1 Seasonal variation in the secondary chemistry of foliar and reproductive tissue of
2 *Delphinium nuttallianum*

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31 Abstract

32 Plant secondary compounds are critical in affecting interactions between plants and their
33 herbivores. The norditerpene alkaloids are secondary compounds in *Delphinium* (larkspur)
34 species which are divided into two classes: the *N*-(methylsuccinimido) anthranoyllycoctonine
35 (MSAL-type) and non MSAL-type, and are known to be toxic to herbivorous insects and
36 livestock. Alkaloid concentrations were measured in a whole plant context in vegetative and
37 reproductive tissues in *D. nuttallianum* at different stages of plant maturity at two locations to
38 explore how plant maturity affected alkaloid concentrations within a growing season. Alkaloid
39 concentrations differed between vegetative and reproductive tissues, with vegetative tissues
40 having significantly lower alkaloid concentrations than reproductive tissues. However, no
41 systematic differences in alkaloid concentrations were observed at different plant maturity stages
42 across the growing season. Based on the data we suggest that alkaloid allocation in different
43 plant parts of *D. nuttallianum* is influenced by life history of the plant, consistent with plant
44 defense theory. At one location, as pods mature the qualitative alkaloid composition changed
45 through structural diversification of the alkaloids present. The ecological significance of this
46 structural diversification awaits further exploration.

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50 Keywords-Larkspur; *Delphinium*; norditerpene alkaloids; plant parts; life history.

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1. Introduction

Plant secondary compounds play an important role in affecting interactions between plants and a diverse group of insects and other animals (McIntyre et al. 1981; Rosenthal and Berenbaum, 1991; Adler and Kittelson 2004). Despite the overwhelming importance of secondary compounds in mediating species interactions, plant species can be highly variable in their secondary chemistry. A variety of factors can contribute to this variation in secondary chemistry, including genetics (Orians and Fritz, 1995; Orians, 2000), resource availability (Bryant et al. 1987; Orians et al. 2003), and previous patterns of damage (Hartley and Lawton, 1987; Baldwin, 1988). There is also recognition that plant maturity or ontogeny can contribute to intraspecific variation in plant secondary chemistry (Barton and Koricheva, 2010). Optimal defense theory predicts that plant tissues more closely tied to fitness should be most heavily defended against herbivores or other antagonists (McKey 1974; 1979). In a whole-plant context, this prediction could be extended such that as plants mature within a growing and flowering season, their defense investment should be tied to the life-history stages most closely tied to fitness and as a function of damage (Barton and Koricheva, 2010). However, optimal levels of defense may be constrained by plant growth and the availability of carbon and other nutrients used for secondary compound production (Bryant et al. 1987; Dudt and Shure, 1994; Orians et al. 2003). A number of studies have reported that secondary compound concentrations in fruits are equal to or greater than in leaves, consistent with optimal defense theory predictions (e.g., Zangerl and Rutledge 1996; Gardner and Pfister, 2000). However, the degree to which plant ontogeny or maturity affects the expression of secondary compounds has yielded mixed results (Barton and Koricheva, 2010). The goal of this study was to explore how plant maturity affected

the expression of plant secondary chemistry in vegetative and reproductive tissues of the toxic rangeland plant *Delphinium nuttallianum* (Ranunculaceae).

The norditerpene alkaloids are the dominant secondary compounds in *Delphinium* (larkspur) species which are divided into two classes: the *N*-(methylsuccinimido) anthranoyllycoctonine (MSAL-type) and the non-MSAL-type (Pfister et al. 1999) (Figure 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 experimentation with cattle (Jennings et al. 1986; Manners et al. 1995; Cook et al. 2011).

Alkaloid profiles in *Delphinium* spp. have been shown to differ among species, populations, and plant parts (Gardner et al. 2002; Cook et al. 2009; 2015). These differences can influence the toxicity to grazing livestock and may influence plant interactions with insects and other animals (Pfister et al. 2002; Welch et al. 2012; 2015).

