepias* species were positively correlated as predicted by nonadaptive hypotheses. However, significant differences in the identities and concentrations of individual cardenolides between nectar and leaves suggest that the production or allocation of cardenolides may be independently regulated at each plant part. In addition, cardenolide concentrations in leaves and nectar exhibited no phylogenetic signal.

5. Surprisingly, bumblebees did not demonstrate an aversion to digoxin-rich nectar, which may indicate that nectar cardenolides have little effect on pollination.

6. Although the idea that discordant patterns of secondary metabolites across tissue types may signal adaptive functions is attractive, there is evidence to suggest constraint contributes to nectar secondary chemistry. Further work testing the ecological impacts of such patterns will be critical in determining the functional significance of nectar cardenolides.

Key-words: *Asclepias*, cardenolide, milkweeds, optimal defence theory, pollinator preference, secondary metabolites, toxic nectar

Introduction

To defend themselves against herbivores, many plants produce noxious compounds that are distasteful, deterrent and often deleterious to consumers (Rosenthal & Berenbaum 1991). Although secondary metabolites have been identified and quantified in the leaves of a vast number of plant species, these compounds are also common in roots, stems and flowers (Van der Putten *et al.* 2001; McCall & Irwin 2006), where they similarly appear to function as defences against herbivores. Secondary metabolites are also frequently found in floral nectar, where their function is more ambiguous.

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Nectar secondary metabolites are a widespread if paradoxical phenomenon reported in at least 21 angiosperm families (Adler 2000). The consequences of consuming these 'toxic' nectars for nectarivores, such as insects and birds, range from putative addiction (e.g. when alkaloids such as nicotine and caffeine are found in trace amounts; Singaravelan *et al.* 2005) to death (e.g. after a honey bee consumed 20 μL of nectar from *Saphora microphylla* flowers; Clinch, Forster & Palmerjo 1972). There are several hypothesized adaptive functions for secondary metabolites in nectar: they may defend nectar from inefficient pollinators and nectar robbers, prevent nectar spoilage because of microbial growth, or increase visitation by specialized pollinators (Baker & Baker 1975; Rhoades & Bergdahl 1981; Adler 2000). Alternatively, secondary metabolites may occur in nectar from passive leakage of compounds into the nectary during vascular transport, the systemic regulation of phytochemical biosynthesis, or because of genetic correlations with leaf secondary metabolites, making their presence in nectar a nonadaptive consequence of foliar defence (*sensu* Adler 2000).

By comparing the secondary metabolite profiles of different plant parts, we can identify patterns of regulation and allocation, which will help to tease apart adaptive vs. nonadaptive hypotheses for their presence (McKey 1974, 1979; Kessler & Halitschke 2009). Although recent work has adopted a more comparative whole-plant approach to understanding plant chemical defences (e.g. Van der Putten *et al.* 2001; Bezemer & van Dam 2005; Rasmann *et al.* 2009), few studies have included the secondary chemistry of floral structures and floral rewards (but see Detzel & Wink 1993; Adler 2000; Adler *et al.* 2006; Kessler, Gase & Baldwin 2008; Kessler & Halitschke 2009).

In this study, we survey the patterns of nectar cardenolides across 12 species of *Asclepias*. Cardenolides are toxic steroids that bind to the sodium–potassium pumps necessary for the functioning of animal cells and are considered an effective plant chemical defence against herbivores (Malcolm 1991; Agrawal *et al.* 2012). We quantified the concentrations of individual cardenolides, along with the sum total of these cardenolide concentrations, in the nectar, leaves and flowers of species from a monophyletic group known to vary in leaf cardenolide concentration (Agrawal, Lajeunesse & Fishbein 2008). We then used cardenolide profiles to evaluate the correlations of the identities and concentrations of cardenolides across plant parts in the 12 species. We interpret correlations between nectar and other plant parts in the concentrations and identities of compounds as supporting a nonadaptive hypothesis, likely indicative of physiological, morphological or phylogenetic constraints (Adler *et al.* 2006; Kessler & Halitschke 2009). Alternatively, differences in cardenolide profiles between plant parts, such as the presence of compounds unique to nectar, may indicate localized regulation of cardenolide production or allocation, suggestive of adaptive function (Rhoades & Bergdahl 1981; Strauss *et al.* 1999; Adler *et al.* 2006). Using phylogenetic information, we can also examine whether patterns in cardenolide concentrations exhibit a phylogenetic signal, or if instead these patterns are independent of phylogenetic relationships. Finally, we used artificial nectar enriched with a commercially available cardenolide to evaluate its effect on pollinator behaviour and determine whether nectar cardenolides have an ecological impact of deterring pollinators.

Specifically, we asked the following questions: (i) How do total cardenolide concentrations vary across the plant parts of 12 *Asclepias* species and are these concentrations positively correlated?, (ii) How do individual nectar cardenolide concentrations and composition compare between *Asclepias* nectar and leaves?, (iii) Are differences in the concentrations of cardenolides between leaves and nectar constrained by evolutionary history in a monophyletic clade? and (iv) Does nectar containing the cardenolide digoxin affect the foraging behaviour of bumblebees, a generalist pollinator of *Asclepias*?

Materials and methods

STUDY SYSTEM

Milkweeds (*Asclepias* spp.) are a classic system for studying plant chemical defences. This plant family has evolved a number of physical and chemical defences against a suite of generalist and specialist herbivores (Agrawal & Fishbein 2006). Milkweeds are often defended by cardenolides, bitter-tasting compounds that elicit aversive or emetic responses in both vertebrates (Brower *et al.* 1968) and invertebrates (Dussourd & Hoyle 2000). Cardenolides affect animals by inhibiting the ubiquitous sodium–potassium cellular pump Na+/K-ATPase (Malcolm 1991). They are poisonous to generalists at very low doses, yet can be tolerated and even co-opted for use as a chemical defence by specialists (Dussourd & Hoyle 2000; Agrawal *et al.* 2012).

