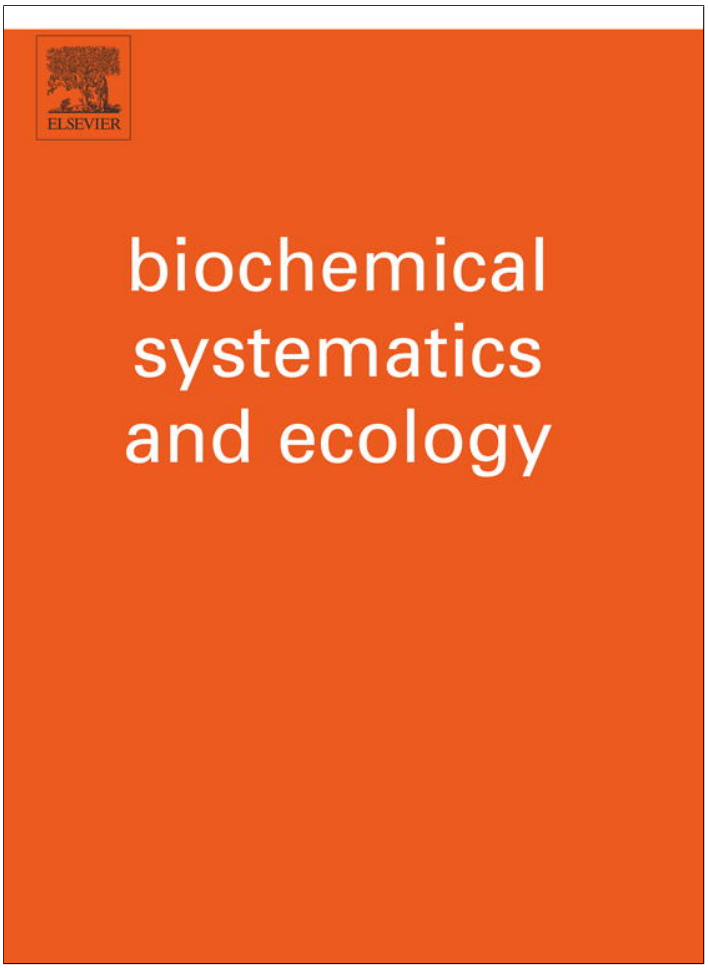
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Norditerpene alkaloid concentrations in tissues and floral rewards of larkspurs and impacts on pollinators

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ABSTRACT

Plant secondary compounds mediate interactions with insects and other animals. The norditerpene alkaloids are significant secondary compounds in *Delphinium* (larkspur) species which are divided into two classes: the 7, 8-methylenedioxylycoctonine (MDL-type) and *N*-(methylsuccinimido) anthranoyllycoctonine (MSAL-type), and are known to be toxic to herbivorous insects and livestock. Alkaloid concentrations were measured in a whole plant context in vegetative and floral tissues as well as rewards (pollen and nectar) in *Delphinium barbeyi* and *Delphinium nuttallianum*. Alkaloid concentrations differed between vegetative tissues, floral tissues and floral rewards. Alkaloid concentrations in floral parts were consistent with optimal defense theory, with tissues more closely tied to plant fitness, such as fruits, being more heavily defended than foliage. However, alkaloid concentrations were significantly lower in nectar compared to other tissues. The norditerpene alkaloids influenced the activity of bumble bees, the dominant pollinator of larkspur, but the effects were concentration dependent. Alkaloids in nectar are found at concentrations that have no effect on bee activity; however, if alkaloid concentrations in nectar were similar to those in foliage bee activity would be reduced significantly. These results suggest that nectar with low alkaloid concentrations may be beneficial to plant fitness by limiting adverse effects on pollinator activity.

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1. Introduction

Plant secondary compounds mediate interactions with a diverse suite of insects and other animals (McIntyre et al., 1981; Agrawal, 2000; Adler and Kittelson, 2004). The majority of studies that have measured plant secondary compounds have focused on their identities and concentrations in leaves or other vegetative material, and many of these secondary compounds have well-known effects of conferring plant resistance to herbivores (Rosenthal and Berenbaum, 1991). Comparatively fewer studies have measured plant secondary compounds in plant reproductive tissue, such as flowers, pollen, and fruits, as well as accessory floral rewards, such as nectar. Studies that have measured secondary compounds in