Several studies have investigated norditerpene alkaloid concentrations in tall larkspurs such as *D. barbeyi*, *D. glaucum*, and *D. occidentale* as a function of plant maturity and in response to different environmental perturbations (Ralphs et al. 1998; Gardner and Pfister, 2000; Ralphs and Gardner 2003). In tall larkspur species, the norditerpene alkaloid concentrations are at their highest concentration early in the growing season and those concentrations decrease as the plant grows and matures (Ralphs et al. 1997; Gardner and Pfister, 2000; Ralphs and Gardner, 2003). Similarly, in individual plant parts in tall larkspur, alkaloid concentrations are at their highest in young immature tissues and decrease as the tissues mature and grow (Gardner and Pfister, 2000; Ralphs et al. 2000). Additionally, norditerpene alkaloid concentrations vary little among vegetative and floral parts at any given phenological stage, with norditerpene alkaloid

concentrations generally only 1-2.5 times greater in fruits compared to leaves (Gardner and Pfister, 2000; Ralphs and Gardner, 2003; Cook et al. 2013).

In comparison, recent research in the low larkspur *D. nuttallianum* demonstrated that alkaloid concentrations differed dramatically among plant parts. For example, concentrations were approximately 6 times greater in fruits than leaves, compared to only approximately 2 times different in *D. barbeyi*. However, these data reflected plant tissues collected at a single time-point when plants were fully mature, had open flowers, and maturing fruits (Cook et al. 2013). On the one hand, these differences between the two species could be due to insufficient sampling of *D. nuttallianum* and sampling at a time of year when differences are maximized. Moreover, these differences in chemistry between the two species may be due to different life histories of *D. nuttallianum* compared to tall larkspur species, such as *D. barbeyi*. Both *D. nuttallianum* and tall larkspur species emerge as the snow melts; however, *D. nuttallianum* completes its life cycle by setting seed typically early to mid-summer while *D. barbeyi* and other tall larkspur species persist throughout the summer and do not flower until mid summer, generally setting seed in late summer. Due to the relatively short life cycle of *D. nuttallianum*, plant defense theory predicts a low concentration of alkaloids in its leaves relative to other parts and relative to the leaves of longer-lived species such as *D. barbeyi* (reviewed in Endara and Coley 2011). However, to more fully test this hypothesis, measurements of secondary chemistry are needed throughout the growing and flowering season of *D. nuttallianum*.

To better understand how alkaloid concentrations change as a function of plant maturity for plants with different life histories, the objective of this study was to measure the qualitative profiles and concentrations of the norditerpene alkaloids across vegetative and reproductive tissues in *D. nuttallianum* over a growing season. *Delphinium nuttallianum* was investigated

because the alkaloids in this species are well characterized in their vegetative tissue and fruits (Gardner and Pfister, 2009) and has a different life history (Ewan, 1945; Williams et al. 2001) than tall larkspur species such as *D. barbeyi*, *D. glaucum*, and *D. occidentale*. Specifically, the following questions were addressed: (1) what are the qualitative profiles and concentrations of the norditerpene alkaloids in vegetative and reproductive tissues and how do they vary seasonally between two populations of *D. nuttallianum*, and (2) is there any evidence that the qualitative norditerpene alkaloid composition changes as a function of plant part or maturity?

2. Material and methods

2.1. Plant Materials

Analytical samples were prepared from *D. nuttallianum* Pritz plant material collected near Gothic, CO (N 38° 58.264' W 106° 59.791') and Logan, UT (N 41° 55.697' W 111° 27.028'). Ten plants of *D. nuttallianum* were collected at regular intervals (approximately weekly) from early flower to late pod over a 5-week period in 2011. At each collection interval, plants were separated into the following parts: stems, leaves, flowers, and fruits. Plant parts were air dried at room temperature and then ground using a Wiley mill or a Retsch mixer mill MM301 (Haan, Germany).

2.2. Sample Extraction and Alkaloid Analysis

Plant parts (stems, leaves, flowers, fruits) were extracted and analyzed by electrospray mass spectrometry using procedures previously described (Gardner et al. 1999). A measured quantity of plant material (50 mg) was extracted in 1.5 ml of methanol for 16 h. Reserpine (150 µg) 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.

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 1). 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.

Samples from both locations as well as the pods from early pod and full pod phenological stages were analyzed by reverse-phase HPLC-esi(+) MS using methods previously described (Gardner et al. 2009). This enabled identification of the protonated molecules (m/z 669 and 711) in the different plants as a function of maturity.