Cardenolide biosynthesis, in which sterol precursors are modified into the **5b** cardenolide genins that form the backbone of each compound, has not been fully characterized in plants and is hypothesized to depend on more than one pathway (reviewed in Agrawal *et al.* 2012). Cardenolide activity is primarily determined by the steroid nucleus and its stereochemistry, but the lactone and sugar side chains can modify compound selectivity and interactions with Na+/K-ATPase (Repke 1985; Paula, Tabet & Ball 2005). Structural differences, such as the number of glucose, CH_3 or OH groups, between individual compounds affect polarity, toxicity and rate of absorption by animals postconsumption (Malcolm 1991; Agrawal *et al.* 2012).

The concentrations of cardenolides in leaves vary substantially across the genus *Asclepias* (Agrawal & Fishbein 2006; Agrawal, Lajeunesse & Fishbein 2008) and can also vary among plant parts such as the leaves, roots, pith and epidermis within the same plant (Nelson, Seiber & Brower 1981; Fordyce & Malcolm 2000; Rasmann *et al.* 2009). Although *Asclepias* nectar is reportedly toxic to honeybees (Pryce-Jones 1942), cardenolides have not previously been reported in the floral nectar.

The genus *Asclepias* is a monophyletic group (Agrawal & Fishbein 2008) composed of about 135 species found in the Americas (Woodson 1954; Agrawal & Fishbein 2008; Fishbein *et al.* 2011). We selected 12 closely related species in the series Incarnatae (Fig. 1, Table 1), as this is a monophyletic group with relatively well-resolved phylogenetic relationships (Agrawal & Fishbein 2008) and significant variation in leaf cardenolide concentrations among species (Agrawal, Lajeunesse & Fishbein

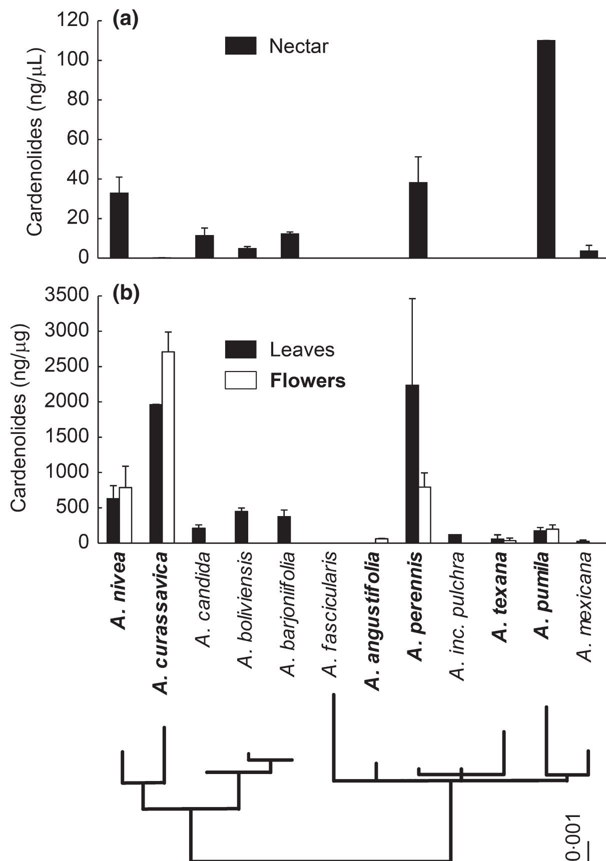

Fig. 1. Average gross cardenolide concentrations (±SE) for (a) nectar, and (b) leaves (black bars) and flowers (white bars). Species names in bold indicate that flowers were collected and analysed for cardenolides. Sample sizes can be found in Table 1. Below the graphs is a molecular phylogeny of the 12 focal *Asclepias* species based on data from Agrawal & Fishbein 2008, where posterior probabilities can also be found.

Table 1. The number of individual cardenolides found in nectar and leaf samples from the 12 focal *Asclepias* spp., along with number of compounds that are found in both plant parts

Species	Nectar cardenolides (*n*)	Leaf cardenolides (*n*)	Shared cardenolides
A. angustifolia	0 (5)	0 (2)	N/A
A. barjoniifolia	4 (3)	5 (2)	3
A. boliviensis	5 (6)	7 (2)	3
A. candida	4 (3)	7 (2)	3
A. curassavica	0 (8)	11 (2)	N/A
A. fascicularis	0 (7)	0 (1)	N/A
A. incarnata ssp. *pulchra*	0 (2)	1 (1)	N/A
A. mexicana	1 (2)	1 (2)	0
A. nivea	11 (7)	13 (2)	9
A. perennis	9 (4)	16 (2)	8
A. pumila	7 (1)	6 (2)	4
A. texana	0 (4)	2 (3)	N/A

n indicates the number of samples analysed; note that nectar samples represent pooled nectar collections (see Materials and methods).

2008). Each plant was grown from seed and maintained in one of two greenhouse collections at Cornell University; seeds were collected by the authors, colleagues or from native plant nurseries and, once in bloom, we used flowers to verify species identity.

CARDENOLIDE SAMPLING AND QUANTIFICATION

We collected samples to analyse constitutive cardenolide concentrations on five occasions between July 2007 and August 2008. The frequency and timing of nectar collections depended on the flowering time of the different species. We collected nectar from all plants in flower, making collections one to three times per species over the course of the sampling period. We also collected whole mature leaves from all 12 species. Leaves were collected after taking nectar samples to avoid potential induction of cardenolides in nectar or leaves as a result of the leaf removal. After completing nectar sampling, we collected whole flowers in six of the 12 species. We were unable to collect flowers from all species because although nectar collection rarely resulted in damage that affected floral nectar quality, manipulation of flowers often led to petal damage that may have altered cardenolides in floral tissue.

