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Post-ingestive effects of nectar alkaloids depend on dominance status of bumblebees

JESSAMYN S. MANSON and JAMES D. THOMSON Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario, Canada

Abstract. 1. Secondary metabolites have acute or chronic post-ingestive effects on animals, ranging from death to growth inhibition to reduced nutrient assimilation.

2. Although characterised as toxic, the nectar of *Gelsemium sempervirens* is not lethal to pollinators, even when the concentration of the nectar alkaloid gelsemine is very high. However, little is known about the sublethal costs of nectar alkaloids.

3. Using a microcolony assay and paired worker bumblebees, the present study measured the effects of artificial nectar containing gelsemine on oocyte development. Oocytes are a sensitive indicator of protein utilisation and general metabolic processes. We also calculated carbohydrate concentrations in the haemolymph to examine energetic costs of gelsemine consumption.

4. High concentrations of gelsemine significantly reduced mean oocyte width in subordinate bees, while dominant bees showed only a trend towards oocyte inhibition. Gelsemine consumption did not reduce carbohydrate concentrations in haemolymph.

5. The cost of ingesting gelsemine may be due to direct toxicity of alkaloids or may be an expense associated with detoxifying gelsemine. Detoxification of alkaloids can require reallocation of resources away from essential metabolic functions like reproduction. The risks associated with nectar alkaloid consumption are tied to both the social and nutritional status of the bee.

Key words. *Bombus impatiens*, gelsemine, *Gelsemium sempervirens*, nectar alkaloids, oocyte development, sublethal costs, toxic nectar.

Introduction

Plant secondary metabolites are believed to have evolved as chemical defences against herbivorous animals (Whittaker & Feeny, 1971; Janzen, 1973; Feeny, 1992; Berenbaum, 1995). Acute toxicity, resulting in death, is reported in many of the major secondary metabolite families (e.g. alkaloids, phenolics, glycosides; Berenbaum & Rosenthal, 1992). Although lower concentrations of a secondary metabolite may not be lethal, they can reduce the overall health and fitness of an animal (chronic toxicity; Berenbaum *et al.*, 1986).

Such chronic effects are subtle and highly variable. Typically, bioassays of growth, development, or reproduction are used. Zangerl and Berenbaum (1993) showed decreased growth of parsnip webworm (*Depressaria pastinacella*) larvae, when fed on wild parsnip (*Pastinaca sativa*) umbels with high furanocoumarin levels. Similarly, winter moth caterpillars (*Operophtera brumata*) fed on oak leaves with high tannin concentrations had reduced larval and pupal weights, along with reduced adult emergence (Feeny, 1970). Tannic acid caused developmental malformations in tent caterpillar (*Malacosoma disstria*) pupae (Karowe, 1989), while phenolic glycoside concentrations were negatively correlated with fecundity in gypsy moth, *Lymantria dispar* (Osier *et al.*, 2000).

Nearly all studies on plant chemical defences focus on secondary metabolites in the shoots or roots; however, these compounds are also paradoxically found in floral nectar. Although the functional significance of nectar secondary metabolites is not fully understood (but see Adler, 2000 for a review of hypotheses), studies do suggest that this so-called 'toxic' nectar can have deleterious consequences for nectar-collecting floral visitors. Honey bees have died after consuming artificial nectar containing very low concentrations of alkaloids and glycosides (Detzel & Wink, 1993). In other cases, ingesting 'toxic' nectar has less severe consequences. For example, Palestine sunbirds consuming nectar containing pyridine alkaloids were less able to

Correspondence: Jessamyn S. Manson, Department of Ecology and Evolutionary Biology, University of Toronto, 25 Willcocks Street, Toronto, ON M5S 3B2, Canada. E-mail: jessamyn.manson@utoronto.ca

assimilate sugar from their diet (Tadmor-Melamed *et al.*, 2004). Secondary metabolites in nectar, particularly alkaloids and phenolics, have been shown to deter pollinators and reduce number of flower visits (Adler & Irwin, 2005; Singaravelan *et al.*, 2005; Johnson *et al.*, 2006; Gegear *et al.*, 2007). Despite multiple reports of the distastefulness of 'toxic' nectar, few studies have correlated behavioural responses with effects of nectar secondary metabolite consumption on floral visitors.