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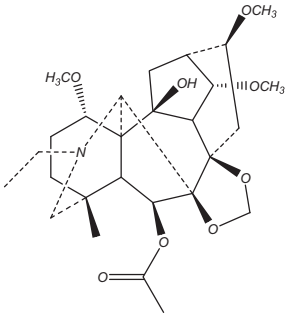
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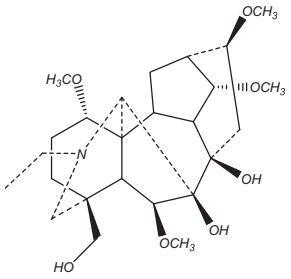
reproductive tissues or rewards have found that, in some cases, secondary compound concentrations in reproductive tissue can rival those in vegetative tissue (Ohnmeiss and Baldwin, 2000; Strauss et al., 2004; Frolich et al., 2006; Asplund et al., 2010). However, too few studies have measured secondary compound concentrations across tissue and reward types within plants to make broad generalizations (Kessler and Halitschke, 2009; McCall and Fordyce, 2010).

Optimal defense theory predicts that plant tissues directly tied to fitness should be most heavily defended against herbivores or other antagonists (McKey, 1974, 1979). There are a growing number of studies suggesting that secondary compound concentrations in fruits are equal or greater than in leaves consistent with optimal defense theory predictions (e.g., Zangerl and Rutledge, 1996; Gardner and Pfister, 2000). However, optimal defense theory does not make explicit predictions about secondary compound concentrations in rewards, such as nectar, or structures that have a dual-purpose of reward and gamete, such as pollen. On the one hand, there may be strong selection for plants to allocate secondary compounds in nectar and pollen to prevent removal by herbivores or larcenous floral visitors (Irwin et al., 2004). On the other hand, the accumulation of secondary compounds in nectar and pollen may come at a cost in terms of reduced pollinator visitation (Adler and Irwin, 2005; Kessler and Baldwin, 2007) or performance of pollinators (Manson and Thomson, 2009; Köhler et al., 2012). The accumulation of secondary compounds in nectar and pollen must be measured in a whole-plant context to understand whether the concentration and composition of these compounds in floral rewards are positively associated with concentrations and composition in other tissue types, and thus likely a consequence of plant defense (Adler, 2000), or whether the plant may control the distribution and accumulation of secondary metabolites in floral rewards, which may indicate an adaptive response.

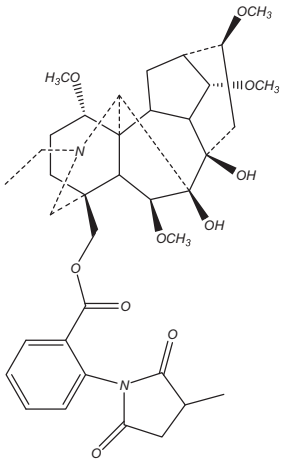
The dominant secondary compounds in *Delphinium* (larkspur) species are the norditerpene alkaloids which are divided into two classes: the 7, 8-methylenedioxylycoctonine (MDL-type) and *N*-(methylsuccinimido) anthranoyllycoctonine (MSAL-type) (Pfister et al., 1999) (Fig. 1). The MSAL-type alkaloids are significantly more toxic based upon assays with the generalist herbivores, *Spodoptera eridania* and *Musca domestica*, as well as in a mouse model and cattle (Jennings et al., 1986; Manners et al., 1993, 1995; Cook et al., 2011; Welch et al., 2012). Because the amounts of the norditerpene alkaloids in nectar and pollen are unknown as are their effects on the primary pollinators of *Delphinium*, the objective of this study was to measure the identities and concentrations of the norditerpene alkaloids across vegetative and reproductive tissues as well as floral rewards in two *Delphinium* species and to assess the bioactivity of these compounds on floral visitors. Two larkspurs, *Delphinium nuttallianum* and *Delphinium barbeyi*, were investigated because the alkaloids in these species are well



Deltaline (MDL-type)



Lycoctonine (MDL-type)



Methyllycaconitine/MLA (MSAL-type)

Fig. 1. Structure of select norditerpene alkaloids in *Delphinium* species.

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characterized in their vegetative tissue and fruits (Gardner and Pfister, 2000; Ralphs and Gardner, 2003), they are widely used in studies of pollination biology (e.g., Inouye, 1978; Waser and Price, 1983; Elliott and Irwin, 2009), and they have different life histories (Ewan, 1945; Williams et al., 2001). Specifically the following questions were addressed: (1) what are the identities and concentrations of the norditerpene alkaloids in vegetative and reproductive tissues and how do they compare to those found in nectar and pollen, (2) how do the identities and concentrations of the norditerpene alkaloids in the different tissue types vary between the two species, and (3) do the norditerpene alkaloids affect the activity of the dominant floral visitors, bumble bees, of *Delphinium*?

