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Dose-dependent effects of nectar alkaloids in a montane plant–pollinator community

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Summary

1. Although secondary metabolites are prevalent in floral nectar, the ecological consequences for pollinators and pollination remain relatively unexplored. While often deterrent to pollinators at high concentrations, secondary metabolite concentrations in nectar tend to be much lower than secondary metabolite concentrations in leaves and flowers; yet, they may still affect the maintenance of pollination mutualisms.

2. *Delphinium barbeyi*, a common montane herb, contains norditerpene alkaloids in its nectar but at concentrations that are substantially lower than those found in its leaves or flowers. By manipulating nectar alkaloid concentrations in the field and laboratory, we assessed the degree to which varying concentrations of alkaloids in nectar influenced pollinator behaviour and activity and plant reproduction.

3. In the field, nectar alkaloids significantly reduced both the number of flower visits and the time spent per flower by free-flying bumblebee pollinators, but we only observed effects at alkaloid concentrations 50 times that of natural nectar. When we supplemented *D. barbeyi* nectar with alkaloids at concentrations almost 15 times that of natural nectar, we found no evidence for direct or pollinator-mediated indirect effects on female plant reproduction.

4. In the laboratory, the direct consumptive effects of nectar alkaloids on bumblebee pollinators were also concentration dependent. Bumblebees exhibited reduced mobility and vigour but only at alkaloid concentrations more than 25 times higher than those found in natural nectar.

5. *Synthesis.* We found that nectar alkaloids have dose-dependent effects on pollinator behaviour and activity. While concentrations of nectar alkaloids rivalling those found in leaves would negatively affect pollinator behaviour and pollination services, the natural concentrations of nectar alkaloids in *D. barbeyi* have no negative direct or pollinator-mediated indirect effects on plant reproduction. These results provide experimental insight into the dose-dependent ecological consequences of nectar secondary metabolites for pollinators and pollination, suggesting that low nectar alkaloid concentrations incurred no ecological costs for *D. barbeyi*.

Key-words: *Bombus*, bumblebee, *Delphinium barbeyi*, female plant fitness, pollinator preference, reproductive ecology, secondary metabolites, toxic nectar

Introduction

Plant secondary metabolites affect a diversity of plant–animal interactions (e.g. Adler 2000; Theis & Lerdau 2003; Wink 2003), but the majority of studies have focused on foliar herbivores, their preference and performance, and subsequent effects on plant fitness (Fraenkel 1959; Rosenthal & Berenbaum 1991; Bennett & Wallsgrove 1994). However, plant secondary metabolites are not restricted to foliage and can also be found in reproductive tissues and floral rewards, such as nectar (e.g. Adler & Irwin 2012; Kessler *et al.* 2012; Manson *et al.* 2012). Secondary metabolites have been found in the nectar of species from at least 21 plant families (reviewed in Adler 2000) and include alkaloids, iridoid glycosides, phenolics and cardenolides. Nectar secondary metabolites can elicit a range of behavioural responses from floral visitors, from attraction to avoidance (Adler & Irwin 2005; Johnson, Hargreaves & Brown 2006; Gegear, Manson & Thomson 2007), and a range of physiological responses as well, from positive to negative (Tadmor-Melamed *et al.* 2004;

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Manson & Thomson 2009; Wright *et al.* 2013), with the direction and magnitude of effect often being dependent upon secondary metabolite identity and/or concentration. The functional significance of these compounds in nectar remains unclear, but hypotheses include deterring inefficient pollinators, encouraging specialist pollinators and defending nectar from nectar robbers and microbes (Rhoades & Bergdahl 1981; Adler 2000). Alternatively, nectar secondary metabolites may simply be a pleiotropic consequence of chemical defences in other plant parts and may sometimes represent an ecological cost rather than adaptive advantage to flowering plants (Adler 2000; Strauss & Whittall 2006). Despite the adaptive and non-adaptive hypotheses proposed for the existence of nectar secondary metabolites, there are still surprisingly few studies that have measured their plant fitness consequences in the field (but see Adler & Irwin 2005; Kessler, Gase & Baldwin 2008; Adler & Irwin 2012).

Secondary metabolite concentrations vary widely among plant parts and rewards, with nectar consistently exhibiting lower concentrations than leaf or flower tissue (Adler *et al.* 2012; Manson *et al.* 2012; Cook *et al.* 2013). In some cases, these differences can be dramatic (Manson *et al.* 2012; Cook *et al.* 2013). This dissimilarity in concentration between secondary metabolites in leaves and nectar may be due to physiological or genetic constraints or allocation costs associated with their transport or production (Adler *et al.* 2006; Kessler & Halitschke 2009; Manson *et al.* 2012). Conversely, selection could have driven the reduction in or absence of secondary metabolites in nectar if plants experience ecological costs of high concentrations (i.e. pollinator deterrence) or ecological benefits associated with low concentrations (i.e. pollinator attraction). Low or no secondary metabolites in the nectar of otherwise heavily defended plants could presumably be adaptive, but testing this hypothesis requires directly manipulating nectar secondary metabolite concentrations and evaluating subsequent costs and benefits to pollination and plant fitness.

