

**Direct and indirect effects of frost on growth and plant-pollinator interactions in
*Delphinium nuttallianum***

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Abstract

Global climate change-driven changes in phenology affect populations of alpine meadow wildflowers and their pollinators through direct and indirect effects. Earlier snowmelt in montane regions, caused by a warming climate, results in earlier blooming date and longer blooming period of wildflowers such as *D. nuttallianum*, an early-blooming larkspur that flowers shortly after snowmelt. In addition to temporal mismatch of plant-pollinator pairs, this change in phenology can also result in frost damage to wildflower reproductive tissues such as buds and flowers. To test the direct and indirect effects of frost on *D. nuttallianum*, a two-way factorial experiment was employed. Plants were exposed to a simulated frost event in one treatment and hand-pollinated to control for pollen limitation in another. This study shows a direct negative effect of frost treatment on *D. nuttallianum* spur length, as well as a delay in peak bloom time and no significant correlation between frost and growth of other tissues, pollinator interactions, or reproductive success.

Introduction

The global temperature rise of 0.6°C over the past 100 years has had great effects on organisms and their interactions with each other.¹ Important interactions affected by this climate change include plant-pollinator interactions, which are primarily affected by phenology changes, range shifts, community shifts, and other changes to the life histories of the species involved.¹ One way that climate change affects phenology is earlier snowmelt, which results in earlier blooming times and earlier pollinator activity in alpine meadow regions.^{2,3} The temporal mismatch of plant and pollinator species caused by earlier spring activity can result in local extinctions as pollinators lose a source of floral and nesting resources and plants lose a method of reproduction, and may have long-term impacts on populations of both participants in the interaction.^{2,3} The earlier blooming times of plants as a result of early snowmelt also have been shown to result in increased plant mortality due to increased risk of early spring frost damage.^{4,5} The earlier blooming of some flower species can expose younger plant tissues such as buds and flowers to frost events that damage these tissues, which are essential for reproduction and pollinator interactions.⁶ Frost damage may also indirectly affect pollinators visiting the plants. For example, plants exposed to frost might lose their attractiveness to pollinators if they have fewer floral and nectar rewards to offer them. Research on the effects of climate change and frost on the flowering phenology of montane wildflowers is prevalent, but research on the physiological growth and pollinator-interactions of these plants is not. Further, while the importance of frost as a factor in climate driven changes in plant growth and plant-pollinator interactions has been recognized, it has not yet been widely studied and can use further investigation. For this study, we ask the following questions:

- 1) What are the direct effects of frost on *Delphinium nuttallianum* growth and reproduction?

2) How do pollinators respond to *D. nuttallianum* plants that have been exposed to frost?

Methods

We conducted our study on a population of *D. nuttallianum* located north of the Rocky Mountain Biological Laboratory in Gothic, Colorado (3020m elevation, Fig. 1). *D. nuttallianum* plants bloom early after snowmelt from late May to late June, and so are often exposed to multiple frost events each spring.^{5, 7} *D. nuttallianum* is pollinated primarily by hummingbirds and bees.^{8, 9} From a preliminary census of pollinators around the site using bee bowls, as well as visual observation of the site, we determined that bees, and not flies or hummingbirds, were the primary pollinators in the area.

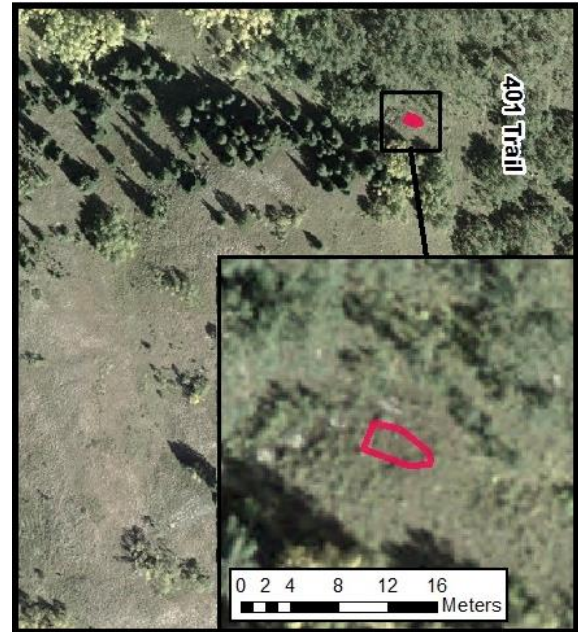


Fig. 1: Map of *D. nuttallianum* site, summer 2014

To investigate the effects of freezing events on plant performance and plant-pollinator interactions, we conducted a two-way factorial experiment. We manipulated freezing events and pollination in this population to examine the direct effects of frost on plant growth and reproduction and the indirect effects of frost on plant-pollinator interactions, utilizing four treatments (1) frost and added pollen, 2) no frost and added pollen, 3) frost and no added pollen, and 4) no frost and no added pollen) applied to 15 plants each, for a total of 60 plants. We simulated freezing events using a foam cooler divided into an upper and lower section by a steel mesh. We then placed two plastic bags with one bag of 650g of dry ice and one bag of 300g ice and 30g of rock salt to insulate the dry ice, in the upper section of the cooler and placed it over the plants for four hours from 6AM-10AM. The temperature within the coolers was measured using temperature data loggers to determine that all coolers reached below 0°C. Plants in the no-frost treatment were covered with unmodified foam coolers as a control. All plants were covered with sheets on nights with predicted freezing temperatures to prevent damage from natural freezing events.