The chemical arsenal of *Gelsemium sempervirens* L. (Carolina jessamine) includes the indole alkaloid gelsemine, a compound found in the roots, shoots, flowers and floral nectar of the plant. *Gelsemium sempervirens* is a perennial vine native to the southeastern U.S.A. Its fragrant yellow flowers open in the early spring and attract several flower visitors (Adler & Irwin, 2005; Pascarella, 2007; J. S. Manson, pers. obs.), including bumblebees (*Bombus bimaculatus*, *B. impatiens*), honeybees (*Apis mellifera*), carpenter bees (*Xylocopa virginica*), and solitary bees (*Osmia lignaria*, *Habropoda laboriosa*). The consequences of ingesting flowers or leaves from *Gelsemium* spp. are severe for mammals and include psychosis, respiratory failure, severe convulsions, and death (Blaw *et al.*, 1979; Ott, 1998; Rujjanawate *et al.*, 2003; Fung *et al.*, 2007). In contrast, adult bumblebees exposed to high levels of gelsemine experience no acute effects, even when gelsemine concentrations are 20 times higher than natural levels (J. S. Manson, pers. obs.). Similarly, Elliott *et al.* (2008) reported no effect of gelsemine on the number or survivorship of offspring produced by the megachilid solitary bee, *Osmia lignaria*. However, laboratory assays indicate that bees prefer to feed on sucrose-only nectar rather than a solution of gelsemine and sucrose (Gegear *et al.*, 2007), while enriching *G. sempervirens* nectar with gelsemine deterred visitors in nature (Adler & Irwin, 2005), implying that there are consequences to the ingestion of alkaloid-rich nectar.

Given the absence of acute effects, we tested for sublethal costs of nectar alkaloids by feeding bumblebee workers (*Bombus impatiens* Cresson) artificial nectar containing gelsemine, and measuring the development of their oocytes. Oocyte development provides a good bioassay, because it is a defined metabolic challenge that can be induced by pairing worker bees without a queen (Cnaani *et al.*, 2002, 2007). Furthermore, it is a complex and costly physiological process which we predict to be sensitive to toxins for several reasons. First, oocyte development is highly correlated with protein utilisation in worker bees (Lin & Winston, 1998; Pernal & Currie, 2000). Although there are several ways for insects to cope with secondary metabolites, a common mechanism is to detoxify these compounds into less hazardous ones (Slansky, 1992). Detoxification requires the production of specific enzymes from dietary proteins. If this mechanism is used by bumblebee workers, protein could be reallocated towards the construction of enzymes and away from oocyte production. Second, alkaloid processing requires energy, so carbohydrates used for normal metabolic processes may be redirected to alkaloid metabolism, leaving less energy for the formation of reproductive structures. In addition, alkaloids may directly interfere with the absorption of nutrients by inhibiting digestive enzymes or forming nutrient–allelochemical complexes (Slansky, 1992). We therefore hypothesise that metabolic costs associated with nectar alkaloid consumption will result in reduced oocyte development. We also evaluated whether alkaloid processing directly reduces available carbohydrates, by measuring carbohydrate levels in bee haemolymph 24 hours after ingestion. We discuss our findings with a focus on possible mechanisms for alkaloid tolerance in pollinators.

Methods

Oocyte development

Oocyte development in *Bombus* spp. depends on social circumstances. If multiple workers are kept in queenless colonies, one of them frequently assumes a queen-like role, becoming the dominant worker and developing oocytes (Cnaani *et al.*, 2002, 2007). When two bumblebee workers interact in a queenless colony, the dominant worker will develop its ovaries at an optimal rate while suppressing the ovary development rate of the subordinate worker. We took advantage of this developmental strategy, building 'microcolonies' from worker bees to assess how gelsemine consumption affects ovary development under optimal and suboptimal conditions.