2.4. Data Analysis

For all comparisons of alkaloid concentrations in different parts, concentrations were log-transformed to improve normality. To assess if alkaloid concentrations varied with plant part and time, we performed a two-way ANOVA in SAS 9.3 (SAS Institute, Cary, NC, USA). Separate analyses were performed for each site (Logan, UT and Gothic, CO). Least square means were calculated in SAS for the analysis; however, all means presented in this paper are

unadjusted and non-transformed. When significant interactions ($P < 0.05$) were detected, the PDIFF option with a Tukey adjustment was used for mean comparisons.

3. Results and Discussion

The qualitative norditerpene alkaloid composition differed between the two collection sites, with the rank order of the individual norditerpene alkaloids varying between sites (Table 1). The dominant MSAL-type alkaloids at Logan, UT were nudicauline (m/z 711), methyllycaconitine (m/z 683), and 14-deacetylnudicauline (m/z 669) while the dominant MSAL-type alkaloids at Gothic, CO were 14-deacetylnudicauline (m/z 669), barbinine (m/z 667), and nudicauline (m/z 711). The protonated molecules at m/z 468, 466, 454, likely non-MSAL type alkaloids, represented other dominant alkaloids at both collection sites. The alkaloid profiles reported here for *D. nuttallianum* are consistent with previous observations for this species at the same locations (Gardner and Pfister, 2009). Differences in the qualitative MSAL-type alkaloid profiles between these sites may offer additional tools to study the relative role of other MSAL-type alkaloids and their relative toxicity. The population at Gothic, CO is of unique interest because the concentration of methyllycaconitine (MLA, m/z 683) is very low. All of the studies to date where tall larkspurs have been dosed to cattle, the dominant MSAL-type alkaloid is methyllycaconitine (Welch et al. 2012).

Total alkaloid concentrations showed a part x time interaction at Logan, UT and Gothic, CO ($P < 0.001$ in both sites). Total alkaloid concentrations differed between stems, leaves, and reproductive tissues (Table 2). Fruits and/or reproductive tissues generally had the highest alkaloid concentration at both locations at all time points (Table 2). Stems were intermediate in their alkaloid concentrations while the leaves were the lowest at both locations at all time points (Table 2). The concentrations of alkaloids in the different vegetative and reproductive tissues

were consistent with optimal defense theory predictions (McKey 1974; 1979). Furthermore, the concentrations reported here in leaves and reproductive tissues are biologically active against herbivorous insects and mammals (Jennings et al. 1986; Pfister et al. 2002). In summary, fruits and/or reproductive tissues were most heavily defended as they contained the greatest concentrations of total alkaloids at both locations.

Alkaloid concentrations have been reported to decrease over time in tall larkspurs on a whole plant basis and in individual parts (Gardner and Pfister, 2000; Ralphs and Gardner, 2003). For example, the MSAL-type alkaloids in leaves of *D. barbeyi* range from 18 mg/g early in the summer to 1 mg/g at the end of the summer. This is thought to be due to changes in plant size, resulting in diluting of the alkaloids as the plants grow, and maturity of the tissues, with older tissues containing less defensive compounds than younger tissues. Alkaloid concentrations in *D. nuttallianum* showed some changes between time points within parts at both locations, although there were no consistent trends that occurred across all plant parts and sites (Table 2). For example, alkaloid concentrations in leaves showed significant declines across some time periods in both sites, whereas stems showed increased and then decreased alkaloid concentrations across the season, but only at one site (Gothic, CO). In addition, flowers and fruits showed no statistically significant differences in alkaloid concentrations among time periods at either site (Table 2). By separating plants into their constituent parts, we gained insight into the degree to which individual parts were changing over time, albeit idiosyncratically. In comparison, Gardner and Pfister (2007) observed few changes in the MSAL-type alkaloid concentrations measured on a whole plant basis in *D. nuttallianum* at different stages of maturation within a flowering season.