We primarily collected nectar from flowers using 5-μL graduated microcapillary tubes, although several species (*Asclepias angustifolia*, *A. curassavica* and *A. nivea*) produced nectar so copiously that we used a 200-μL microcapillary tube for sample collection. We took every precaution to ensure that we caused no damage to the nectary, as this could induce cardenolide production; fortunately, damage can be visually detected in *Asclepias* because of the exudation of latex, which is rich in cardenolides (Zalucki, Brower & Alonso 2001). On the few occasions where damage did occur, samples were discarded. Because most plants produce very little nectar per flower, we pooled nectar; samples were pooled daily within individual plants, with each sample containing between 20 and 230 μL of nectar, representing tens to hundreds of individual flowers. Nectar was added to 500 μL of 70% ethanol (following Blüthgen, Gottsberger & Fiedler 2004) and stored at −80 °C prior to analysis. After nectar samples were pooled, we had between one and eight samples per species from 1 to 2 individual plants each. We then harvested leaves and flowers from a subset of plants also used for nectar sampling (for a total of 1 to 3 leaf and flower samples per species, collected as single leaves or umbels, respectively) and froze them immediately at −80 °C. Because our nectar samples typically lack independence (although temporally separated, they were often collected from the same plants), they cannot be considered true replicates. Therefore, although we analysed each sample separately, the data were combined to create composite profiles of cardenolide identities and concentrations for each of the 12 focal species.

We used high-performance liquid chromatography (HPLC) to quantify cardenolides in nectar, leaf and flower samples, adapting a protocol from Zehnder & Hunter (2007). We prepared nectar samples for extraction by drying down all water and ethanol from the stored samples using a rotary evaporator (Labconco, Kansas City, MO, USA). We extracted the residuum with 1 mL of 100% methanol and added 10 μL of a 0·2 g/L solution of the cardenolide digitoxin as an internal standard, which allowed for the direct comparison of unknown cardenolide peaks to a known cardenolide of predetermined quantity. Digitoxin is a common standard used when quantifying cardenolides using chromatography and is not known to be produced by *Asclepias*. We let the samples shake gently for 24 h and then centrifuged them for 30 min at 19722 rcf and 15 °C, removing the supernatant containing the dissolved cardenolides for further analysis. In some cases, preliminary analysis of nectar samples had very low cardenolide yield, so where necessary, we pooled material yet again to increase our power to detect cardenolides. For leaf and flower samples, we ground fresh

tissue in liquid nitrogen and added 10 μL of 2 g/L digitoxin to each sample, at *c.* 100 mg of tissue (fresh mass). We extracted the samples with 1 mL of 100% methanol using FastPrep® homogenization (MP Biomedicals, Solon, OH, USA) to rapidly lyse cells set at 5·0 m/s for 30 s. Flower and leaf samples were spun down as with nectar samples, but we did not further concentrate the material.

Cardenolides were analysed on an Agilent 1100 series HPLC using a Gemini C18 reversed-phase column (3 mm, 150 × 4·6 mm; Phenomenex, Torrance, CA, USA). We injected 15 mL of extract, which was eluted at a constant flow rate of 0·7 mL/min with a solvent gradient of 0·25% phosphoric acid in water and acetonitrile as follows: 0–5 min with 20% acetonitrile, followed by a constant increase to 70% acetonitrile until 20 min; steady elution for 20–25 min with 70% acetonitrile; followed by a constant increase to 95% acetonitrile until 30 min; and hold at 95% acetonitrile until 35 min. UV absorption spectra were recorded from 200 to 400 nm and cardenolides were quantified by integrating the peak area at 218 nm. Each HPLC run included several blanks, containing only methanol and controls, comprising of methanol plus a known concentration of digitoxin. Cardenolides are identifiable from other compounds by a single symmetrical peak that absorbs between 217 and 222 nm (Zehnder & Hunter 2007; Rasmann, Johnson & Agrawal 2009), and we confirmed this by checking the shape and absorption of the peak in control samples containing only digitoxin. The amounts of cardenolides present were then calculated relative to the peak area of the digitoxin internal standard.

Because the concentrations of cardenolides in nectar are often very low, true peaks can be difficult to distinguish from noise. Therefore, we defined a detection threshold and considered all peaks with uncorrected peak areas of more than 15 absorbance units, representing >50 ng of cardenolides, as true peaks. Peaks that fell below this threshold were only considered true peaks if they met the following conditions: (i) the peak shape was extremely symmetrical, and (ii) a peak had been detected at that retention time in the nectar of at least two other species. Final cardenolide concentration estimates were calculated as nanograms of cardenolides per microlitre of nectar or per microgram of fresh tissue collected.

POLLINATOR BEHAVIOUR

Large-bodied Lepidoptera and Hymenoptera, such as monarch butterflies, honeybees and bumblebees, are effective pollen vectors for most milkweed species (Woodson 1954; Wyatt & Broyles 1994). We used a commercially supplied *Bombus impatiens* (Biobest Canada, Leamington, ON, USA) colony for our behaviour experiments. We created an artificial nectar solution by mixing 30% w/w sucrose with the cardenolide digoxin (92% HPLC grade; Sigma, St. Louis, MO USA). Ideally, we would employ cardenolides from *Asclepias* for these experiments, but this was not feasible because of limited availability of plant tissue and the prohibitive costs associated with purifying cardenolides. We therefore used digoxin, an affordable, commercially produced cardenolide, as a proxy for *Asclepias* nectar cardenolides. Digoxin, found in *Digitalis* spp., causes 50% mortality in honeybees when ingested at concentrations of 0·5% or approximately 5 ng/μL (nectar consumed *ad libitum* for 48 h), but did not deter honeybees at concentrations of 1% or *c.* 10 ng/μL (Detzel & Wink 1993). Digoxin differs from digitoxin, the standard used in HPLC analyses, in that it is a more polar compound and is therefore more representative of the cardenolides found in *Asclepias* nectar.

Preliminary studies showed that bumblebees did not find digoxin deterrent at concentrations of 10 or 50 ng/μL (J. S. Manson, unpublished data). We therefore prepared three solutions for testing pollinator response to cardenolides in nectar: 100, 250 and 1000 ng/μL digoxin. We mixed nectar solutions every 2 days, refrigerating unused portions at 4 °C for no more than 24 h.