We obtained pupal clumps of *Bombus impatiens* from Biobest Canada Ltd (Leamington, Ontario). Bumblebee microcolonies were composed of two unfed callow workers (<24 h old). We created a size dichotomy in each container in an effort to enhance differences between dominant and subordinate bees, as previous work suggests that larger bees are more likely to be dominant (Ayasse *et al.*, 1995). Each pair of bees was housed in a closed 500 ml clear plastic food container, lined with paper to absorb faeces, and equipped with holes for ventilation (along the sides) and nectar access (on the base). This container was nested in a second food container, which held a small Petri dish of artificial nectar, made accessible by a cotton wick that led up to the holes on the base of the first container. This arrangement reduced spilling and contamination of the nectar by preventing direct contact between the nectar and the bees. Individuals were divided evenly among treatments so that we had a total of 28, 29, and 28 pairs of bees in control, moderate, and high gelsemine treatments, respectively, after three replicates, which were run at three separate dates.

A 30% w/w solution of sucrose was used as artificial nectar, which fell well within the range of natural sugar levels reported in *G. sempervirens* flowers (Leege & Wolfe, 2002; Adler & Irwin, 2005), to which we added gelsemine hydrochloride (hereafter referred to as gelsemine; Chromadex, Santa Ana, California). We used three diet treatments: control, composed of sucrose only; moderate gelsemine, a solution of sucrose plus 50 ng μl^{-1} gelsemine; and high gelsemine, a solution of sucrose plus 250 ng μl^{-1} gelsemine. The two gelsemine treatments simulate the mean and maximum concentrations, respectively, of gelsemine found in the nectar of natural *G. sempervirens* populations (Adler & Irwin, 2005). Bees avoid nectar of both of these concentrations if control nectar is available (Gegear *et al.*, 2007). We supplied 1.5 ml of artificial nectar daily, as well as commercially available pollen *ad libitum*. We provided new pollen every day. Pollen lumps were weighed before and after they were provided to a container, to determine daily pollen consumption by

the pair of bees. Microcolonies were maintained for 6 days under controlled environmental conditions (23–27°C in the dark, except for during feeding), which is the estimated time needed for *B. impatiens* oocytes to mature (Cnaani *et al.*, 2002, 2007). After 6 days, we froze the bees and dissected them in distilled water to determine ovary development. Using a scaled ocular, we measured the length and width of the largest oocyte in each of the two paired ovaries using the average of the two in our analyses. Development in the two tended to be symmetrical. We also recorded the length of the radial cell in the front right wing as a proxy for bee size (Harder, 1982).

We compared oocyte length and width between treatments with ANCOVA, using radial cell length as a covariate. We chose to analyse length and width separately to pinpoint the effects of gelsemine consumption on each of these size parameters. Previous work on oocyte development has used either a subjective size 'score' (Pernal & Currie, 2000) or measured length alone (Bloch & Hefetz, 1999; Cnaani *et al.*, 2007), which may have overlooked possible variation in oocyte width. Dominance was assigned to the bee within each pair with the larger oocytes, estimated as length times width, and we analysed dominant and subordinate bees separately. Data from the three experimental replicates were pooled, as the data did not significantly differ between replicates. Four pairs of bees were removed from the analysis, because one of the pair died before the experiment was completed, changing the social environment of the remaining bee. These pairs were spread across treatments. We also removed two subordinate bee outliers with extremely small oocytes.

To assess possible differences in protein intake between treatments, we analysed daily pollen consumption by microcolonies using repeated measures ANOVA. When necessary, data were transformed to meet assumptions for normality and homogeneity of variance.

Haemolymph carbohydrates

We removed pupal clumps from individual commercial colonies and isolated unfed callow bees (<24 h old) in individual vials. We provided bees with 500 μl of one of the three treatments: control (30% w/w sucrose), moderate gelsemine (50 ng μl^{-1} gelsemine in 30% sucrose), or high gelsemine (250 ng μl^{-1} gelsemine in 30% sucrose). After 24 h, when nearly all the nectar was consumed, we refrigerated the bees and then decapitated them. We took haemolymph samples by separating the ventral terga with forceps and gently inserting a 5 μl microcapillary tube into the lower abdominal cavity. We estimated the volume of each haemolymph sample and stored them individually in 1 ml of 80% ethanol. We analysed carbohydrates in 18, 17, and 17 individuals in the control, moderate and high gelsemine treatments, respectively.