2. Material and methods

2.1. Plant materials

Analytical samples were prepared from plant material collected near Gothic, CO (N 38° 58.264′ W 106° 59.791′) representing *D. barbeyi* Huth and *D. nuttallianum* Pritz. Ten plants of *D. barbeyi* and twenty plants of *D. nuttallianum* in full flower to early fruit were collected. Plants were separated into the following plant parts: stems, leaves, flowers, and fruits. Additionally, anthers (including pollen and pollen sac) and nectar were collected from each plant. Plant parts and anthers were oven dried at 40 °C. Plant parts were ground using a Wiley mill or a Retsch mixer mill MM301 (Haan, Germany). Nectar was frozen at −80 °C until it was analyzed.

In addition, because anther collections included pollen as well as pollen sac, corbicular pollen loads were also collected from bumble bees foraging on *D. barbeyi* and *D. nuttallianum* to assess whether pollen loads also contained norditerpene alkaloids. One corbicular pollen load was collected from each of 12 bees foraging on *D. barbeyi* and 10 bees foraging on *D. nuttallianum*. Corbicular pollen loads can contain a mixture of pollen from multiple species. To ensure that we were analyzing pollen loads that contained primarily the *Delphiniums* of interest, two subsamples of pollen from each pollen load were stained using fuchsin dye (Kearns and Inouye, 1993) and examined under a microscope. The two *Delphinium* species do not overlap in flowering phenology and have distinct pollen coats from other co-occurring flowering species. The pollen subsamples were scored for the proportion of *Delphinium* pollen grains, and pollen loads that had >80% *Delphinium* pollen were analyzed. Pollen loads were stored at −20 °C until chemical analysis.

2.2. Sample extraction and alkaloid analysis

Plant parts (stems, leaves, flowers, fruits, anthers, and pollen loads) were extracted and analyzed by electrospray mass spectrometry using procedures previously described (Gardner et al., 1999). In summary, a measured quantity of plant material (50 mg) was extracted in 1.5 ml of methanol for 16 h. Reserpine (250 μg for *D. barbeyi*, 125 μg for *D. nuttallianum*) was added as an internal reference after extraction. The sample was mixed and then centrifuged. An aliquot of the extracted sample was diluted into 1:1 methanol/1% acetic acid for a total volume of 1 ml for analysis. For samples with less than 50 mg of material, the volume of methanol and amount of reserpine were adjusted accordingly.

Nectar samples were analyzed by reverse-phase HPLC-esi(+) MS using methods previously described (Gardner and Pfister, 2009). In summary, a measured volume of nectar (10 μl) was extracted in 90 μl of 1:1 methanol/1% acetic acid. Reserpine (0.1 μg for *D. barbeyi* and *D. nuttallianum*) was added as an internal reference. The samples were mixed and then centrifuged, and an aliquot was removed for analysis.

Mass spectra were recorded for each sample over a range of 150–800 *m/z* and averaged across all scans taken at 40% of peak height (total ion current). Data were calculated by recording the abundance of all ions above a relative area of 0.1%. The amount of a compound (as represented by a single mass unit) detected was calculated based on the relative abundance of the internal standard reserpine ($MH^+ = 609$). The resulting mass spectral data were reduced and tabulated to a final set of quantitative values for 18 different protonated molecules (*m/z* 414, 428, 454, 466, 468, 482, 494, 496, 508, 536, 552, 564, 578, 667, 669, 683, 697, and 711) using a method similar to that reported by Gardner et al. (2002) (Table 2). All alkaloid amounts were expressed as μg alkaloid/100 mg plant material. Alkaloid concentrations reported here are relative as they have been normalized to an internal reserpine standard.

Delphinium crude alkaloid extract (used in Section 2.3 below) 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′) using the methods described in Cook et al. (2011). The individual *Delphinium* alkaloids, deltaline and methyllycaconitine (MLA), were reference standards at the Poisonous Plant Research Laboratory previously extracted from *D. barbeyi* (Pelletier et al., 1989).

2.3. Alkaloid affects on bumble bees

To measure the effects of alkaloid consumption on bumble bees, workers from commercial colonies of *Bombus impatiens* (Biobest, Leamington, Canada) were fed artificial nectar enriched with *D. barbeyi* alkaloids. *Bombus impatiens* was used because it is a commercially available generalist species in the large subgenus *Pyrobombus*, and some bumble bee species that forage on *D. barbeyi*, such as *Bombus flavifrons* (Inouye, 1978), are also in the same subgenus; thus the reported results are applicable to some visitors of *D. barbeyi*. Furthermore, worker bees were used because *D. barbeyi* is an abundant floral resource that is frequently a focal species for foraging worker bumble bees (Inouye, 1978; Elliott, 2008). Artificial nectar

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Table 1
Alkaloid (MDL, MSAL, and total) amounts in vegetative tissues, floral tissues, and rewards of *Delphinium barbeyi* (A) and *Delphinium nuttallianum* (B).