We examined pollinator behaviour using methods reported by Gegear, Manson & Thomson (2007). In short, marked worker bees were trained to associate artificial flower colour (either blue or yellow) with one of two nectar types, either artificial nectar composed of 30% sucrose or containing both sucrose and digoxin. Artificial flowers were constructed from microcentrifuge tubes with the caps removed and spray-painted polystyrene squares measuring *c.* 3 × 3 cm. Bees foraged freely as a group on alternating monotypic training arrays of each flower type, but experiments were conducted with single foragers. The association between flower colour and nectar condition was randomized among bees to control for any potential bias because of innate colour preferences. Immediately following training, individual bees foraged on a mixed array with 30 flowers of each type for at least 80 flower visits. We filled flowers with 2 μL of nectar and refilled each flower immediately after it was drained during the trial. After an individual worker had completed the minimum visit number, she was captured and terminated. We replaced flowers between individual bees to remove any scent marks left on artificial flowers after foraging, which might deter nectar collection. We evaluated a total of 24 individual foragers at three digoxin concentrations: 100 ng/μL (n = 10), 250 ng/μL (n = 8) and 1000 ng/μL (n = 6). All foraging bouts were videotaped and subsequently analysed using JWatcher Video Version 1.0 (Blumstein & Daniel 2007).

We assessed the effect of nectar cardenolides on two behaviour parameters, pollinator preference and flower-handling proficiency. We quantified preference by counting the number of visits to flowers with cardenolide-enriched and cardenolide-free nectar. A visit was defined as an event where a bee entered and imbibed nectar from an artificial flower. To evaluate the effect of nectar cardenolides on flower-handling proficiency, we identified bees with a significant preference for either nectar cardenolides or control nectar, as determined by G-tests and examined a series of sequential visits from these individual bees, calculating both the average visit duration and the rate of flower visitation.

DATA MANIPULATION

We analysed quantitative differences in cardenolides between plant parts by summing the concentrations of all individual cardenolides within each sample, then averaging this summed total across all within-species samples for each plant part, creating an average gross cardenolide concentration (ng/μg for leaves and flowers or ng/μL for nectar). Sampling methods differed for each plant part. Leaf and flower samples were only collected from plants in flower and were taken either from two different plants on the same date or at two collection dates months apart. To minimize pooling of nectar, so as to have as many opportunities as possible to capture cardenolide diversity, nectar samples were often taken from different inflorescences on the same plant or from the same plant during different flowering periods. Despite this lack of independence, using an average over all samples provides the most representative quantitative data for total nectar cardenolides. Quantitative data are therefore reported and analysed as means per species, with variances calculated across all available samples from that species.

Different samples often varied in the identity and concentration of individual compounds. Cardenolide peaks were considered to represent the same compound when their retention times were within 0·1 min of each other. For our primary qualitative analysis, we created 'cardenolide profiles' for the nectar and leaves of each of the 12 species examined. Due to the limited number of species we were able to sample, we omitted flowers from these analyses. We created these profiles by pooling all within-species samples of nectar and leaves. We then summed the mass of each individual cardenolide across those samples and divide each sum by the total

cardenolides within the samples, calculating the proportion that each individual cardenolide comprises for the total nectar or leaf cardenolides of each species (See Supporting information). The cardenolide profiles then locate each species' nectar and foliage in a multidimensional space, such that plant parts with similar profiles are close together.

STATISTICAL ANALYSIS

Quantitative cardenolide analysis

Because many individual cardenolides were not present in all samples, the data set contains many zeros. The distribution of estimated concentrations could not be made normal via data transformation, so we chose nonparametric analyses. First, we assessed whether the average gross nectar cardenolides differed between the 12 species using a Kruskal–Wallis test. We then tested for positive correlations in average gross cardenolide concentrations between leaves, nectar and flowers of the same species using Kendall's rank correlation.

Phylogenetic signal, evaluated using Pagel's l (Pagel 1999, 2007), was estimated using methods outlined in Rasmann *et al.* (2009). A value of 1 indicates phylogenetic signal consistent with the tree topology and a random walk model (i.e. trait similarity is directly proportional to the extent of shared evolutionary history), while a value of 0 indicates no influence of shared ancestry on trait values (i.e. independence of the trait from the phylogeny). A value of l is estimated using maximum likelihood in a generalized least squares framework, with the estimated value compared statistically with models where it is set to either 0 or 1 (Pagel & Meade 2007).

Qualitative cardenolide analysis

We compared the total number of individual cardenolides detected in leaves and nectar in the 12 species (Table 1) using a generalized linear model with a Poisson error distribution and tested for a correlation in compound number between plant parts using Kendall's rank correlation. We then examined differences in chemical polarity, a characteristic that affects the mobility and absorbency of compounds. Highly polar cardenolides are poorly absorbed by animals and less mobile within plants, while less polar cardenolides are absorbed quickly and are highly mobile (Malcolm 1991); because reverse phase HPLC filters compounds based on chemical polarity, retention time is a reasonable surrogate for their polarity. Highly polar compounds have short retention times and less polar compounds have longer retention times (Rasmann, Johnson & Agrawal 2009). We examined the average retention time of species with detectable cardenolides in both leaves and nectar, comparing differences across species and between plant parts. We weighted the retention times of individual compounds by the proportion of the sample's total cardenolide concentration that each compound represented (Fordyce & Malcolm 2000) and analysed the weighted retention times using a generalized linear model with a Gaussian distribution.