Studies that have manipulated nectar secondary metabolites have typically manipulated single compounds either via additions/dilutions or by silencing their production (e.g. Adler & Irwin 2005; Kessler *et al.* 2012). Nonetheless, the nectar of most plant species studied contains a suite of secondary metabolites (e.g. Manson *et al.* 2012; Cook *et al.* 2013), and there is recognition that pollinator preferences and foraging are a function of associations among multiple traits (Campbell 2009). While manipulations of single secondary metabolites in nectar can isolate the effects of those single compounds, they may over- or under-estimate the effects of natural nectar secondary metabolite composition, especially given that metabolites can have different ecological, and sometimes non-additive, effects (e.g. Panter *et al.* 2002; Dyer *et al.* 2003). Thus, experiments need to move beyond manipulating single compounds to reflect the effects of the complexity of natural nectar secondary metabolite composition on pollinators and pollination.

In this study, we examined the role of nectar alkaloids on plants, pollinators and their interactions. We focused on *Delphinium barbeyi*, a plant that has high concentrations of norditerpene alkaloids in all its parts (up to 2% dry weight) with known toxicity to vertebrates and invertebrates (Manners *et al.* 1993; Welch *et al.* 2012). *Delphinium barbeyi* also contains norditerpene alkaloids in its nectar but at substantially lower concentrations than those found in leaves and flowers (Cook *et al.* 2013). By manipulating nectar alkaloid concentrations in the field using an alkaloid solution that mimicked the suite of compounds found in *D. barbeyi* nectar, we assessed the degree to which low nectar alkaloid concentrations may be adaptive for pollination and plant reproduction. In addition, we complemented our field study with controlled laboratory experiments to examine the mechanisms behind pollinator responses to nectar enriched with alkaloids. Specifically, we asked the following questions: (i) To what degree does the concentration of nectar alkaloids affect pollinator foraging behaviour? (ii) What are the direct and pollinator-mediated indirect effects of nectar alkaloids on pollinator behaviour and plant reproduction? and (iii) What are the post-consumptive effects of alkaloid-enriched nectar on pollinators?

Materials and methods

STUDY SYSTEM

Delphinium barbeyi (Ranunculaceae) is a long-lived herbaceous perennial common to moist subalpine meadows in the Rocky Mountains, USA, and around the Rocky Mountain Biological Laboratory (RMBL) in Gothic, CO, USA. Plants grow in large clusters and are one of the dominant flowering species in mid to late summer (Williams *et al.* 2001; Inouye, Morales & Dodge 2002; Elliott & Irwin 2009). *Delphinium barbeyi* produces an average of 13.6 $\pm$ 0.5 flower stalks per plant, each bearing an inflorescence averaging 25.4 $\pm$ 0.8 flowers (Elliott & Irwin 2009). The hermaphroditic, protandrous flowers have two nectar spurs contained within the fused upper petals. The nectar standing crop is approximately 1.8 $\pm$ 0.05 μL per flower in the morning before pollinator visits (n = 512 flowers, Elliott 2008) with a sugar concentration of 44 $\pm$ 3% (n = 18 flowers from the year 2000 in 1 *D. barbeyi* population near the RMBL; R. E. Irwin, unpublished). Though self-compatible, flowers produce very few seeds through autogamous self-pollination (Williams *et al.* 2001), so pollinators are required to carry pollen even within flowers and plants. Moreover, in a natural population of *D. barbeyi* near the RMBL, more seeds were produced through outcrossed than selfed pollen (Williams *et al.* 2001), suggesting that pollinator visitation is important for plant reproduction.

A diverse community pollinates the flowers of *D. barbeyi*, with bumblebees as the dominant pollinator. The most common pollinator is *Bombus appositus* (Inouye 1978; Graham & Jones 1996; Elliott 2008), but flowers are also frequented by *B. flavifrons*, *B. bifarius*, *B. nevadensis* and *B. occidentalis* as well as hummingbirds (*Selasphorus platycercus* and *S. rufus*), hawk moths (*Hyles lineata*) and other small bees and flies (Waser 1982; Elliott 2008). Bumblebees collect both nectar and pollen from *D. barbeyi*. Elliott (2009) reports that 91.2% of pollen collected by *B. appositus* was from *D. barbeyi* and that 94.6% of *B. appositus* visits were to *D. barbeyi* flowers in meadows where this flowering species is common. Individual *D. barbeyi* vary in the degree to which they are pollen limited for seed production (Williams *et al.* 2001), although in some sites and years, no pollen limitation of fruit or seed set occurs (Elliott & Irwin 2009). Exclusion of bumblebee, hummingbird and hawk moth pollinators, however, significantly reduces plant reproduction (Elliott 2008).

Delphinium barbeyi produces norditerpene alkaloids, which can be found in all plant parts, including leaves, stems, flowers, anthers and fruits (Cook *et al.* 2013; Appendix S1 and Table S1 in Supporting Information). With the exception of occasional grazing by livestock, there is little natural herbivory reported on *D. barbeyi* (Inouye, Morales & Dodge 2002), potentially due to these norditerpene alkaloids. Alkaloid concentrations range from 790 ± 38 μg alkaloid/100 mg dry weight (DW) of stems to 3867 ± 315 μg alkaloid/100 mg DW of fruits. Alkaloids can also be found in the nectar of *D. barbeyi* but at concentrations over 1000 times lower than leaf, flower and anther tissue and 2000 times lower than fruits (nectar: 1.7 ± 0.4 μg alkaloid/100 mg or 0.017 μg/μL; Cook *et al.* 2013). The norditerpene alkaloid profiles between nectar and vegetative parts are qualitatively similar; thus, nectar does not appear to contain or lack common norditerpene alkaloids relative to above-ground plant parts (Cook *et al.* 2013; Appendix S1 and Table S1). The norditerpene alkaloids of *D. barbeyi* are divided into two main classes: 7, 8-methylenedioxylycoctonine (MDL type) and N-(methylsuccinimido) anthranoyllycoctonine (MSAL type) (Pfister *et al.* 1999). Based on bioassays with generalist invertebrate herbivores (*Spodoptera eridania*), mice and cattle, the MSAL-type alkaloids are significantly more toxic than the MDL type (Jennings, Brown & Wright 1986; Cook *et al.* 2011). In all above-ground *D. barbeyi* plant parts and nectar sampled at sites near the RMBL, the MDL-type alkaloids make up a higher proportion of total alkaloid content than the MSAL type (Cook *et al.* 2013). Further, the three dominant alkaloids, deltaline, 14-acetlydictyocarpine and methyllycaconitine, represent between 66.9 and 89.2% of the total alkaloids found in *D. barbeyi* plant parts (Appendix S1 and Table S1).