A.			
D. barbeyi ($n = 10$)	μg alkaloid/100 mg[a,b]		
	MDL	MSAL	Total
Leaves	1629 ± 136^{2}	446 ± 52^{1}	2075 ± 126^{2}
Stem	370 ± 38^{3}	420 ± 33^{1}	790 ± 38^{3}
Flowers	1842 ± 109^{2}	210 ± 34^{2}	2052 ± 105^{2}
Fruits	3333 ± 290^{1}	533 ± 85^{1}	3867 ± 315^{1}
Anthers	$2069 \pm 157^{1,2}$	$287 \pm 54^{1,2}$	2357 ± 164^{2}
Pollen Loads	230 ± 17^{3}	90 ± 9^{3}	321 ± 25^{4}
Nectar	1.4 ± 0.4^{4}	0.3 ± 0.08^{4}	1.7 ± 0.4^{5}
B.			
D. nuttallianum ($n = 20$)	μg alkaloid/100 mg[a,b]		
	MDL	MSAL	Total
Leaves	190 ± 22^{2}	203 ± 27^{4}	393 ± 45^{3}
Stem	135 ± 13^{2}	420 ± 49^{3}	555 ± 57^{3}
Flowers	620 ± 45^{1}	737 ± 47^{2}	1357 ± 74^{2}
Fruits	883 ± 56^{1}	1487 ± 105^{1}	2370 ± 137^{1}
Anthers	672 ± 45^{1}	870 ± 71^{2}	1541 ± 102^{2}
Pollen loads	113 ± 13^{2}	79 ± 9^{5}	192 ± 22^{4}
Nectar	0.2 ± 0.03^{3}	0.5 ± 0.08^{6}	0.7 ± 0.1^{5}

[a] Different numbers within a column represent statistical significance ($P < 0.05$) between tissues and rewards.
[b] Relative alkaloid concentrations per 100 mg of plant material. Concentrations were normalized to an internal reserpine standard.

enriched with *D. barbeyi* alkaloids was prepared from a crude alkaloid extract as well as the dominant alkaloids deltaline (an MDL-type alkaloid) and methyllycaconitine (an MSAL-type alkaloid). Bees were provided with artificial nectar comprised of 45% sucrose solution (Control), a 45% sucrose solution enriched with alkaloids at 4 concentrations (0.1, 1, 2, and 4 mg/ml), or a "no nectar" treatment to determine whether the observed behavioral responses were due to the consumption of alkaloids or were a result of potential reduced food intake in the alkaloid treatments. In the crude alkaloids experiments, 10 bees per treatment were used and in the individual alkaloid experiments, 5 bees per treatment were used. Bees were taken from three colonies to ensure results were not colony specific, and bees were mixed haphazardly across treatments to avoid any colony by treatment interactions.

Individual workers were placed in vials, starved for 2 h, and then fed 500 μl of sucrose solution of the appropriate treatment, except in the "no nectar" treatment, where bees received no sucrose solution. After 24 h, individual bees were transferred to a clear plastic container fitted with a removable lid with multiple perforations. Bees were agitated by blowing on them for 90 s, and simultaneously, their behavior was recorded with a digital camcorder to measure their response to negative stimuli. Bees were freeze-killed after their activity was recorded.

Videos of each bee were scored for nine activities, including tarsal or wing movements, aggression (attempted stinging) and flight, using a subjective scale of 0–5, with 0 indicating the behavior in question was not observed and 5 indicating that the behavior was nearly continuous. Activities were scored based on both frequency and intensity. For each bee, the scores were summed to obtain a general metric of bee activity (the summed scores ranged from 0 to 45).

2.4. Data analysis

For all comparisons of alkaloid concentrations in different tissues, concentrations were log-transformed and a one-way ANOVA was performed using Sigma Stat 3.1 with a post-hoc test of significance using a Bonferroni correction. A p-value of <0.05 was considered to be statistically significant. Mean values reported are $\pm$ the standard error. To analyze bee activity, a non-parametric Kruskal–Wallis test was used to test the effects of alkaloids on bee activity. Significant Kruskal–Wallis tests were followed by non-parametric multiple comparison tests using the R package npmcI (R version 2.10.1).