Comparing the concentrations of individual cardenolides across species and plant parts required a multivariate approach. Because of fundamental differences in the physical structure of nectar (solution) and leaves (plant tissue), we converted raw cardenolide concentrations into relative concentrations, or the proportion that the cardenolide contributed to the total cardenolide concentration of the sample, thus reducing spurious associations driven by absolute concentration differences in plant parts. We used a two-dimensional nonmetric multidimensional scaling (NMDS) ordination to order nectar and leaf tissues from each species (entities) by the similarity of their cardenolide profiles (attributes). The ordination used a Bray–Curtis dissimilarity index and was conducted in R's vegan package (Oksanen 2009). We visually evaluated stress plots of ordinations with increased dimensions to assess the optimal dimensionality of the ordination. We removed samples that had no detectable cardenolides, as their positions in an ordination are undefined; these samples included not only the leaves and nectar of *A. angustifolia* and *A. fascicularis*, but also the nectar samples of *A. curassavica*, *A. incarnata* ssp. *pulchra* and *A. texana*, leaving some of the leaf samples in the ordination unpaired. The NMDS therefore includes ten species where leaf cardenolides were detected and seven species where nectar cardenolides were detected. To statistically compare leaf and nectar cardenolide composition, we conducted two separate paired Wilcoxon signed rank tests for the seven species' axis scores on the first and second ordination axes.

Pollinator behaviour analysis

To assess pollinator preference for flowers with or without nectar cardenolides using G-tests, we evaluated whether the visitation rate of individual bees deviated from a random visit frequency of 0·5. We then performed a G-test on the visitation rates of all individuals within each of the three cardenolide concentrations to assess the heterogeneity of the preferences across each treatment (Sokal & Rohlf 1995).

We examined the effect of nectar cardenolides on the foraging speed of bees with significant individual preferences for either sucrose-only nectar ($n = 9$) or nectar-containing digoxin ($n = 8$) using the average length of 6–10 consecutive visits to the same flower type and comparing flower-handling time and foraging rate (visits/min). Because there were no significant effects of cardenolide concentration on mean visit length or foraging rate, we pooled data across the three cardenolide treatments, allowing us to compare differences in foraging efficiencies between nectar with and without cardenolides. We next analysed these data using a generalized linear model, with radial cell length, a proxy for bee size, as a covariate (bee size is known to affect foraging; Pyke 1978). Data were transformed to meet assumptions of normality when necessary. Analyses were performed in R (version 2.10.1; 2009).

Results

QUANTITATIVE CARDENOLIDE ANALYSIS

The concentration of average gross cardenolides per microlitre of *Asclepias* nectar differed substantially among species ($\chi^2 = 37{\cdot}7$, d.f. = 11, $P < 0{\cdot}001$; Fig. 1a), with five of the 12 species having no detectable nectar cardenolides. Species that had quantifiable nectar cardenolides showed a 30-fold difference among species. Only seven of the 104 nectar cardenolide peaks that are reported in this study fell below our detection threshold but still met our criteria for true peaks, making exceptions to our threshold rare (see Materials and methods). Neither the nectar nor leaf samples showed any evidence for phylogenetic signal; the maximum likelihood estimate of λ was 0 for both traits, and these estimates were significantly <1 ($P < 0{\cdot}01$).

The cardenolide concentration for nectar samples, averaged across all 12 species, was $14{\cdot}71 \pm 3{\cdot}22$ ng/μL, while the average cardenolide concentration was $0{\cdot}54 \pm 0{\cdot}18$ ng/μg in leaves and $0{\cdot}76 \pm 0{\cdot}41$ ng/μg in flowers (Fig. 1b). Comparing total cardenolide concentrations

between nectar and other plant parts is difficult because of the inherent physical differences between nectar and leaf or flower tissue. However, if we crudely estimate that 1 μL of nectar weighs 1000 μg (based on the mass to volume conversion for water), gross nectar cardenolides are approximately 0·015 ng/μg, or 35-fold lower in concentration than gross leaf cardenolides.

There was a significant positive correlation between average gross cardenolide concentrations in the nectar and leaves among the 12 species examined ($n = 12$, $\tau = 0{\cdot}48$, $P = 0{\cdot}02$, Fig. 2). This correlation is not driven by the high nectar cardenolide concentration in *A. pumila*, nor is it driven by the high leaf cardenolide concentration of *A. perennis*, as the relationship maintains significance when *A. pumilla*, *A. perennis*, or both outliers are removed (without *A. pumilla*: $n = 11$, $\tau = 0{\cdot}60$, $P = 0{\cdot}006$; without *A. perennis*: $n = 11$, $\tau = 0{\cdot}41$, $P = 0{\cdot}046$; without *A. perennis* and *A. pumilla*: $n = 10$, $\tau = 0{\cdot}51$, $P = 0{\cdot}03$). Although we found a significant correlation between cardenolides in leaves and flowers ($n = 6$, $\tau = 0{\cdot}73$, $P = 0{\cdot}03$), there was no correlation between nectar and flower cardenolides ($n = 6$, $\tau = 0{\cdot}10$, $P = 0{\cdot}38$), but this may be due to the small number of flower samples. Leaf and nectar cardenolide concentrations also showed positive correlated evolution (Phylogenetic generalized least squares analysis: $n = 12$, $r = 0{\cdot}593$, Likelihood Ratio = 5·2, $P = 0{\cdot}023$).

QUALITATIVE CARDENOLIDE ANALYSIS

Across the 12 species in this study, and of the initial 104 cardenolide peaks, we identified thirty unique cardenolides, as defined by their retention time (see Table S1, Supporting information). Nectar contained fewer cardenolides than leaves ($F_{1,10} = 14{\cdot}53$, $P < 0{\cdot}01$), but the number of distinct compounds was positively correlated between the plant parts ($n = 12$, $\tau = 0{\cdot}59$, $P = 0{\cdot}006$); in other words, species with high diversity of unique compounds (e.g. *A. nivea* and *A. perennis*) also had many leaf and nectar cardenolides, while species with no detectable leaf cardenolides (*A. angustifolia* and *A. fascicularis*) also lacked nectar cardenolides (Table 1).

