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Plant-pollinator interactions and environmental change: effects of experimental changes in phenology and water availability on a montane wildflower

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PLANT–POLLINATOR INTERACTIONS AND ENVIRONMENTAL CHANGE: EFFECTS OF EXPERIMENTAL CHANGES IN PHENOLOGY AND WATER AVAILABILITY ON A MONTANE WILDFLOWER

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Ecology and Evolutionary Biology

by

Megan Katherine Gallagher

Dissertation Committee:
Professor Diane R. Campbell, Chair
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2017

DEDICATION

“You must do something to make the world more beautiful.”
— *Ms. Rumphius*, by Barbara Cooney

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ABSTRACT OF THE DISSERTATION

Plant–pollinator interactions and environmental change:
effects of experimental changes in phenology and water availability on a montane wildflower

By

Megan Katherine Gallagher

Doctor of Philosophy in Ecology and Evolutionary Biology

University of California, Irvine, 2017

Professor Diane R. Campbell, Chair

With an estimated 87% of angiosperms depending on animal-mediated pollination, plant-pollinator mutualisms play a key role in maintaining ecosystems and supporting global crop production. Climate-driven changes in flowering phenology and water availability, however, may disrupt these ecologically and economically important relationships. This dissertation investigates the mechanisms through which altered water availability and flowering phenology impact pollination and plant reproductive success of *Mertensia ciliata* (Boraginaceae).

First, I tested the hypothesis that impacts of climate on plant-pollinator interactions operate through changes in water availability, and specifically that such effects occur through alteration of floral attractants. Through a multi-year water manipulation experiment, I found that changes in water availability can impact pollinator visitation through pollinator responses to differences in floral attractants, and that the effects of water on visitation can be non-linear. Seed set, however, increased linearly with water

(Chapter 1). By manipulating the onset of flowering, I tested the hypothesis that phenological shifts alter pollination and seed set through changes in the frequency and composition of pollinator visitors. I found that despite a five-fold decrease in pollinator visitation over four weeks, there was no significant difference in conspecific pollen receipt or seed set among phenology treatments. Measurements of single-visit pollinator effectiveness revealed that, on a per-visit basis, each bumblebee transferred more conspecific pollen than did a solitary bee or a fly. Thus, while the total pollinator visitation rate declined over the season, because the proportion of visits by more effective worker bumblebees increased, differences in flowering phenology had no effect on seed set (Chapter 2). Because changes in phenology and water availability are often co-occurring responses to environmental change, I hypothesized that they might have interactive effects on pollination and seed set. Through a factorial experimental manipulation, I found that changes in water interacted with differences in phenology in their effects on pollinator taxonomic composition, but their effects on pollinator visitation and seed set were additive (Chapter 3). Taken together, these results highlight the importance of determining the separate and potential interactive effects that co-occurring ecological responses to environmental change may have on pollination and plant reproductive success.

INTRODUCTION

Plant-pollinator mutualisms play a key role in maintaining global biodiversity, ecosystem functioning, and agricultural security (Kearns et al. 1998; Klein et al. 2007; Potts et al. 2010; Garibaldi et al. 2013; Hanley et al. 2015). Recent changes in climate, however, may disrupt these important relationships (Winfree et al. 2013; Gornish and Tylianakis 2013). Rising temperatures and changing precipitation patterns associated with climate change are affecting water availability during the growing season (Saunders et al. 2008; Blankinship et al. 2014) and altering the seasonal timing (i.e. phenology) of flower production and of pollinator activity (Parmesan and Yohe 2003; Parmesan 2006; Marshall et al. 2008; Hegland et al. 2009; Bartomeus et al. 2011; Wielgolaski and Inouye 2013; CaraDonna et al. 2014). Both shifts in water availability and phenology may affect plant-pollinator interactions, and thus may have important consequences for plant reproductive success.

Differences in water availability during the growing season can have direct effects on plant reproduction by altering resources for leaves, flowers, fruits, and seeds (Galen et al. 1999; Caruso et al. 2005; Burkle and Irwin 2009; Berdanier and Klein 2011; Barber and Soper Gorden 2014). In turn, plant physiological responses to changes in water availability may impact reproduction by altering the plant and floral traits that mediate species interactions, both with antagonists (Jamieson et al. 2012) and mutualists (Blankinship et al. 2011), including pollinators. Altered precipitation patterns associated with anthropogenic climate change are expected to have many direct effects on plants and insect pollinators, however, it is unknown if effects on pollination are mediated by changes in water availability.

Changes in the temporal overlap among interacting species can also affect plant reproductive success by altering the overlap of both antagonists (Brody 1997; Pilson 2000; Lay et al. 2011; Parsche et al. 2011; Ehrlén et al. 2015) and mutualist pollinators (Kudo et al. 2004; Memmott et al. 2007; Hegland et al. 2009; Thomson 2010). These phenological shifts can alter pollination success through changes in the frequency of pollinator visits (Parsche et al. 2011; Rafferty and Ives 2011; Gezon et al. 2016) and the effectiveness of different floral visitors in depositing a sufficient quantity of quality pollen (Ne'eman et al. 2010). Changes in pollinator effectiveness may occur both through compositional changes in pollinator taxa or changes in effectiveness within pollinator taxa over time (Rafferty and Ives 2012). Each of these potential changes in pollination are expected to affect seed set through changes in pollen receipt, but as yet, the extent to which each mechanism actually does affect pollen receipt remains unexplored.