3. Results and discussion

The MDL-type, MSAL-type, and total alkaloids differed between vegetative tissues, floral tissues, and rewards in *D. barbeyi* and *D. nuttallianum* ($P < 0.001$) (Table 1). Fruits had the highest concentrations of each alkaloid class and total alkaloids in both species (Table 1A and B). The rank order of alkaloid concentrations, MDL, MSAL, and total, in other tissues varied between species (Table 1A and B). For example, total alkaloid concentrations in leaves of *D. barbeyi* were 1.9 times less than those in fruits; however, in *D. nuttallianum* concentrations in leaves were 6 times less than those found in fruits. Total alkaloid concentrations in anthers in both species were at least 7 times higher than concentrations in pollen loads (Table 1A and B). The rewards, pollen loads and nectar, had the smallest total concentrations of alkaloids in both species, respectively (Table 1A and B), with nectar having at least 189 times less alkaloid than pollen loads.

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Table 2
Amounts of individual protonated ions in vegetative tissues, floral tissues, and rewards of *Delphinium barbeyi* (A) and *Delphinium nuttallianum* (B).

m/z[a]	Possible alkaloids[a]	µg alkaloid/100 mg[b]						
		Alkaloid type	Leaves	Stem	Flowers	Fruits	Anthers	Pollen loads	Nectar
A.									
414	11,13-Diacetyhetisine	MDL	8	1	9	15	12	3	0.01
428	Geyerine	MDL	0	0	0	3	3	0	0.01
454	Delcosine, Delectinine	MDL	17	5	58	139	96	15	0.05
466	Deltamine, 14-Dehydrobrowniine	MDL	18	9	21	37	66	4	0.07
468	Delsoline, Lycoctonine	MDL	16	3	65	84	238	48	0.10
482	Delphatine	MDL	4	0	2	5	10	0	0.02
494	Dictyocarpine	MDL	73	26	55	57	37	5	0.03
496	14-Acetyldelectinine, 6-Epi-pubescenine	MDL	8	0	8	10	23	11	0.01
508	Deltaline	MDL	1023	267	1101	2043	957	101	0.63
536	14-Acetlydictyocarpine	MDL	431	57	490	859	473	31	0.34
552	Glaucedine	MDL	1	1	4	8	37	2	0.03
564	Glaucerine	MDL	3	0	5	11	39	3	0.01
578	Barbisine, Glaucenine	MDL	27	2	24	63	80	9	0.04
667	Barbinine	MSAL	11	9	9	16	17	0	0.05
669	14-Deacetylnudicauline, 16-Deacetylgeyerline	MSAL	53	29	43	68	72	23	0.10
683	Methyllycaconitine	MSAL	381	382	157	447	191	66	0.16
697	Bearline and isomers	MSAL	1	0	0	3	4	0	0
711	Geyerline, Nudicauline, Acetylgrandifluorine	MSAL	1	0	1	0	4	1	0.03
B.									
414	11,13-Diacetyhetisine	MDL	0	0	0	1	3	0	0
428	Geyerine	MDL	0	0	0	1	4	0	0
454	Delcosine, Delectinine	MDL	43	33	102	161	166	22	0
466	Deltamine, 14-Dehydrobrowniine	MDL	20	14	58	74	40	0	0
468	Delsoline, Lycoctonine	MDL	71	55	330	444	316	54	0.12
482	Delphatine	MDL	26	12	68	85	57	4	0.03
494	Dictyocarpine	MDL	0	0	1	7	5	0	0
496	14-Acetyldelectinine, 6-Epi-pubescenine	MDL	17	16	40	75	44	32	0.04
508	Deltaline	MDL	1	1	17	25	19	1	0
536	14-Acetlydictyocarpine	MDL	0	0	0	2	3	0	0
552	Glaucedine	MDL	0	0	0	1	2	0	0
564	Glaucerine	MDL	14	4	4	6	9	0	0
578	Barbisine, Glaucenine	MDL	0	0	0	1	3	0	0
667	Barbinine	MSAL	31	72	100	166	83	4	0
669	14-Deacetylnudicauline, 16-Deacetylgeyerline	MSAL	148	224	514	934	589	44	0.44
683	Methyllycaconitine	MSAL	1	8	5	10	9	8	0.01
697	Bearline and isomers	MSAL	16	21	35	77	32	0	0.02
711	Geyerline, Nudicauline, Acetylgrandifluorine	MSAL	8	95	84	300	156	24	0.07

[a] Alkaloid identification taken from Pelletier et al. (1981, 1989); Joshi et al. (1988, 1989); Kulanthaivel et al. (1988, 1990); Manners et al. (1996, 1998).
[b] Relative alkaloid concentrations per 100 mg of plant material. Concentrations were normalized to an internal reserpine standard.