We calculated carbohydrate concentrations, expressed as micrograms of trehalose equivalents per microlitre of haemolymph, using the anthrone method (modified from Siegert, 1987). Since the carbohydrate data were not normally distributed, we tested for differences in carbohydrate concentration between gelsemine treatments using a non-parametric Kruskal–Wallis test.

All statistical analyses were performed in R (version 2.6.0).

Results

Protein metabolism

Nectar gelsemine concentration did not affect oocyte length of dominant or subordinate bees (Table 1, Fig. 1). However, high levels of gelsemine did significantly reduce oocyte width in subordinate bees (*post hoc* Tukey tests using the multcomp package in R, Fig. 1), while there was a trend towards smaller widths in oocytes of dominant bees in the high gelsemine treatment. An unexpected element of the experiment was that dominance was not reliably predicted based on bee size; that is to say, the largest bees did not always have the largest oocytes. However, there was still a positive relationship between oocyte size ($L \times W$) and radial cell length ($R^2 = 0.2$, $F = 42.36$, d.f. = 162, $P < 0.001$). Therefore, radial cell was kept in the analyses and did contribute to the explanatory power of each model.

The reduction in oocyte size was not correlated with reduced protein intake, as pollen consumption did not differ between treatments (Table 2). Pollen consumption did vary significantly between days within each treatment, with bees eating the most on the second day of the assay, followed by a decline in appetite and a resurgence in pollen consumption by day 6. There was no interaction between treatment and day.

Haemolymph carbohydrates

Gelsemine did not affect the concentration of carbohydrates found in bee haemolymph (Kruskal–Wallis test, $\chi^2 = 3.014$, d.f. = 2, $P = 0.222$). Although the data are highly variable (Fig. 2), removing outliers did not reveal differences between treatments (analysis not shown).

Discussion

The nectar alkaloid gelsemine significantly inhibits oocyte development in subordinate bees, but is only marginally effective at reducing oocyte size in dominant bees. This effect was detectable

Table 1. The effect of zero, moderate (50 ng μl^{-1}), and high (250 ng μl^{-1}) gelsemine on oocyte length and width, on dominant and subordinate bees. All analyses are ANCOVAS with Type III SS and radial cell length (a proxy for bee size) as a covariate. Significant effects of the gelsemine treatment are in bold.

	Oocyte length				Oocyte width			
Source	d.f.	SS	*F*	*P*	d.f.	SS	F	*P*
Dominant bees								
Treatment	2	0.01	0.39	0.68	2	0.01	1.60	0.21
Radial cell	1	0.09	4.98	0.03	1	0.02	4.87	0.03
Subordinate bees								
Treatment	2	0.04	1.57	0.22	2	0.03	4.80	**0.01**
Radial cell	1	0.12	10.11	<0.001	1	0.02	6.92	0.01

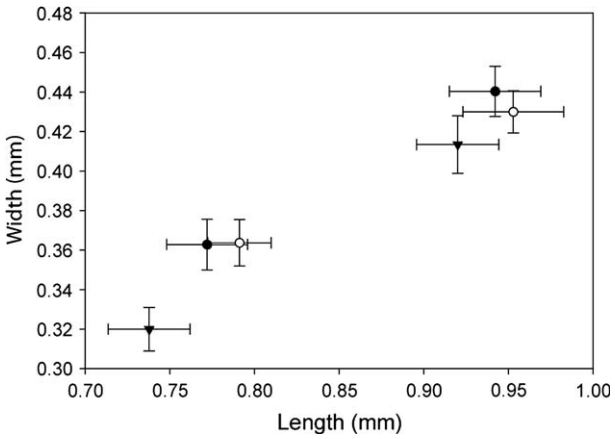

Fig. 1. Mean oocyte size, plotted as length against width, in dominant and subordinate bees fed 0 ng μl^{-1} (●), 50 ng μl^{-1} (○), or 250 ng μl^{-1} (▼) gelsemine. Dominant bees have larger oocytes and clump together in the upper right corner of the graph, while the smaller oocytes of subordinate bees fall in the lower left corner. The graph indicates the SE of both length and width measurements.