208 In *D. barbeyi*, alkaloid concentrations in leaves and reproductive tissues at early to late
209 maturity stages differ between 1-2.5 fold (Gardner and Pfister, 2000; Ralphs and Gardner, 2003).
210 Previously we reported that total alkaloid concentrations in leaves of *D. barbeyi* were 1.9 times
211 less than those in fruits; however, in *D. nuttallianum* concentrations in leaves were 6 times less
212 than those found in fruits at a single collection time. We hypothesized that *D. nuttallianum* may
213 have much lower alkaloid concentrations in leaves compared *D. barbeyi*, a tall larkspur species,
214 due to differences in life history. This hypothesis is consistent with the Resource Availability
215 Hypothesis, which predicts that the leaves of longer-lived species would be more heavily
216 defended than shorter-lived species (Coley et al. 1985). *D. nuttallianum* is a single stemmed
217 plant growing up to 0.7 meters with 2-6 leaves and 4-48 flowers per plant, while *D. barbeyi* is
218 often a multi-stemmed plant growing up to 2.5 meters with 8-24 leaves per stalk and 10-50
219 flowers per stalk, often resulting in hundreds of flowers per plant (Ewan, 1945; Warnock, 1997;
220 Williams et al. 2001). Both species emerge as the snow melts; however, *D. nuttallianum*
221 completes its life cycle by setting seed typically within 2 months of snow melt while *D. barbeyi*
222 persists throughout the summer and does not flower until early to mid summer, generally setting
223 seed in late summer. Results reported here for *D. nuttallianum* showed that alkaloid
224 concentrations were always greater in reproductive tissues. Differences range from
225 approximately 2 fold at early phenological stages to greater than 10 fold at later phenological
226 stages (Table 2). The data reported here representing changes in *D. nuttallianum* maturity within
227 a flowering season support our hypothesis that the differences in life history, specifically the
228 time for the plant to emerge at snow melt and set seed, is associated with the differences in
229 allocation of alkaloids across tissues within *D. nuttallianum*.

Pyrrolizidine alkaloids are thought to undergo major structural diversification in the shoots after translocation of an N-oxide to the shoots from the roots (Hartmann, 1999). To date there is no evidence of structural diversification of the norditerpene alkaloids in different tissues of *Delphinium* spp. as a function of plant maturity. Previously we reported that the qualitative alkaloid profiles in *D. barbeyi* and *D. nuttallianum* were similar among the different tissues of each respective species. Here, the qualitative profile of alkaloids was similar among the different tissues at the different collection periods with one exception. In the pods collected at Gothic, CO, 14-deacetylnudicauline (m/z 669) was the dominant MSAL-type alkaloid at the initial collection. Initial concentrations of 14-deacetylnudicauline were 269 ± 120 $\mu\text{g}/100$ mg at the early pod stage. In each collection thereafter 14-deacetylnudicauline concentrations decreased to a concentration of 11 ± 5 $\mu\text{g}/100$ mg at the full pod stage. As the concentration of 14-deacetylnudicauline decreased a corresponding increase in nudicauline (m/z 669) was observed. Initial concentrations of nudicauline were 29 ± 10 $\mu\text{g}/100$ mg at the early pod stage and 164 ± 19 $\mu\text{g}/100$ mg at the full pod stage. These data suggest that as pods mature 14-deacetylnudicauline is acetylated to form nudicauline. Furthermore, it provides the first example of structural diversification of the norditerpene alkaloids in *Delphinium* spp. The ecological significance of this structural diversification is unknown and warrants further investigation. Similar observations were not made in *D. nuttallianum* collected at Logan, UT as nudicauline was the dominant alkaloid at all phenological stages.

In conclusion, norditerpene alkaloid concentrations were measured in a whole plant context including vegetative and reproductive tissues in *D. nuttallianum*. Alkaloid concentrations were consistent with predictions made by defense theory. Furthermore, the data support our hypothesis that alkaloid allocation in different tissues may be influenced by the life

253 history strategy of the plant. Lastly, we present data suggesting that as pods mature the
254 qualitative alkaloid composition may change through structural diversification of the alkaloids
255 present at some rangeland locations.

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Table 1. Representative alkaloid profile showing relative concentrations of individual protonated ions from *Delphinium nuttallianum* collected at Gothic, CO and Logan, UT