The concentrations of alkaloids in the different vegetative and floral tissues were consistent with what one may predict for optimal defense theory (McKey, 1974, 1979). Fruits were most heavily defended as they accumulated the greatest concentrations of MDL, MSAL, and total alkaloids in both species. Other floral tissues, including flowers and anthers, also accumulated significant concentrations of alkaloids in both species consistent with optimal defense theory predictions. The concentrations of alkaloids observed in fruits, flowers, and anthers are biologically active against herbivorous insects and mammals (Jennings et al., 1986; Pfister et al., 2002).

Differences in alkaloid allocation in different plant parts between species, such as those found in leaves and fruits, may be due to the different life histories of *D. barbeyi* and *D. nuttallianum*, with insights gained from theories of plant defense (McKey, 1974, 1979; Coley et al., 1985). *D. barbeyi* is often a multi-stemmed plant growing up to 2.5 m with 8–24 leaves per stalk and 10–50 flowers per stalk resulting in often hundreds of flowers per plant; in comparison, *D. nuttallianum* a single stemmed plant growing up to 0.7 m with 2–6 leaves and 4–48 flowers per plant (Ewan, 1945; Warnock, 1997; Williams et al., 2001). Both species emerge as the snow melts; however, *D. nuttallianum* completes its life cycle by setting seed typically within 2 months of snow melt while *D. barbeyi* persists throughout the summer and does not flower until early to mid summer, generally setting seed in late summer. As *D. barbeyi* has a longer life cycle and is a much more robust plant than *D. nuttallianum*, one may expect more similar alkaloid concentrations in leaves and fruits because it is exposed to external biotic stressors for a longer period of time. However, in *D. nuttallianum* one may expect higher concentrations in fruits relative to leaves due to its relatively shorter life cycle and potentially high fitness value of fruits and seeds. Furthermore, the Resource Availability hypothesis would predict the observation that the leaves of longer-lived species would be more heavily defended than shorter-lived species (Coley et al., 1985). In summary, the differences in life history specifically the time for the plant to emerge at snow melt and set seed may explain the allocation of alkaloids across tissues within the two species.

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We predicted that concentrations of alkaloids found in anthers may be representative of alkaloid concentrations found in pollen. However, concentrations of the MDL, MSAL and total alkaloids differed significantly between anthers and bee pollen loads in both species ($P < 0.001$, Table 1). Anthers typically contained 8 times more total alkaloids than pollen loads. Because pollen loads were not pure *Delphinium* pollen and rather were a mixture of pollen (>80% *Delphinium* pollen) and nectar (which serves as the matrix that holds bee pollen loads together), it seems probable that the concentrations of total alkaloids as well as the two structural classes in *Delphinium* pollen may be significantly underestimated using bee pollen loads. Studies are needed that collect granular pollen directly from the plant to verify this speculation. Nonetheless, research documenting secondary compounds in stamens are increasing in number and typically find that concentrations of compounds in stamens are higher than in nectar (Detzel and Wink, 1993; London-Shafir et al., 2003) and in some cases can be higher on a per-gram basis fresh weight than that reported in leaves (Frolich et al., 2006). While the ecological costs and benefits of secondary compounds in nectar, particularly as they pertain to pollinators, is currently receiving attention (reviewed in Adler, 2000), assessing their costs and benefits in pollen has lagged behind and studies are warranted.

The qualitative alkaloid profile differed significantly between the two species ($P < 0.001$). The alkaloid profiles reported here for *D. barbeyi* and *D. nuttallianum* are consistent with previous observations for these species at the same locations (Gardner et al., 2002; Gardner and Pfister, 2009). Deltaline and methyllycaconitine (MLA) were the dominant MDL-type alkaloid and MSAL-type alkaloid in *D. barbeyi*, respectively (Table 2A). Deltaline and MLA comprised greater than 60% of the total alkaloids in *D. barbeyi* in all tissues and rewards analyzed. In contrast, deltaline and MLA were minor alkaloids in the overall alkaloid composition in *D. nuttallianum*. The protonated molecules at *m/z* 468 and 669 were the dominant MDL-type and MSAL-type alkaloid(s) in *D. nuttallianum*, respectively, where they comprised 50–60% of the total alkaloids in the tissues analyzed (Table 2B). In *D. barbeyi*, the MDL-type alkaloids were at least 2.5 times greater than the MSAL-type alkaloids in all tissues and rewards other than stems. In contrast, the MSAL-type alkaloids in *D. nuttallianum* were equivalent or greater than the MDL-type alkaloids in all tissues including the rewards. The qualitative profile of alkaloids was similar within tissues and rewards within a species but differed quantitatively (Table 2A and B). Lastly, the data suggests that no novel norditerpene alkaloids appeared in the different tissues or rewards.