Growth

During the blooming period, we took weekly plant measurements of plant height, number of buds, number of flowers in bloom, spur length, flower head width, and the length and width of one petal per flower head, for up to three flowers. Flowers and petals were chosen arbitrarily. Additionally, we recorded the first day of flowering, day of peak bloom, and bloom duration per plant.

We used MANOVAs to test the direct effects of the frost treatment on plant growth as estimated by average plant height, flower width, spur length, petal width, and petal length, and on bloom phenology as estimated by day of first bloom, day of peak bloom, and bloom duration.

Pollinator Interactions

To examine the indirect effects of frost on plant-pollinator interactions, we observed pollinator interactions three times a week for one hour, followed by netting of bees for 30 minutes, once in the morning and again in the afternoon. During observations we recorded any visitor that landed on the plant, noting the number of flowers visited per visitor and the duration of each visit. We separated pollinators into six groups based on physical traits that are characteristic of various species found in the area; bumblebees, small and dark, red-bodied, blue/green, hairy-legged, and non-bee/fly. To aid identification of the bees, we collected a reference collection of solitary bees found in the study area. Bumblebees were not collected, but were identified, marked, and released during netting so as not to remove queens gathering resources for nests.

We used ANOVAs to test the indirect effects of frost on pollinator interactions by comparing treatment type with the average number of visits per plant, proportion of flowers visited, and average time of visit per flower.

Reproductive Success

To determine effects of pollen limitation as a result of frost events on plant reproductive success, we ensured that randomly selected plants received maximum pollination by applying dehiscent anthers from the same species gathered from plants 5-10 meters away to the stigmas of flowers within the pollen supplementation treatments every other day. Flowers not in the pollen supplementation treatment were handled, but no pollen was supplemented. When the plants senesced, we collected mature fruit from each plant several days a week for two weeks, and then air-dried the fruit in marked coin envelopes for two to three days. To estimate reproductive success, we counted the seeds and fruit per plant, as well as aborted seeds and fruit to determine proportion seed and fruit set.

To test the effects of frost treatments on female *D. nuttallianum* reproductive success, we used ANOVAs of average seeds per plant, average seeds per fruit, and proportion fruit set against frost and pollen treatment type.

The data were log transformed for all categories to account for non-normality, and proportions were arcsine root-squared transformed. We completed all of the above analyses using R.¹⁰

Results

Growth

We found no significant difference in plant height, flower width, petal length, and petal width between frost and no frost treatments; however there was a significant negative correlation

between spur length and frost treatment (Table 1, mean spur length for frost treatment = 13.73, for no frost treatment = 14.47). Similarly, we found no significant difference in date of first

Table 1: Multivariate analysis of variance (MANOVA) of *D. nuttallianum* growth between frost and no frost treatments . Asterisk denotes significance.

Dependent Variable	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Plant Height	1	0.0024	0.0024018	0.2358	0.6277
Spur Length	1	0.02545	0.0254539	8.9365	0.003092*
Flower Width	1	0.0007	0.0006759	0.0428	0.8362
Petal Length	1	0.00026	0.0002552	0.0266	0.8706
Petal Width	1	0.0001	0.0001013	0.008	0.9288

Table 2: Multivariate analysis of variance (MANOVA) of *D. nuttallianum* phenology estimates between frost and no frost treatments. Asterisk denotes significance.

Dependent Variable	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
First Bloom	1	4.27	4.2745	0.6395	0.4244
Peak Bloom	1	134.2	134.214	14.196	0.000194*
Bloom Duration	1	24.0	24.046	1.7318	0.1891

bloom and bloom duration between treatments, but we did find that plants in the frost treatments reached peak bloom approximately 1.5 days later than those in the no frost treatment (Table 2, Fig. 2, mean peak bloom date for frost treatment = 179.02, for no frost treatment = 177.76).