Although there was substantial overlap in the cardenolide composition of nectar and leaves, differences in individual compound identity and concentration were common (Table 1, Fig. S2, Supporting information). A single cardenolide, detected as a peak at 14·1 min, was unique to *Asclepias* nectar and found in both *A. pumila* and *A. perennis*. Conversely, nearly one-third of the compounds (9 of 30) found in leaves were not detected in nectar samples. The compounds that were missing from nectar were scattered throughout the range of cardenolide retention times and were found at a range of concentrations in leaves, suggesting that compounds were not simply produced in low concentrations throughout the plant. Eighteen individual cardenolides could be detected in both leaves and nectar, but the cardenolide profiles of nectar overlapped with those of leaves by only 68%, on average (calculated as the sum of all compounds present in nectar that are also present in leaves, divided by the total amount of cardenolides present in nectar samples and averaged across species).

The average weighted retention time, which estimates polarity, did not differ between leaves and nectar ($F_{1,14} = 2{\cdot}10$, $P = 0{\cdot}17$). There was also no difference in average weighted retention time between the seven species with detectable cardenolides in both their nectar and leaves ($F_{6,9} = 1{\cdot}925$, $P = 0{\cdot}19$).

Our NMDS ordination analysis revealed that the cardenolide profiles of nectar were, in general, more similar to each other than to the cardenolide profiles of leaves from the same species; in other words, nectar cardenolides appear to differ qualitatively from leaf cardenolides (Fig. 3). When we analysed the paired values for each species on X and Y ordination axes, we found that the differentiation between leaves and nectar was statistically significant for each axis (Wilcoxon rank sum test:

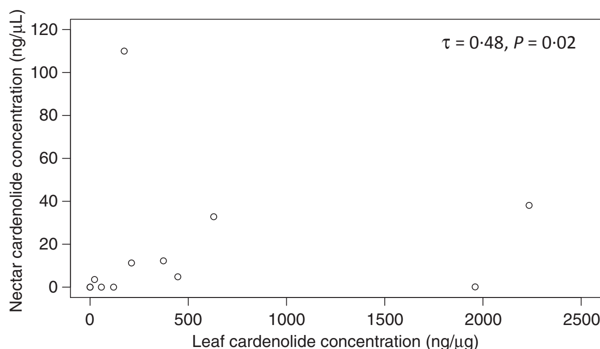

Fig. 2. Correlation between leaf and nectar cardenolide concentrations. Each point represents paired concentration data for the average cardenolide concentrations of leaf and nectar samples from a single plant.

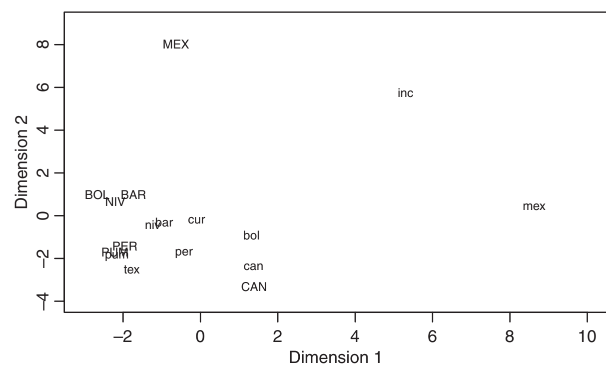

Fig. 3. Nonmetric multidimensional scaling two-dimensional ordination of the individual carndenolide concentrations of *Asclepias* nectar and leaves. Nectar samples are denoted by upper case letters, while lower case denotes leaf samples (see Fig. 1 for species abbreviations). Leaves and nectar with no detectable cardenolides could not be included in the ordination (see Materials and methods for more information).

Dimension 1: $Z = -2{\cdot}197$, $P = 0{\cdot}028$, Dimension 2: $Z = 2{\cdot}028$, $P = 0{\cdot}043$).

Five of the seven species with both leaf and nectar data have consistent directionality, with nectar samples closer to the axes and leaf samples clumped centrally in the plot; this trend is driven by the reduction in the number of individual compounds found in nectar relative to leaves. A sixth species, *A. pumila*, is represented by nectar and leaf samples that sit nearly on top of each other; this is because of an equal number of compounds in the species' nectar and leaves. The three points that fall near the edge of the ordination represent species with only a single detectable cardenolide. Interestingly, the leaf samples from *A. mexicana* and its paired nectar sample differ in their single cardenolide, but the two compounds were not unique to this species.

POLLINATOR BEHAVIOUR ANALYSIS

Seventeen of the 24 individual bees in the behaviour experiment foraged nonrandomly with respect to nectar cardenolides, but some actually preferred them, suggesting that cardenolides were not distasteful as we predicted (Fig. S1, Supporting information). When presented with artificial nectar containing 100 ng/μL, three individuals demonstrated a significant preference for sucrose-only nectar, two individuals preferred digoxin-rich nectar and five individuals foraged randomly; these bees were highly heterogeneous in their preferences ($G_H = 56{\cdot}01$, d.f. = 9, $P < 0{\cdot}001$).

For bees choosing from either nectar containing 250 ng/μL digoxin or nectar containing only sucrose, three individuals had a significant preference for control nectar, while five individuals preferentially foraged on digoxin-rich nectar; in this treatment, the overall behavioural responses were also extremely heterogeneous ($G_H = 400{\cdot}25$, d.f. = 7, $P < 0{\cdot}001$). Finally, when bees had the option of either sucrose-only artificial nectar or nectar spiked with 1000 ng/μL digoxin, three bees chose to forage on control nectar, two bees foraged randomly and a single bee showed a preference for nectar cardenolides; bees in this treatment were very heterogeneous in their foraging decisions ($G_H = 136{\cdot}90$, d.f. = 5, $P < 0{\cdot}001$).

Foraging preferences did not affect foraging rate or flower-handling time. Neither mean visit length ($F_{1,15} = 1{\cdot}3$, $P = 0{\cdot}21$) nor number of visits per minute ($F_{1,15} = 1{\cdot}12$, $P = 0{\cdot}28$) differed between bees that preferred control nectar and bees that instead preferred nectar-containing digoxin.