Alkaloid amounts found in the nectar of both species differed quantitatively but not qualitatively compared to other tissues and pollen ($P < 0.001$). Although, the alkaloid amounts detected in nectar were rather small (<1.7 μg/g total alkaloid), it is unlikely that they are due to contamination from the nectar sampling process as there was no mechanical damage to the flower tissue during the sampling. Given their low concentrations, the alkaloid amounts found in nectar are unlikely to provide any defense to the plant against nectar robbers or thieves (Irwin and Maloof, 2002; Burkle et al., 2007). Robbing rates in *D. nuttallianum* range from 0 to 100% of available flowers robbed with a peak mean robbing level of 56.5% ± 8.5% (mean ± SE) of flowers robbed (Irwin and Maloof, 2002). Rather, the results may suggest that the *Delphinium* species investigated may exclude the alkaloids from nectar as they may come at a cost in terms of reduced pollinator visitation (Adler and Irwin, 2005). An alternative explanation is that at the time of flowering, alkaloids may have already been provisioned into leaves and flower parts and there is little alkaloid in phloem (given that many alkaloids such as the quinolizidine alkaloids are translocated in phloem, Lee et al., 2007). Studies measuring alkaloid concentrations in nectar as well as phloem at different developmental stages would provide additional insights.

To test if increased alkaloid amounts in nectar could affect pollinator activity, the activity of the dominant floral visitors, bumble bees, were evaluated after consumption of nectar enriched with norditerpene alkaloids of *D. barbeyi*. Bees that consumed the nectar enriched with the *D. barbeyi* crude alkaloid extract had a significant decrease in bee activity ($P < 0.001$). Overall activity declined as the concentration of crude alkaloids in nectar increased. There was a significant reduction in activity for bees fed the nectar enriched with the 4 mg/ml crude alkaloid extract compared to the control nectar solution (Fig. 2A). In general, bee activity tended to fall into one of two categories: high activity level, observed in bees fed the control solution, nectar enriched with the 0.1 mg/ml crude alkaloid extracts, and bees that received no nectar, and decreased or low activity level, observed in bees fed nectar enriched with 1, 2, and 4 mg/ml crude alkaloid extract. Lastly, no differences in post consumptive bee activity were observed between the control nectar solution and no nectar treatments (Fig. 2A). These results suggest that the changes observed in bee activity were due to the alkaloids, and not simply the result of low energy due to reduced consumption of sucrose solutions with alkaloids.

In addition, both deltaline and MLA, the dominant MDL and MSAL-type alkaloids in *D. barbeyi*, significantly affected post-consumption activity in bumble bees ($P < 0.001$). As was observed with the crude alkaloid extract, reduced bee activity was observed as the concentrations in both deltaline and MLA enriched nectar increased (Fig. 2B). Lastly, no differences in post consumptive bee activity were observed when comparing bees fed similar concentrations of the deltaline and the MLA enriched nectar solutions (Fig. 2B). As observed with the crude alkaloid extracts, bee activity tended to fall into one of two categories: high activity level, observed in bees fed the control solution, nectar enriched with the 0.1 mg/ml deltaline and MLA, and bees that received no nectar, and decreased or low activity level, observed in bees fed nectar enriched with 1, 2, and 4 mg/ml deltaline or MLA. Although the reduced bee activity was observed with the crude *D. barbeyi* alkaloid extract and its dominant MDL and MSAL-type alkaloids, it is probable that similar results would be observed with a crude extract and purified alkaloids from *D. nuttallianum*. Individual MDL-type alkaloids have similar bioactivities, as do individual MSAL-type alkaloids based upon LD_{50} studies in a mouse model (Manners et al., 1995, 1998).