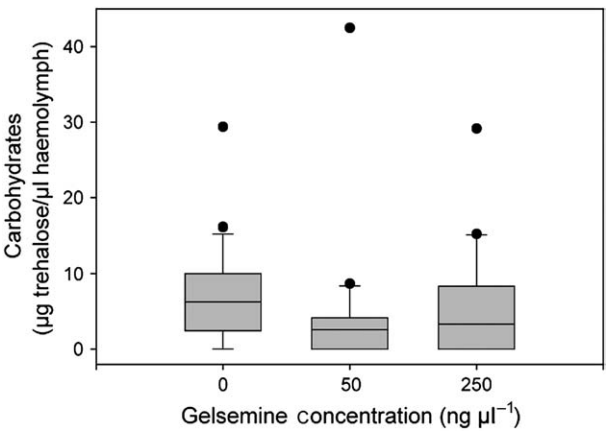

Fig. 2. Median carbohydrate concentrations in haemolymph 24 h after bees consumed artificial nectar with 0 ng μl^{-1}, 50 ng μl^{-1}, or 250 ng μl^{-1} gelsemine. Carbohydrates are expressed as micrograms of trehalose equivalents per microlitre haemolymph. The boxes represent the 25th and 75th percentiles, while the whiskers indicate roughly two SD.

at ecologically relevant concentrations, suggesting that ingestion of nectar alkaloids can incur a cost to pollinators. The severity of this cost, however, appears to depend on the condition of the bumblebee and the concentration of the alkaloid. Overall, the mean concentration of gelsemine found in nature may be largely innocuous to healthy bees. Under suboptimal circumstances, however, the ingestion of nectar alkaloids might be chronically deleterious to pollinators.

Sublethal effects of nectar alkaloids on bumblebees may arise in various ways. First, the alkaloids may not be toxic enough to kill bees, but they may be toxic enough to compromise nutrient absorption, alter neurohormonal processes, or damage internal organs (see Slansky, 1992 for review). These outcomes may lead to protein excretion or increased protein investment in immune function, reducing oocyte size. Another explanation for inhibited oocyte development is that detoxifying alkaloids is costly. Detoxification of secondary metabolites is a common process, whereby compounds are metabolised into less toxic components, and it is often accompanied by rapid excretion. This process requires protein to build detoxification enzymes and energy to process the deconstruction of the secondary metabolites; it is therefore assumed to be metabolically expensive. The assumption of costliness is supported by the observation that many detoxification mechanisms are induced only after the consumption of a secondary metabolite. Inducibility is interpreted as an energy-saving strategy (Berenbaum & Zangerl, 1994). Although this explanation is attractive, empirical evidence is divided on whether detoxification is a significant expense. Detoxification of alkaloids is reported to reduce digestive efficiency in the southern armyworm, *Spodoptera eridania* (Cresswell *et al.*, 1992), while parsnip webworms (*Depressaria pastinacella*) shunt energy away from growth to metabolise furanocoumarins (Berenbaum & Zangerl, 1994). In contrast, alkaloid detoxification in *Helicoverpa zea* required a negligible amount of energy compared to that spent on regular metabolic activity (Neal, 1987). Evidence of metabolic costs due to 'toxic' nectar ingestion are sparse. We know that Palestine sunbirds experienced reduced sucrose assimilation after consuming nectar containing the alkaloids nicotine and anabasine (Tadmor-Melamed *et al.*, 2004), but whether this was the result of reallocation to alkaloid detoxification was not identified. In our study, we did not find that gelsemine affected carbohydrate levels (see Fig. 2). However, carbohydrate levels in insect haemolymph are reportedly highly variable and may lack the resolution necessary to detect the energetic costs associated with alkaloid detoxification (Thompson, 2003).

Table 2. Daily pollen consumption for 6-day microcolony assay, compared between the three gelsemine treatments using a repeated measures ANOVA. Note that because bees were raised in pairs, each measurement represents pollen consumed for one dominant and one subordinate bee.