Discussion

Nectar cardenolides among the 12 studied *Asclepias* species vary in concentration, identity, number and chemical polarity. Despite correlations between cardenolide concentrations across plant parts, differences in total and individual cardenolide concentrations, as well as the identity of nectar cardenolides imply that mechanisms located within the nectary itself or adjacent structures drive production or allocation of these toxins in floral nectar. In every species where nectar cardenolides were detected, their concentrations in nectar were lower than those found in leaves and flowers (Fig. 1). However, variation in the identity and concentration of *individual* cardenolides found in nectar and leaves of the same plant (Table 1) indicate that nectar has a distinct chemical composition, which may be indicative of different selection pressures for cardenolide expression in nectar. Thus, the similarities and differences in the cardenolide profiles of *Asclepias* nectar, leaves and flowers strongly suggest that secondary metabolites in nectar are the results of both adaptive and nonadaptive factors.

ARE NECTAR CARDENOLIDES ADAPTIVELY REGULATED?

Individual cardenolides found in the nectar but absent from the leaves represented a large fraction of the total cardenolide concentration in each nectar sample. In addition, a single cardenolide found in *A. pumila* and *A. perennis* was detected in floral nectar but was not found in leaves or flowers and may therefore have a novel biosynthetic source or function. This unique nectar cardenolide falls well above the detection threshold and represented more than 10% of total nectar cardenolides in samples where it was present, making it unlikely that the compound was simply too depauperate to be detected in leaves. In addition, the unique nectar cardenolide was detected in samples collected 7 months apart, suggesting that compound production was not anomalous. McKey (1974, 1979) suggested that the heterogeneous allocation of secondary metabolites within a plant is the signature of regulated distribution of these compounds, likely in response to selection by an antagonist and the relative value of each plant part. We therefore speculate that, in spite of the reductions in quantity and diversity, the patterns observed in cardenolide profiles provide preliminary evidence for independent regulation and expression of secondary metabolites at or near the nectary.

Phylogenetic analyses indicate that evolutionary history does not constrain the relationship between total constitutive leaf and nectar cardenolides in the 12 *Asclepias* species examined here. This result is consistent with two previous studies that found no phylogenetic signal in *Asclepias* leaf cardenolides (Agrawal, Lajeunesse & Fishbein 2008; Rasmann *et al.* 2009), but inconsistent with two other studies using larger samples sizes that found a phylogenetic signal in the leaf cardenolide concentrations of 35 (Agrawal, Salminen & Fishbein 2009) and 49 *Asclepias* species (Rasmann & Agrawal 2011), respectively. Owing to the limited number of species examined here, our own work may lack the power required to detect a significant phylogenetic signal in leaf cardenolides (see Rasmann *et al.* 2009). Nonetheless, a closer examination of evolutionary patterns in nectar cardenolides reveals that three pairs of

very close relatives have highly divergent nectar cardenolide concentrations (Fig. 1). While *A. pumila*, *A. perennis* and *A. nivea* are rich in nectar cardenolides, their closest relatives in this study, *A. mexicana*, *A. incarnata* and *A. curassavica*, respectively, have some of the lowest detectable nectar cardenolide levels of the 12 species examined. This large disparity between closely related taxa may indicate rapid evolution of nectar defences because of divergent selection regimes.

EVIDENCE THAT NECTAR CARDENOLIDES ARE NONADAPTIVE

Despite the suggestion of evolutionary divergence in nectar cardenolides discussed previously, we found positive raw correlations between the gross cardenolide concentrations in nectar, flowers and leaves, suggesting shared regulation of cardenolide biosynthesis or allocation (Fig. 2). Nonetheless, little is known about cardenolide biosynthesis in *Asclepias* (Agrawal *et al.* 2012), and thus, it is unclear how changes in particular structures could be achieved while being constrained by overall flux through the biochemical pathways. The notion of shared regulation is supported by a large amount of overlap in the identity of individual cardenolides in the leaves and nectar of nearly all *Asclepias* species (Table 1). Taken together, it appears that nectar cardenolides are like most traits: at some level constrained by either physiology or evolutionary history, but also divergent in ways that suggest adaptation.

Differences in cardenolide profiles between plant parts, such as the general reduction in number of compounds in nectar, may be due to physical barriers such as membrane porosity, the molecular size or mobility of specific compounds. For example, the distribution of individual cardenolides in the roots, leaves, stems and latex of *A. eriocarpa* (Nelson, Seiber & Brower 1981) has been attributed to differences in compound polarity and the resulting mobility of compounds within plants (Malcolm 1991). In *Digitalis*, enzymes glucosylate cardenolides and then actively transport these compounds into plant vacuoles (Kreis & May 1990). Differences in the composition of cardenolides between plant parts may therefore be influenced by enzyme-mediated structural changes in cardenolides that facilitate transport of modified compounds to other plant parts (Agrawal *et al.* 2012). Overall, the relationship between cardenolide concentration and composition in *Asclepias* shoots, nectar, roots, stems, latex and flowers suggests that chemical defences in milkweeds may be independently regulated in each plant part but physiologically linked through either shared biosynthesis, common vasculature, genetic linkage or an underlying systemic defence strategy.

NECTAR CARDENOLIDES AS AN OPTIMAL DEFENCE

As the primary reward for pollination services, floral nectar is very important for plant fitness and should therefore be protected from illegitimate nectar consumers. Optimal defence theory (ODT) predicts that chemical defences should be allocated to plant parts in proportion to the contribution that each part makes to plant fitness (McKey 1979). The concentrations of secondary metabolites in reproductive parts generally supports ODT (McKey 1974; Zangerl & Rutledge 1996; but see McCall & Fordyce 2010); chemical defences of flowers and floral parts, which are indirectly responsible for reproduction in many plants, are often but not always higher than leaves (McKey 1974; Strauss & Irwin 2004). Data comparing nectar secondary metabolites to the chemical defences of other plant parts are scarce, limited to nectar alkaloids and do not support predictions based on ODT. For example, Detzel & Wink (1993) examined the chemistry of five alkaloid-rich plant species and found that alkaloid concentrations, measured as μg/g of fresh weight, were generally lower in nectar than in leaves. Adler *et al.* (2006) found that nectar alkaloid concentrations in *Nicotiana tabacum* were much lower than leaf alkaloid concentrations (inferred based on mass to volume conversion). Our results also conform to this pattern of reduced secondary metabolite concentrations in nectar relative to leaves. Nonetheless, there has been little rigorous analysis of nectar chemistry in the context of ODT.