The effects of crude and purified alkaloids on bees that were observed match studies showing that secondary compounds in nectar can influence pollinator activity or visitation patterns within and/or among flowers and plants (Detzel and Wink, 1993; Tadmor-Melamed et al., 2004; Adler and Irwin, 2005; Gegear et al., 2007; Kessler and Baldwin, 2007; Sharp et al.,

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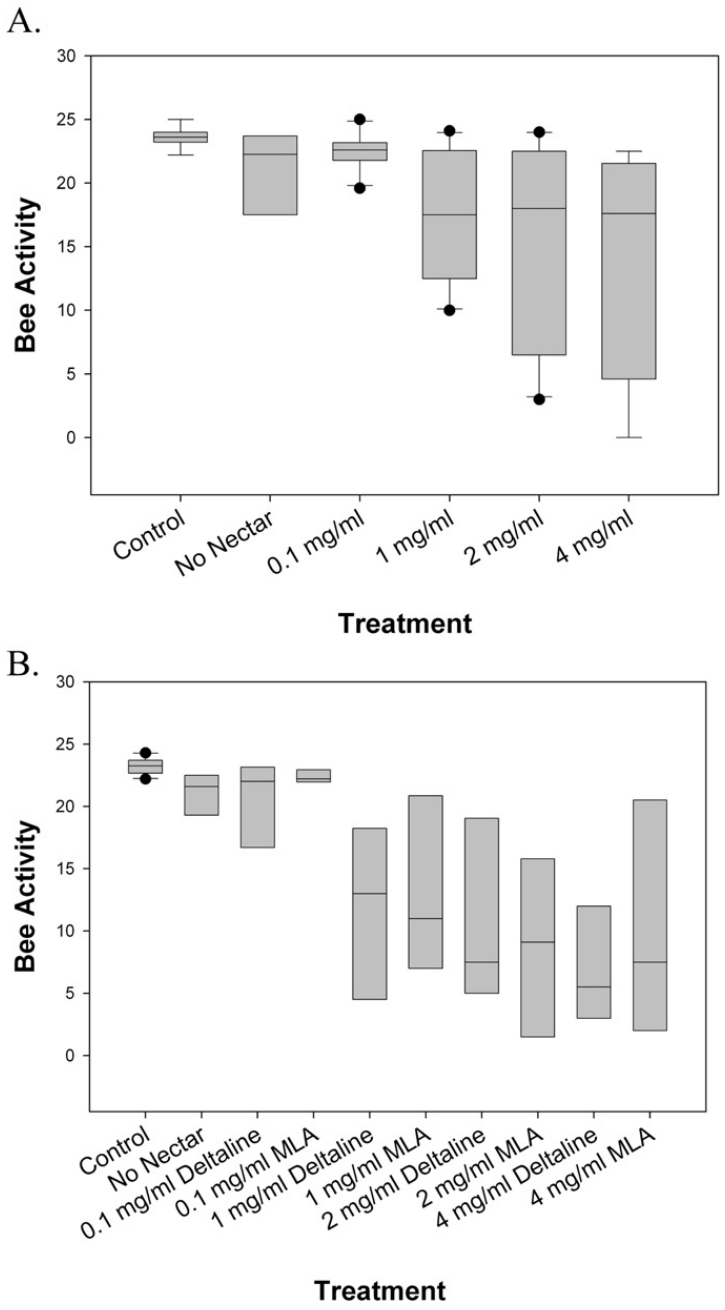


Fig. 2. Box-whisker plot showing post consumptive bee activity for bees fed (A) treatments of crude alkaloid extract at concentrations ranging from 0.1 mg/ml to 4 mg/ml and (B) treatments of deltaline and MLA purified extracts at concentrations ranging from 0.1 mg/ml to 4 mg/ml.

2009). These studies were performed on a diversity of pollinators, including honey and bumble bees, solitary bees, hawk-moths, hummingbirds, and sunbirds. It remains to be explored whether the patterns reported here of alkaloid effects on bee activity hold for the diversity of bumble bee visitors and other bird and insect visitors to *Delphinium*. Moreover, the results we report also mirror studies showing that the effects of secondary compounds in nectar can be concentration dependent (Detzel and Wink, 1993; Hagler and Buchmann, 1993; Tadmor-Melamed et al., 2004). Our results suggest that if the norditerpene alkaloids accumulated in nectar to concentrations similar to those found in vegetative and floral tissue, then there could be negative effects on pollinator foraging and potentially plant fitness, assuming plants are pollinator- and pollen-limited for fruit and seed set. To test this hypothesis further would require manipulations of nectar alkaloids to plants in the field and measurement of pollinator visitation and plant reproduction.

In conclusion, norditerpene alkaloid concentrations were measured in a whole plant context including vegetative tissues, reproductive tissues, and rewards in *D. barbeyi* and *D. nuttallianum*. Alkaloid concentrations were consistent with predictions made by the optimal defense theory. Furthermore, we suggest that alkaloid allocation in different tissues may be influenced by the life history strategy of the plant. Lastly, both *Delphinium* species had low amounts of alkaloids in nectar relative to vegetative and floral tissues, and feeding experiments with bumble bees suggest that plants may benefit from low nectar alkaloids given the negative concentration-dependent effects of nectar alkaloids on bee activity.

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