NECTAR ALKALOID MANIPULATIONS

Because the alkaloid profile in nectar mirrors that of *D. barbeyi* plants (Appendix S1), we extracted alkaloids from above-ground plant material to add to nectar to test how varying concentrations of alkaloids in nectar affected pollinators and pollination. The alkaloid extract was prepared from *D. barbeyi* collected near Gothic, CO (N 38° 58.264′ W 106° 59.791′), and Montrose, CO (N 38° 18.255′ W 108° 12.071′). Plant material was collected from populations distinct from our experimental field sites in the year prior to conducting our study. Plant material was air-dried and alkaloids were extracted using methods outlined in Appendix S1 and in Cook *et al.* (2011). It is important to note that the extract contained only alkaloids (Appendix S1).

To create alkaloid-enriched artificial nectar, we dissolved the alkaloids in water acidified with 0.1 M ascorbic acid (ascorbic acid can occur naturally in nectar; Baker 1977). Once the alkaloids were dissolved, we added this aqueous solution to a 45–50% sucrose solution, creating artificial nectar with a known sucrose and alkaloid concentration and a neutral pH. Control artificial nectar was 45–50% sucrose solution supplemented with trace amounts of ascorbic acid. We used 45–50% sucrose solution because it is within the range of natural nectar sugar concentration of *D. barbeyi* (R. E. Irwin unpublished). Hereafter, we refer to these solutions as alkaloid enriched and control, respectively.

EXPERIMENTAL METHODS

To what degree does the concentration of nectar alkaloids affect pollinator foraging behaviour?

We assessed the behavioural responses of pollinators to a range of alkaloid concentrations using free-flying bumblebees foraging in a *D. barbeyi* population near the RMBL. We used alkaloid-enriched solutions composed of sucrose solution and the following alkaloid concentrations: 0 μg/μL (control), 0.1 μg/μL, 1 μg/μL, 2 μg/μL and 4 μg/μL. We selected this concentration range for two reasons. First, the concentration range is relevant within a whole-plant context as concentrations are similar to those found in other plant parts including leaves and flowers (Cook *et al.* 2013). Secondly, preliminary laboratory experiments with free-flying workers showed deterrence by nectar enriched with 0.1 μg/μL alkaloids (Appendix S2) and avoidance at 4 μg/μL alkaloid-enriched nectar (J. S. Manson, D. Cook and R. E. Irwin unpublished data), suggesting we would be using a concentration range that captured variation in behavioural responses.

We clipped *D. barbeyi* inflorescences, trimmed them until they had 10 open flowers per stalk and placed each stalk in individual florist water picks. Each stalk of 10 flowers, hereafter called an 'interview stick', was assigned an alkaloid concentration, and we supplemented each flower with 2 μL of alkaloid solution using blunt-point Hamilton syringes, injecting approx. 1 μL of solution into each spur. Nectar treatments were added to standing nectar volumes; to avoid damaging floral tissue, we did not remove the nectar from flowers (similar to Adler & Irwin 2005). Thus, our treatments represent dilutions and augmentations of nectar alkaloids that are naturally present in nectar (Table 1). The control treatment had nectar alkaloid concentrations that were up to 3 times lower than untreated flowers, while the highest alkaloid treatment had concentrations that were over 150 times higher than untreated flowers (Table 1). All flowers on an interview stick received the same treatment, and treatments were applied to interview sticks just prior to offering them to pollinators.

To present interview sticks to free-flying pollinators, we used the following protocol. We approached a bee foraging on non-experimental *D. barbeyi* and presented them with an interview stick. We positioned the interview stick within the proximity of the foraging individual such that the bee might interpret the interview stick as the next available inflorescence. Once a bee began to forage on an interview stick, we positioned another interview stick with a different nectar treatment nearby to encourage the bee to continue visiting treated interview sticks. We allowed bees to visit as many treated interview sticks, and flowers per stick, as desired, which led to some individuals visiting multiple interview sticks. We randomized the order in which treatments were presented to individual bees, and each bee was presented with up to five interview sticks, all with different treatments. Because visiting alkaloid-enriched flowers often led to a rapid departure of individuals, we were

Table 1. Alkaloid concentrations added to *Delphinium barbeyi* nectar to dilute or augment natural nectar alkaloids and the resulting alkaloid concentrations available to free-flying pollinators. We assumed that *D. barbeyi* nectar had on average 0.017 μg/μL alkaloids (Cook *et al.* 2013) and individual flowers had 1–2.5 μL standing crop of nectar (Elliott 2009)

Nectar alkaloid treatment (μg/μL)	Actual alkaloid concentration range (μg/μL)
1) To what degree does the concentration of nectar alkaloids affect pollinator foraging behaviour?	
0	0.0057–0.0094
0.1	0.054–0.072
1	0.453–0.673
2	0.897–1.340
4	1.785–2.674
2) What are the direct and pollinator-mediated indirect effects of nectar alkaloids on pollinator behaviour and plant reproduction?	
0	0057–0.0094
0.05	0.231–0.339

unable to capture and mark bees that foraged on interview sticks. As such, it is possible that some subsequent visitors may not have been naïve to our treatments, although our field site was large and visitors had many alternative flowers to visit of *D. barbeyi* as well as other flowering species. We used digital voice recorders (Olympus WS-600S) to record how many flowers were visited per interview stick and the time spent per flower. We also recorded the species and caste (worker vs. queen) of each bee and noted when individuals visited more than one treatment. The 10-flower inflorescences used for interview sticks were not reused between bees; they were discarded after a bee visit. Sample sizes ranged from 62 to 82 interview sticks per treatment.