A shortcoming of ODT is that it does not consider how differences in secondary metabolite *identity* between various plant parts contribute to a plant's defence strategy. Detzel & Wink (1993), who characterized the nectar alkaloids of five highly toxic plants, observed a limited series of nectar alkaloids and described this difference in alkaloid composition between nectar and leaves as 'selective secretion'. As effective nectar defences must deter unwanted nectar consumers without discouraging pollinators, the presence or the absence of a certain secondary metabolite could be determined by its functional specificity. The chemical polarity of a cardenolide is linked to compound toxicity; low-polarity compounds are bitter and acutely toxic after consumption, whereas highly polar compounds are harder to taste and are cumulatively toxic (Malcolm 1991; Rasmann, Johnson & Agrawal 2009). Previous comparative studies on *Asclepias* chemistry have found that the chemical polarity of cardenolides can differ significantly between leaves and roots (Nelson, Seiber & Brower 1981), and between leaves and stems (Fordyce & Malcolm 2000), suggesting that polarity might account for the presence or the absence of individual cardenolides in floral nectar. However, the cardenolides present in nectar have a range of polarities; we did not detect a shift in weighted average retention time, indicating no trend towards either high- or low-polarity compounds in nectar relative to leaves.

Nectar cardenolides could have many functions in addition to defence. For example, secondary metabolites frequently act as foraging cues or feeding stimulants for specialist herbivores (Dethier 1980); pollinators may similarly use cardenolides as a signal to identify *Asclepias*

nectar as a floral resource. Conversely, nectar cardenolides may filter out generalist pollinators that are less effective at vectoring intraspecific pollen (Rhoades & Bergdahl 1981). Nectar plays a unique role in *Asclepias* reproduction as a medium for pollen tube growth (Kevan, Eisikowitch & Rathwell 1989) and the presence of yeasts in floral nectar can physically disrupt the expansion of pollen tubes in *A. syriaca*, significantly reducing seed set (Eisikowitch *et al.* 1990). The antimicrobial properties of cardenolides (Lefevre *et al.* 2010) may therefore act to both protect fertilization events in *Ascleplias* and preserve the nutritional integrity of nectar for pollinators. Future work evaluating the effect of nectar cardenolides on antagonists, microbes and generalist pollinators will be critical to determining the functional significance of secondary metabolites in floral rewards.

POLLINATOR RESPONSE TO CARDENOLIDE-ENRICHED NECTAR

While pollinator aversion has been demonstrated in studies of nectar alkaloids (e.g. Adler & Irwin 2005; Gegear, Manson & Thomson 2007), results from our behavioural assays suggest that bumblebee workers have no consistent reaction to digoxin at the concentrations tested. In fact, over 47% of visits, on average, were to flowers containing nectar cardenolides and 9 of the 24 foragers actually preferred to collect digoxin-rich nectar (Fig. S1, Supporting information). In addition, collecting nectar with digoxin had no acute toxicity for bees, nor did it alter foraging efficiency. Previous work found that nectar containing digoxin had no effect on honeybee foraging preferences at concentrations of up to 10 ng/μL (Detzel & Wink 1993). In a recent study, honeybees consumed nectar containing distasteful compounds such as quinine and salicine until they had ingested a relatively large volume of these solutions and had experienced so-called 'postingestive malaise' (Ayestaran, Giurfa & de Brito Sanchez 2010). The lack of deterrence in our experiment could therefore be due to foraging workers storing digoxin-rich nectar in their honey crops, preventing the postingestive malaise that may lead to aversion.

The consumption of cardenolides can have a striking effect on pollinator fitness. Cardenolides such as convallatoxin (found in *Convallaria majalis*), ouabain (found in *Strophanthus* spp.) and digoxin can be lethal for honeybee workers, but five other cardenolides had no detectable postconsumptive effects (Detzel & Wink 1993). Pollinator response to nectar secondary metabolites may therefore be compound-specific. Individual cardenolides differ significantly in their toxicity, distastefulness and rate at which postconsumptive responses occur (Malcolm 1991), making it difficult to predict pollinator response to the individual compounds detected in *Asclepias* nectar. Further, our data reveal that nectar can contain a suite of putatively toxic compounds. To date, all studies on nectar secondary metabolites (including our own) have examined the effect of each compound in isolation, ignoring possible synergistic effects between compounds.

Conclusion

On the basis of evidence from both cardenolide concentrations and individual cardenolide profiles of 12 different species, we conclude that *Asclepias* selectively exhibit cardenolides in their floral nectar. However, correlations in cardenolide concentrations across plant parts also suggest that underlying constraints still contribute to nectar cardenolide composition. Comparing chemistry within a phylogenetic framework indicates that this trend is independent of evolutionary history; further, patterns of cardenolide concentrations in closely related species may hint at rapid divergence in nectar chemistry. A growing body of literature suggests that selection by nectar-consuming antagonists may have led to the presence of secondary metabolites in floral nectar (Adler 2000; Strauss & Irwin 2004; Strauss & Whittall 2006; Kessler & Halitschke 2009). Our findings echo other recent studies (Kessler & Halitschke 2009; McCall & Fordyce 2010) that invoke the necessity of a wider lens when determining how floral traits have evolved and are maintained by current agents of selection.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Foraging preferences of bees visiting nectar enriched with digoxin.

Fig. S2. Representative nectar and leaf chromatograms for *Asclepias*.

Table S1. Individual cardenolides found in nectar, leaves and flowers of the 12 *Asclepias* species.

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