m/z ^a	Possible Alkaloids ^a	Alkaloid Type	µg alkaloid/ 100 mg ^b	
			Gothic, CO	Logan, UT
414	11,13- Diacetyhetisine	non-MSAL	1	7
428	Geyerine	non-MSAL	0	25
454	Delcosine, Delectinine	non-MSAL	42	5
466	Deltamine, 14-Dehydrobrowniine	non-MSAL	53	6
468	Delsoline, Lycoctonine	non-MSAL	135	11
482	Delphatine	non-MSAL	28	1
494	Dictyocarpine	non-MSAL	4	9
496	14-Acetyldelectinine, 6-Epi-pubescenine	non-MSAL	5	5
508	Deltaline	non-MSAL	12	3
536	14-Acetyl dictyocarpine	non-MSAL	0	1
552	Glaucedine	non-MSAL	0	0
564	Glaucерine	non-MSAL	3	0
578	Barbisine, Glaucenine	non-MSAL	1	1
667	Barbinine	MSAL	186	19
669	14-Deacetyl nudicauline, 16-Deacetylgeyerline	MSAL	214	37
683	Methyllycaconitine	MSAL	3	33
697	Bearline and isomers	MSAL	24	21
711	Geyerline, Nudicauline, Acetylgrandiflourine	MSAL	12	202

^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.

Table 2. Norditerpene alkaloid concentrations (means $\pm$ SE) in stems, leaves, flowers, and pods of *Delphinium nuttallianum* collected at different phenological stages and locations. (A) Gothic, CO and (B) Logan, UT.

A.

Gothic, CO	μg alkaloid/ 100 mg ^{a,b,c,d}					
	Time 1 (Bud)	Time 2	Time 3 (Early Pod)	Time 4	Time 5	Time 6 (Full Pod)
stem	415 $\pm$ 27 ^{a,1,2}	562 $\pm$ 84 ^{a,1}	690 $\pm$ 130 ^{a,1}	165 $\pm$ 35 ^{b,c,2}	188 $\pm$ 45 ^{b,c,2}	47 $\pm$ 17 ^{b,3}
leaf	229 $\pm$ 50 ^{a,1}	246 $\pm$ 68 ^{a,1}	194 $\pm$ 23 ^{b,1}	52 $\pm$ 15 ^{c,2}	119 $\pm$ 32 ^{c,1,2}	111 $\pm$ 61 ^{b,1,2}
fl ower	341 $\pm$ 39 ^{a,1}	558 $\pm$ 109 ^{a,1}	726 $\pm$ 185 ^{a,b,1}	380 $\pm$ 36 ^{a,b,1}	474 $\pm$ 68 ^{a,b,1}	
fruit			2009 $\pm$ 730 ^{a,1}	648 $\pm$ 63 ^{a,1}	661 $\pm$ 114 ^{a,1}	452 $\pm$ 62 ^{a,1}

B.

Logan, UT	μg alkaloid/ 100 mg ^{a,b,c,d}				
	Time 1 (Bud)	Time 2	Time 3 (Early Pod)	Time 4	Time 5 (Full Pod)
stem	142 $\pm$ 21 ^{a,1}	100 $\pm$ 16 ^{a,1}	104 $\pm$ 23 ^{a,1}	99 $\pm$ 21 ^{a,1}	64 $\pm$ 15 ^{b,1}
leaf	62 $\pm$ 36 ^{b,1}	11 $\pm$ 2 ^{b,2}	16 $\pm$ 9 ^{b,1,2}	4 $\pm$ 1 ^{b,2}	5 $\pm$ 1 ^{c,2}
fl ower/fruit	170 $\pm$ 21 ^{a,1}	160 $\pm$ 18 ^{a,1}	143 $\pm$ 17 ^{a,1}	363 $\pm$ 59 ^{a,1}	408 $\pm$ 38 ^{a,1}

^aDifferent letters within a column represent statistical significance ($P < 0.05$) between different plant parts.

^bDifferent numbers within a row represent statistical significance ($P < 0.05$) between different collection times.

^cEach phenological stage represents 10 plants separated into plant parts.

^dRelative alkaloid concentrations per 100 mg of dry plant material. Concentrations were normalized to an internal reserpine standard.

409
410 Figure Legends
411
412 Figure 1. Structure of select norditerpene alkaloids in *Delphinium* species.
413
414 Figure 2. 14-Deacetylnudicauline and nudicauline concentrations ($\mu\text{g} / 100 \text{ mg}$ dry plant
415 material, means $\pm$ SE) in pods of *Delphinium nuttallianum* at early pod and full pod phenological
416 stages from Gothic, CO. Different letters above each bar represent statistical significance at
417 $P < 0.05$.

Figure 1.

Deltaline (MDL-type)

Lycoctonine (MDL-type)

Methyllycaconitine/MLA (MSAL-type)

464 Figure 2.

465

Alkaloid / Phenological Stage

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