The majority of visits to the interview sticks were from *B. appositus* workers (90% of individuals), and only 10% combined were from *B. californicus* and *B. flavifrons* workers and *B. appositus*, *B. californicus* and *B. nevadensis* queens. Given the dominance of *B. appositus* visitation to the interview sticks, we focused our statistical analyses on the foraging behaviour of *B. appositus* workers only; however, patterns were qualitatively similar when all visitors were analysed (data not shown). We analysed the number of flowers visited per stalk and mean foraging time per flower per stalk using separate ANOVAS with alkaloid treatment as a fixed factor and bee individual as a random factor. Bee individual was included as a random factor to avoid pseudoreplication within individuals when individuals visited multiple interview sticks. Number of flowers probed per stalk was log(x + 1) transformed, and we used *post hoc* Tukey's HSD tests to assess which treatments were significantly different from one another for both analyses. We predicted that nectar alkaloids would modify bee foraging behaviour, but only at the higher alkaloid concentrations. Statistical analyses were performed in JMP (v. 10) here and below, unless otherwise noted.

What are the direct and pollinator-mediated indirect effects of nectar alkaloids on pollinator behaviour and plant reproduction?

We tested the effects of nectar alkaloids on pollinator behaviour and plant reproduction in the same *D. barbeyi* field site as in *Question 1*. We haphazardly chose 21 *D. barbeyi* plants that had mature but unexpanded flower buds. Each plant had between 5 and 28 stalks. We haphazardly chose four stalks per plant and randomly assigned each to one of the four treatments representing a factorial cross of nectar treatment (control or alkaloid enrichment) crossed by hand pollination (open or supplemental pollination). We marked stalks with label tape, using the same colours in different combinations to identify each of the four treatments. Because individual *D. barbeyi* plants can be very large, it was not possible to apply treatments at the whole-plant level. Instead, we applied treatments at the whole-stalk level. Within each plant, there were therefore four experimental stalks, one per treatment, which allowed us to control for maternal plant. Treated stalks were approx. 30 cm or less from each other, while the 21 individual plants were at separated from their nearest treated neighbours by at least 1 m.

Nectar treatments were comprised of either control solution or alkaloid-enriched solution at a concentration of 0.5 μg/μL *D. barbeyi* alkaloids. We injected 2 μL of artificial nectar into every open flower on a stem, as described above. Syringe tips were cleaned with ethanol between flowers to avoid transferring pollen. We performed nectar treatments 5 days per week on all open flowers on every experimental stalk throughout the peak blooming season. The order in which plants were treated was randomized daily to control for potential differences in pollinator activity at different times of day. Because we did not remove nectar from flowers prior to adding solutions, our treatments represent dilutions and augmentations of nectar alkaloids, with the control treatment having one-third to one-half the alkaloid concentrations as naturally occurring *D. barbeyi* nectar and the addition treatment having 14–20 times higher alkaloid concentrations (Table 1). This treatment range is relevant within a whole-plant context (Cook *et al.* 2013) and fell within the concentrations that elicited strong behavioural responses in laboratory assays (Appendix S2).

For our pollination treatments, flowers received either ambient levels of pollination (open pollination) or supplemental hand pollination. We performed hand pollinations by contacting receptive stigmas with dehiscing anthers collected from at least five non-experimental plants growing at least 10 m away. We performed hand pollinations three times during peak flowering and marked the pedicel of each hand-pollinated flower that received supplemental pollination with a small dot of indelible ink (Sharpie™).

Pollinator foraging behaviour

After daily nectar treatments, we spent a total of 27.67 person hrs over 11 days observing pollinator foraging behaviour. The 11 days of observation were spread across the flowering season. We used a digital voice recorder to note all pollinator visits to treated stalks, recording species identity of the pollinator, the number of flowers probed and time spent per flower. Because of the speed at which hummingbirds visit individual flowers, we only recorded time spent per flower for bee and not bird visitors. It was not possible to observe all treated stalks simultaneously for pollinator foraging behaviour due to the spatial arrangement of the plants. Instead, three groups were demarcated within the population, and we watched all plants within each group simultaneously. We rotated the order in which we observed the three groups of plants and the observer at each group to control for temporal and observer bias. For each treated stalk, we calculated the mean number of visits per hour per day (hereafter mean stalk visitation rate), the mean number and proportion of flowers probed and the mean time spent per flower.

Plant reproduction

We estimated plant fitness via female components of reproduction. The number of seed-bearing and aborted fruits were recorded for each stalk, and for mature seed-bearing fruits, we counted the number of seeds per fruit and weighed their seeds. We collected some fruits prior to full maturation to prevent damage to the plants due to grazing cattle, which were introduced to our study site in early August; at this point, seed maturation had progressed far enough to ensure accurate distinction between developing and aborted ovules. For each treated stalk, we calculated proportion fruit set (number of seed-bearing fruits divided by seed-bearing plus aborted capsules), mean number of seeds per successful fruit and mean seed weight.