Source	d.f.	SS	*F*	*P*
Between subjects				
Treatment	2	8.96	1.53	0.29
Day	1	0.41	0.14	0.72
Within subjects				
Treatment	2	7.48	3.74	0.25
Day	5	181.4	13.36	<0.001
Treatment × Day	10	20.10	0.74	0.69

Secondary metabolites have reduced protein utilisation in previous studies on both vertebrates and invertebrates (reviewed in Duffey & Stout, 1996). Oocyte size is a sensitive measure of protein utilisation (Duchateau & Velthuis, 1989; Lin & Winston, 1998; Pernal & Currie, 2000) and smaller oocytes were reported in worker bumblebees infected with the gut pathogen *Crithidia bombi*, suggesting that oocyte size can indeed be an indicator of poor health (Shykoff & Schmid-Hempel, 1991). We must therefore conclude that protein metabolism and, consequently, fecundity in dominant bees is only modestly affected by ingested nectar

alkaloids. This conclusion is supported by work carried out on *Osmia lignaria* (Elliott *et al.*, 2008), which found that gelsemine did not reduce the fecundity of healthy solitary bees. The significant reduction in the oocyte size of subordinate bees in the high gelsemine treatment suggests nectar alkaloids may incur a cost to protein metabolism in individuals of suboptimal condition. Whether the inhibition of oocyte development due to gelsemine results in extended ovary development time or smaller offspring is unknown, but both outcomes could affect fitness.

The microcolony assay was designed to test the direct effects of gelsemine on dominant bees, because previous studies have shown a predictable response on the ovary development of the dominant worker under different social and nutritional environments (Duchateau & Velthuis, 1989; Cnaani *et al.*, 2002, 2007). The role of the subordinate bees in the microcolonies was simply to fulfil the social conditions required for optimal ovary development in their dominant counterparts. However, the significant treatment response by the subordinate bees, which experienced suboptimal conditions for oocyte development, is an unexpected but important result. The response of subordinate bees to the consumption of gelsemine is a complex effect that may involve both metabolism and behaviour. Previous studies on worker oocyte development have reported that bees exert dominance, in part, by monopolising the pollen ball (Cnaani *et al.*, 2007). This behaviour reduces the subordinate bee's access to protein, which likely explains why all subordinate bees have smaller oocytes. In addition to obtaining less dietary protein to support oocyte development, these food-stressed bees may experience heightened costs of detoxification. In fact, Wahl and Ulm (1983) demonstrated that the cost of metabolising pesticides increased when honey bee pollen intake was reduced. Compensatory pollen feeding by the dominant bees in the high-gelsemine treatment might further reduce the amount available to subordinates in those treatments, either directly through consumption by the dominants, or indirectly because dominant bees spend more time at the pollen ball and guard it more stringently. The possibility of competition for access to pollen could also explain why larger bees tended to fare better (significant effect of radial cell length Table 2). We found no effects of gelsemine on pollen consumption (Table 2), but those data include pollen consumption by both bees. We cannot determine whether the allocation of pollen to dominants and subordinates may have differed among treatments. Furthermore, the hygroscopic nature of pollen, coupled with the necessity of using fresh weights, renders the pollen consumption data only approximate.

The effects of nectar alkaloids on pollinators must be interpreted with natural plant–insect interactions in mind. In this study, we found that the inhibition of oocyte development due to the consumption of gelsemine was related to a pollinator's condition. Despite its acute toxicity to mammals, gelsemine seems to be distasteful but largely harmless to bees, except if they are consuming the highest natural concentrations, have little other food to dilute the toxic effects, or are metabolically challenged. These circumstances might apply to bumblebee queens foraging on the early spring flowers of *G. sempervirens.* Queens that have recently emerged from hibernation are developing their ovaries to begin nestmaking, and often have few other nectar and pollen resources to choose from. They may be ingesting substantial amounts of gelsemine-rich nectar while highly food-stressed, and therefore vulnerable to the deleterious consequences of gelsemine. Even a slight sublethal effect of nectar alkaloids may present a subtle but significant impediment to pollinator fitness. Whether that impediment offsets the positive value of the nectar sugars obtained would depend on the dietary choices available. The role that nectar secondary metabolites play in plant–pollinator communities will therefore be shaped by the composition of each community, and future work needs to move beyond the interactions of a single plant and pollinator to include more complex, community-level interactions. Although 'toxic' nectar may be less severe than its name suggests, its deleterious effects still have the potential for widespread consequences to pollinators and the plants they visit.

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