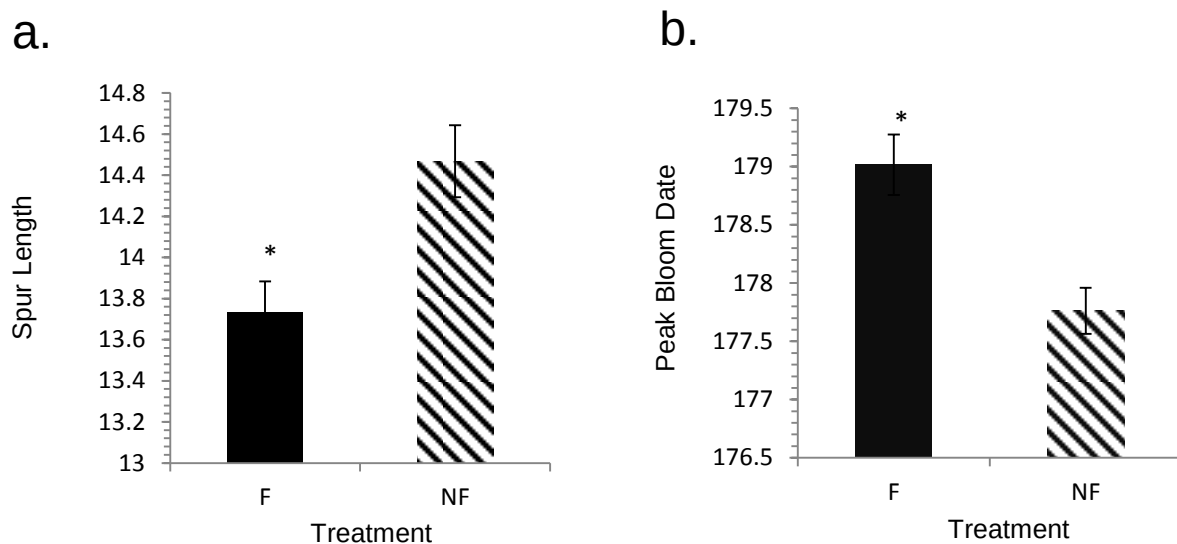


Fig. 2: The relationships between frost (F) and no frost (NF) treatments and spur length (a) and peak bloom date (b) in *D. nuttallianum*. Significance noted with asterisk.

Pollinator Interactions

We found no significant difference between treatments in time spent per flower ($F_1=0.0507$, $P=0.822$), number of plant visits ($F_1=0.7902$, $P=0.3781$), or proportion of flowers visited ($F_1=0.6715$, $P=0.413$).

Reproductive Success

We found no significant difference between frost, no frost, and crossed pollination treatments in average seeds per fruit ($F_1=0.4525$, $P=0.5062$), average seeds per plant ($F_1=0.1074$, $P=0.7444$), and proportion fruit set ($F_1=0.5224$, $P=0.4734$).

Discussion

Our results indicate that an early-blooming wildflower such as *D. nuttallianum* may have adaptations to natural frost events, which may be suited to protect the plant from a frost event. We found that *D. nuttallianum* is resistant to frost in terms of plant height and flower size, however we also found that spur length was negatively correlated with freezing events. The direct negative effect of frost on *D. nuttallianum* spur length could possibly be a result of the plant's phenology: buds are formed the previous year, and were not open during frost treatments, although the spur was exposed.⁷ The protection offered by a closed bud may have prevented damage to the flower while the unprotected spur is damaged. One caveat to our study is that we only exposed the plants in the experimental treatment to one frost event, which might have explained why we didn't see a negative correlation between frost and plant growth. Since this plant would bloom earlier as a result of earlier snowmelt, it follows that it would be exposed to more frost events than usual, suggesting that an investigation into effects of multiple frost events on *D. nuttallianum* might prove informative.

Although there was no significant direct relationship between first and end bloom dates and frost, a significantly later peak bloom date of approximately 1.5 days for frost-treated plants was observed. Later peak bloom could mean less floral resources for pollinators, and would further shorten their window of time for nectar foraging.³

We did not find any indirect effects of frost treatment on pollinator interactions. There was no strong evidence to suggest that number of pollinators, duration of each visit, or proportion of flowers probed differ between plants that were exposed to frost and plants that were not exposed to frost. Further, our results suggest that frost exposed plants were not pollen limited. Our results could be due to several factors. Since we did not observe significant differences in flowers between treatments, except for spur length, there may not have been external cues for bees to discriminate between flowers by. It has been suggested that larger spur and corolla lengths attract larger pollinators, especially in the case of hummingbirds and *Ipomopsis aggregate* corolla tubes, and bees and *D. barbeyi*.¹¹ The correlation of spur length and frost treatment bears some importance in this concept. While our results were inconclusive as to indirect effects of frost on pollinator interactions, further studies investigating the spur length and pollinator preference may provide different results.

We also did not observe much pollinator diversity, which could have contributed to the lack of significant evidence for indirect effects of frost on pollinator interactions. One possible explanation for the low diversity of pollinators observed is competition between these plants and the study group; *D. nuttallianum* and *I. aggregate* have been shown to exhibit competition for hummingbird pollinator interactions.⁸ We observed mostly bumblebees on the *D. nuttallianum* and smaller solitary bees on other flowering species within the site. This potentially skewed our results for pollinator interactions, and further study could be used to determine if competition between *D. nuttallianum* and other plants in the area does affect those interactions. The low abundance of pollinators observed also could have affected results; had we more observations to work with, we might have seen a different trend.

D. nuttallianum shows an overall resistance to frost damage, and the presence of undamaged *D. nuttallianum* flowers in early spring may provide floral resources for pollinators that otherwise would find mostly damaged flowers. In addition to answering our questions of how frost affects *D. nuttallianum* growth and reproduction, this short-term experimental study also furthers our ability to interpret long-term observational data of early-blooming alpine meadow wildflowers, and could contribute to models and further study of wildflower and pollinator population change as a result of climate change-driven early snowmelt.

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