Statistical analyses

To test the effects of nectar treatment on pollinator foraging behaviour, we used ANOVAS with nectar treatment as a fixed factor, plant as a random effect, and mean stalk visitation rate, the mean proportion of flowers probed per stalk and the mean time spent per flower as response variables. We combined all floral visitors into the same analyses, except for visit length analyses, where we examined only bumblebees. Results were qualitatively similar when we broke the analyses down by visitor species and by caste (queen vs. worker) for bumblebees (data not shown).

To test the effects of nectar treatment and pollen supplementation on plant reproduction, we used two-way ANOVAS with nectar treat-

ment, pollination treatment (hand- vs. open-pollinated), and their interaction as factors, plant as a random effect, and proportion fruit set, mean number of seeds per fruit and mean seed weight as response variables. Proportion fruit set was arc-sine square-root transformed. An effect of nectar treatment would indicate that nectar alkaloids affect plant reproduction, and an effect of supplemental pollination would indicate that stalks are pollen limited for reproduction. A significant interaction between nectar and pollination treatments could suggest that the effects of nectar alkaloids are mediated through changes in pollinator behaviour. Finally, an effect of nectar treatment but no effect on pollinator behaviour and no significant interaction term would suggest that nectar alkaloids probably directly affect plant reproduction.

WHAT ARE THE POST-CONSUMPTIVE EFFECTS OF ALKALOID-ENRICHED NECTAR ON BUMBLEBEE POLLINATORS?

To determine the effects of *Delphinium* alkaloid consumption on bumblebee activity, we recorded the post-ingestive effects of consuming control vs. alkaloid-enriched solutions on wild *B. appositus* workers. Our goal here was to assess the direct effects of a range of nectar alkaloid concentrations on bumblebee workers in no-choice feeding assays to estimate physiological consequences of alkaloid consumption for pollinators. We used a concentration range of 0 μg/μL, 0.1 μg/μL, 1 μg/μL, 2 μg/μL and 4 μg/μL *D. barbeyi* alkaloids, similar to that used in *Question 1*. We also included a no-food treatment where bees received no nectar, to determine whether the observed responses were due to the consumption of alkaloids vs. reduced food consumption. *B. appositus* workers were collected approx. 5 km north of our *D. barbeyi* field site but in areas where *D. barbeyi* occurred. We used 10 workers per treatment. We placed individual workers in vials, deprived them of food for 2 h, and then exposed each bee to either 500 μL of control or alkaloid-enriched sucrose solutions. After 24 h, we transferred individual bees to a 500-mL clear plastic container fitted with a removable lid with multiple perforations. To evaluate their motility and vigour, we then agitated the bees by blowing on them for 90 s and video-recorded their response to this negative stimulus with a digital camcorder (Sony Super SteadyShot HDR-SR11).

We scored bee videos for nine activities, including tarsal or wing movements, aggression (attempted stinging) and flight, using a scale of 0–5, with 0 indicating the activity was not observed and 5 indicating that the behaviour was nearly continuous (as in Cook *et al.* 2013). Activities were scored based on both frequency and intensity. For each bee, we summed the scores to obtain an overall metric of activity (the summed scores ranged from 0 to 45). We used a nonparametric Kruskal–Wallis test to assess the effects of alkaloid concentration on bee activity. Because the Kruskal–Wallis test was significant, it was followed by nonparametric multiple comparison tests using the R package npmc (R version 2.10.1).

Results

TO WHAT DEGREE DOES THE CONCENTRATION OF NECTAR ALKALOIDS AFFECT POLLINATOR FORAGING BEHAVIOUR?

Nectar supplemented with the alkaloid extract significantly affected the number of *D. barbeyi* flowers visited per stalk by *B. appositus* workers ($F_{4,224} = 20.86$, $P < 0.001$) as well as the mean time spent per flower ($F_{4,209} = 27.67$, $P < 0.001$), but the effect was dependent upon alkaloid concentration (Fig. 1a,b). There was no difference in bee foraging behaviour between the stalks that were enriched with 0 μg/μL, 0.1 μg/μL and 1 μg/μL alkaloids; however, stalks enriched with 2 μg/μL and 4 μg/μL experienced at least 45% fewer flowers visited per stalk (Fig. 1a), and bees spent at least 40% less time visiting individual flowers (Fig. 1b) relative to 0 μg/μL enrichment. Data suggest that there may be a threshold alkaloid concentration that affects bee behaviour, with a significant reduction in both visit number and length between stalks with 1 μg/μL vs. 2 μg/μL alkaloid enrichment (Fig. 1a,b).

WHAT ARE THE DIRECT AND POLLINATOR-MEDIATED INDIRECT EFFECTS OF NECTAR ALKALOIDS ON POLLINATOR BEHAVIOUR AND PLANT REPRODUCTION?

Pollinator foraging behaviour

We recorded 159 pollinator foraging bouts. Of these, 13 were hummingbirds and the rest were bumblebees. We recorded

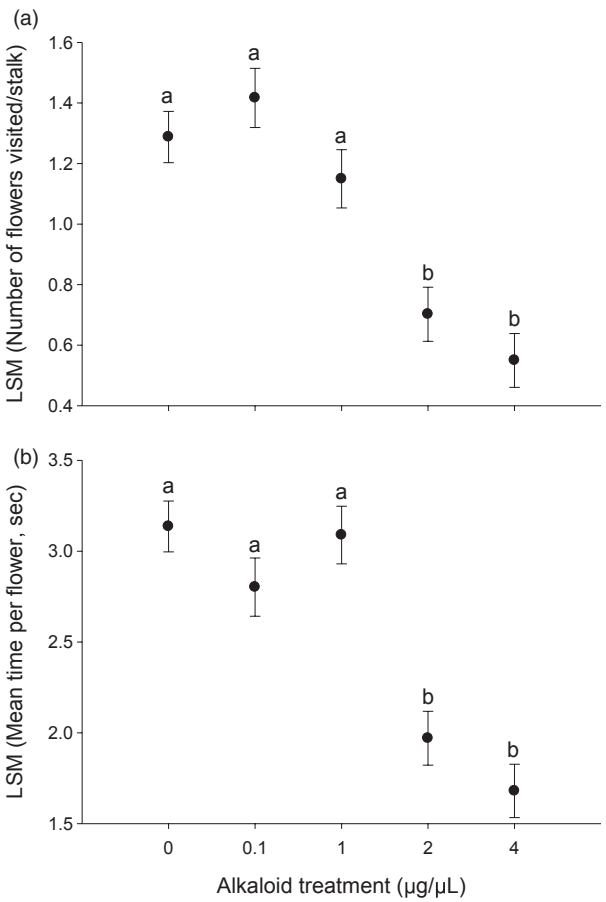

Fig. 1. Behavioural responses of *Bombus appositus* workers foraging on *Delphinium barbeyi* flowers supplemented with five different alkaloid treatments ranging from 0–4 μg/μL alkaloids. Different letters above treatments represent a significant difference in (a) the number of flowers visited per stalk (log(x + 1) transformed) and (b) mean time spent per flower per stalk. Least squares means (LSM) ± SE are reported.

visits from five different bumblebee species: *B. bifarius*, *B. californicus, B. flavifrons*, *B. appositus* and *B. nevadensis*, with 86% of all visitors being from the latter two species. Of the bumblebee visits, workers accounted for nearly 65% of all visits; queens accounted for the remainder.

We found no difference between alkaloid-enriched (0.5 μg/μL) and control stalks in any metric of pollinator visitation, including the mean number of pollinator visits per hour per stalk ($F_{1,60} = 0.16$, $P = 0.69$), the mean proportion of flowers probed per stalk ($F_{1,60} = 1.06$, $P = 0.31$) and mean time per flower ($F_{1,58} = 0.01$, $P = 0.92$). Across nectar treatments, pollinator visitation rate was (mean ± SE) 3.48 ± 0.25 visits per hour per stalk, mean proportion of flowers visited was 0.31 ± 0.02 and bees spent 3.75 ± 0.16 s visiting individual flowers.

Plant reproduction

Our results suggest no direct or pollinator-mediated indirect effects of the alkaloid concentrations used in this study on any metric of female reproduction. We found no significant difference between alkaloid-enriched and control stalks in proportion fruit set ($F_{1,60} = 0.19$, $P = 0.67$), mean number of seeds per fruit ($F_{1,58} = 1.29$, $P = 0.26$) or mean seed weight ($F_{1,50} = 1.32$, $P = 0.26$). We found limited evidence that flowers might be pollen limited for reproduction. Our pollen-supplementation treatment resulted in statistically significantly higher proportion fruit set ($F_{1,60} = 57.51$, $P < 0.0001$), but the magnitude of the effect was small, with hand pollination leading to 99% of fruits maturing successfully vs. 95% for open-pollinated fruits. However, we found no difference between pollen-supplemented and open-pollinated stalks in mean seeds per fruit and mean seed weight ($P > 0.05$ in both cases). Across all treatments, stalks produced (mean ± SE) 31.45 ± 0.97 seeds per fruit with mean seed weight of 1.06 ± 0.03 mg. Finally, we detected no interaction between alkaloid supplementation and hand pollination for any measure of female reproduction ($P > 0.05$ in all cases), suggesting no indirect effects of alkaloid enrichment via changes in pollination for the alkaloid concentrations tested in this experiment.

WHAT ARE THE POST-CONSUMPTIVE EFFECTS OF ALKALOID-ENRICHED NECTAR ON BUMBLEBEE POLLINATORS?

Nectar enriched with the alkaloid extract had a significant effect on the activity of *B. appositus* workers ($\chi^2 = 38.45$, df = 5, $P < 0.001$; Fig. 2). Common activities, such as walking, flying and grooming, and overall vigour were unaffected by the lowest dose of alkaloid extract, but were significantly impaired by alkaloid concentrations higher than 1 μg/μL (Fig. 2). The only mortality observed in the experiment was in the no-food treatment, where 4 bees died after 24 h in captivity and were therefore not included in the activity assays. We hypothesize that these individuals did not have the necessary energetic reserves to survive 24 h without food. However, the six individuals that did survive in the absence of food exhibited activity levels comparable to individuals fed control solution, suggesting that the reduced activity observed in higher alkaloid treatments was due to physiological consequences associated with consuming the alkaloid rather than the result of reduced feeding on alkaloid-enriched nectar.

Discussion

Alkaloids in *D. barbeyi* nectar can have a significant negative effect on pollinator foraging behaviour and activity rates. However, the minimum concentration to elicit these responses was at least 50 times higher than concentrations found in natural *D. barbeyi* nectar, but substantially lower than concentrations found in the plant's flowers and leaves. Indeed, nectar alkaloid concentrations up to 15 times higher than the average did not precipitate changes in pollination and female plant reproduction. Taken together, these results suggest that natural concentrations of alkaloids in *D. barbeyi* nectar have no ecological costs to the plant in terms of pollination services, but that concentrations of nectar alkaloids rivalling those of leaves would significantly reduce pollinator foraging rates and, presumably, plant reproduction in years or sites where plants are pollen limited.

Nectar secondary metabolites have been shown to deter a range of different pollinators (Adler 2000). For example, laboratory behaviour studies using artificial nectar enriched with alkaloids have demonstrated deterrence of honeybees (Koehler, Raubenheimer & Nicolson 2012), hummingbirds (Kessler *et al.* 2012) and bumblebees (Gegear, Manson & Thomson 2007). However, in some cases, deterrence only occurred at concentrations that exceeded those of natural nectar alkaloids (Kessler *et al.* 2012; Koehler, Raubenheimer & Nicolson 2012), similar to the patterns we report in this study. There are at least two mechanisms that can elicit a deterrent response to nectar secondary metabolites by pollinators. First,

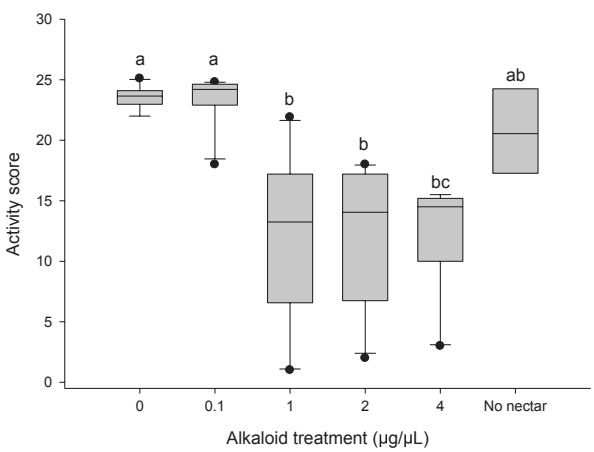

Fig. 2. Post-consumptive effects of *Bombus appositus* workers fed alkaloid-enriched sucrose solution for 24 h. All numbered treatments represent the alkaloid concentration of the sucrose solution, in percentage, whereas the 'no nectar' treatment represents individuals that received no sucrose throughout the experiment. Different letters represent significant differences in activity levels among treatments.

pollinators can be deterred because nectar secondary metabolites are distasteful; in this case, deterrence occurs after pollinators probe nectar (Adler & Irwin 2005). Secondly, deterrence can be due to an external cue, such as nectar colour (Johnson, Hargreaves & Brown 2006) or odour (Kessler & Baldwin 2007), which can inform the pollinator of nectar quality. In both cases, experienced pollinators can learn to avoid flowers with nectar secondary metabolites. We observed differences in both the number of visits and duration of per-flower visits as a function of nectar alkaloids (Fig. 1a,b, respectively). While a decrease in the duration of visits strongly suggests that nectar alkaloids might be distasteful, we cannot currently conclude whether an odour cue or pollinator experience accounts for the decrease in the number of visits. Additional mechanistic studies could determine whether *D. barbeyi* alkaloid extracts, which produce an odour detectable by the human nose, also deter pollinators prior to visits.

Plant fitness costs associated with nectar secondary metabolites have been predicted repeatedly but rarely observed, largely because very few studies have looked beyond the effects of these compounds on pollinator behaviour to the effects on pollination and subsequent plant reproduction (e.g. Adler & Irwin 2005; Kessler, Gase & Baldwin 2008). Those studies that have manipulated nectar secondary metabolites and measured pollination and plant reproduction have reported results ranging from negative to positive effects. For example, in field studies using transgenic *Nicotiana attenuata*, nectar that contained nicotine did not affect plant reproductive success unless the floral volatile benzyl acetone was also present, in which case the two compounds worked in concert to improve both male and female measures of plant fitness (Kessler, Gase & Baldwin 2008). Field studies in *Gelsemium sempervirens* found no effect of alkaloid-enriched nectar on fruit number but a significant reduction in fruit weight, suggesting that nectar alkaloids reduced pollinator visit quality (Adler & Irwin 2012). A reduction in the number of visits and the time per flower also significantly reduced self-pollen transfer in *G. sempervirens* with alkaloid-enriched nectar (Irwin & Adler 2008). Studies on *N. attenuata*, *G. semperpvirens* and *D. barbeyi* have consistently found no direct effects of nectar-alkaloid additions on female plant fitness, as tested through supplemented hand pollinations (Adler & Irwin 2005; Kessler, Gase & Baldwin 2008). To our knowledge, no other published studies have examined the plant fitness consequences of nectar secondary metabolites.

One parsimonious explanation for low alkaloid concentrations in *D. barbeyi* nectar relative to the whole plant is to reduce ecological costs associated with pollinator deterrence and pollination. While we found small but significant pollen limitation of *D. barbeyi* reproduction at our site, previous studies on *D. barbeyi* conducted in different sites and years have found mixed results, suggesting that pollen limitation may vary spatio-temporally (Williams *et al.* 2001; Elliott & Irwin 2009). This pollen limitation could lead to severe reproductive costs should nectar alkaloids affect pollinator behaviour. At the population level, pollinators are the primary selective agents on many nectar traits, including the rate and timing of nectar secretion (Heil 2011) and nectar volumes and sugar ratios (Baker & Baker 1983). A study by Adler *et al.* (2012) comparing nectar chemistry in 32 species of *Nicotiana* found that self-incompatible plants had lower nectar alkaloid concentrations than self-compatible plants, supporting the hypothesis that pollinators select for lower secondary metabolite concentrations. Thus, we can speculate that *D. barbeyi* with significantly higher nectar alkaloid concentrations would receive fewer visits and shorter visits per flower than those with lower nectar alkaloid concentrations, which may select for a reduction in nectar alkaloid concentrations, assuming changes in pollinator foraging and pollination translate into differences in plant fitness. However, studies measuring phenotypic selection on nectar chemical traits in the wild remain rare.

At least three other non-mutually exclusive mechanisms could also explain low nectar alkaloid concentrations in *D. barbeyi*. First, low nectar alkaloid concentrations could be a result of allocation costs associated with provisioning nectar with these compounds. In plant ecology, allocation costs include resource-based trade-offs between secondary metabolite production and plant survival, growth and fitness (Strauss *et al.* 2002). Although no studies to our knowledge have assessed allocation costs to nectar secondary metabolites, costs associated with secondary metabolites in leaves are well documented (reviewed in Bergelson & Purrington 1996; Strauss *et al.* 2002). Secondly, low nectar alkaloid concentrations could be a non-adaptive consequence of systemic chemical defences and leakage of alkaloids into the nectar. This consequence-of-defence hypothesis (Adler 2000) is supported by the qualitatively similar, yet quantitatively lower, alkaloid concentrations in *D. barbeyi* nectar relative to the alkaloid concentrations of leaves, anthers, flowers and stems, which suggests that alkaloid production or allocation is linked across plant parts and rewards (Cook *et al.* 2013). Correlations in the secondary metabolite composition of nectar, leaves and flowers have also been reported in *Nicotiana* spp. (Adler *et al.* 2006, 2012). However, differences in secondary metabolite profiles in *Asclepias* spp. suggest that while some nectar secondary metabolites may be corollaries of herbivore defence, other compounds probably have adaptive function (Manson *et al.* 2012). Thirdly, there could be benefits associated with low nectar alkaloid concentrations that we did not measure in this study. A recent study by Wright *et al.* (2013) found that at natural concentrations, the alkaloid caffeine in the nectar of such plants as *Coffea* increased honeybee memory, which could lead to increased constancy and outcrossing within a species. However, at concentrations higher than the natural range, caffeine was deterrent. Wright *et al.* (2013) argue that pollinators may have driven selection in nectar caffeine towards lower concentrations that are still pharmacologically active but not repellent. They also reported that the actions of caffeine were sensitive to d-Tubocurarine, an acetycholine receptor antagonist. Similar to caffeine, the norditerpene alkaloids of *D. barbeyi* act through a cholinergic mechanism (Green *et al.* 2011), and norditerpene alkaloids may therefore cause a similar neurophysiological response on

bee memory that could benefit pollination. Studies are needed, however, that assess the neurophysiological effects of *D. barbeyi* norditerpene alkaloids at natural concentrations and their effects on pollinator memory and pollen movement.

Our examination into the post-consumptive effects of *D. barbeyi* nectar alkaloids suggests that ingesting alkaloids reduced bumblebee activity and vigour, but only at concentrations well above the natural concentration produced in nectar. Moreover, even at the highest concentrations we tested, we observed no short-term effects on bumblebee mortality. These results are probably general across most bumblebee species. For example, *B. impatiens*, a smaller species not native to areas with *D. barbeyi* and found in a different *Bombus* subgenus than *B. appositus* (subgenus *Pyrobombus* vs. *Subterraneobombus*), also experienced negative effects after consuming high concentrations of *D. barbeyi* alkaloids (Cook *et al.* 2013). However, the direct post-consumptive effects of nectar alkaloids on bee performance in other studies have shown mixed results. For example, ingesting the nectar alkaloid gelsemine had no effect on larval development in the solitary mason bee *Osmia lignaria* under unlimited food conditions (Elliott *et al.* 2008), but consuming gelsemine-enriched sugar solution led to reduced ovary development in subordinate *B. impatiens* workers that had reduced access to food (Manson & Thomson 2009). What remains unknown is the degree to which nectar alkaloids in this and most other systems have sublethal effects on bumblebee colony reproduction. Our results suggest that natural concentrations of *D. barbeyi* nectar alkaloids are unlikely to have effects on worker activity within the hive, but whether alkaloids affect the growth and development of larvae fed *D. barbeyi* nectar, especially in cases when workers or colonies are majoring on *D. barbeyi*, is unknown and warrants further investigation.

There is growing recognition that secondary metabolites may be important in mediating not only plant–herbivore but also plant–pollinator interactions (Kessler, Gase & Baldwin 2008; Kessler & Halitschke 2009; Adler *et al.* 2012). We found that nectar alkaloids have dose-dependent effects on pollinator behaviour and activity. However, natural concentrations of nectar alkaloids in *D. barbeyi* have no negative direct or pollinator-mediated indirect effects on plant reproduction. Our findings highlight the importance of using manipulative experiments in natural plant populations and ecologically relevant treatment parameters within a whole-plant context to determine how secondary metabolite concentrations shape, and are shaped by, interactions between plants, mutualists and antagonists. Further, our results suggest that plants may be able to evolve optimal solutions to maintain pollination services in chemically defended plants.

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Supporting Information

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

Appendix S1. Laboratory protocol for the extraction of alkaloids from *Delphinium barbeyi* and comparison of alkaloid identities and concentrations among different plant parts, including nectar.

Appendix S2. Laboratory deterrence experiments with free-flying *Bombus impatiens*.

Table S1. Percentages of individual protonated ions in vegetative and reproductive tissues, nectar, and the alkaloid extract of *D. barbeyi.*