Shifts in water availability and flowering phenology, however, are potentially co-occurring responses to environmental change and may have important interactive effects on plant-pollinator interactions, and thus on plant reproductive success. For my dissertation research, I investigate the mechanisms through which climate change may affect plant-pollinator interactions and seed set through changes in water availability and flowering phenology. I tested the separate and potential interactive effects of these mechanisms in three chapters, using the subalpine perennial wildflower, *Mertensia ciliata* (Boraginaceae), as a model system.

CHAPTER 1: SHIFTS IN WATER AVAILABILITY MEDIATE PLANT-POLLINATOR INTERACTIONS. This study demonstrates the potential for changes in water availability to impact plant-pollinator interactions through pollinator responses to differences in floral attractants, and that the effects of water on pollinator visitation can be non-linear. Seed set, however, increased linearly with water. Thus, plant responses to changes in resource availability may be an important mechanism by which climate change will affect species interactions.

CHAPTER 2: FLOWERING PHENOLOGY INFLUENCES POLLINATION BY ALTERING VISITATION RATE AND POLLINATOR EFFECTIVENESS. This study determines that phenological shifts can alter both the total pollinator visitation rate and the taxonomic composition of floral visitors. Moreover, this study revealed that phenological shifts can affect the quality of pollinator visits by altering the frequencies of visits made by pollinators of different single-visit effectiveness. The results of this study suggest that a higher pollinator effectiveness of late-season visitors compensates for their lower diversity and visitation rate, such that flowering time has little net effect on reproductive success in this plant species. This work also illustrates the need to consider pollinator effectiveness, both in terms of conspecific and heterospecific pollen deposition as well as seeds produced, along with changes in visitation rates and pollinator assemblage to understand the mechanisms by which shifts in phenology impact levels of pollination in the face of climate change.

CHAPTER 3: EXPERIMENTAL ENVIRONMENTAL CHANGE ALTERS PLANT-POLLINATOR INTERACTIONS AND SEED SET. Through manipulations of flowering phenology and soil moisture in a factorial experiment, this study demonstrates that the effects of phenological shifts on pollinator taxonomic composition can vary with changes in water availability. The effects of water and phenology on pollinator visitation and seed set, however, were additive. In this system, phenological shifts had a greater effect on pollinator visitation rates and pollinator taxonomic composition than did changes in water availability, whereas changes in water availability had a larger effect on seed set than shifts in flowering phenology. The results of this study suggest that the effects of changes in water availably and phenology associated with anthropogenic climate change may be more likely to have interactive effects on pollination of zoophilous plant species that have a wide variety of potential pollinators than species serviced by specialized pollinators.

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CHAPTER 1: SHIFTS IN WATER AVAILABILITY MEDIATE PLANT-POLLINATOR INTERACTIONS

INTRODUCTION

Change in water availability is a key mechanism by which climate change will affect plant performance. The direct effects of changes in water availability on plant physiology are well established. Water limitation can reduce the capacity of plants to maintain turgor and transpiration, or to uptake nutrients, and can alter plant-microbial interactions in the soil, all of which can lead to decreased resources for leaves, flowers, fruits, and seeds (Galen et al. 1999; Caruso et al. 2005; Burkle and Irwin 2009; Berdanier and Klein 2011; Barber and Soper Gorden 2014). Such direct effects on plant physiology may in turn mediate plant interactions with antagonists (Jamieson et al. 2012) and mutualists (Blankinship et al. 2011), including pollinators, but those effects are less well understood (Gornish and Tylianakis 2013).

Shifts in water availability may have a variety of effects on plant-pollinator mutualisms. One relatively well-studied mechanism is changes in the temporal overlap between flowering and pollinator activity associated with reduced snowpack and early snowmelt (reviewed in Forrest 2015), but it is not the only way changes in water may impact these interactions (Strauss and Whittall 2006; Gornish and Tylianakis 2013). Plant responses to differences in water availability may also affect their capacity to attract pollinators via changes in floral attractants. Producing and maintaining flowers is energetically costly, in terms of the initial carbon and nutrients necessary for bud formation (Teixido 2014), transpirational water loss through inflorescences (Nobel 1977; Carroll *et al.* 2001), as well as water diverted from other functions (i.e., photosynthesis) for flower maintenance (Galen *et al.* 1999). Perhaps because these costs increase with corolla size (Galen *et al.* 1999), drought-stressed plants often produce smaller flowers, and may also produce fewer flowers and shorter inflorescences (Mal and Lovett-Doust 2005; Caruso 2006; Strauss and Whittall 2006). In addition, changes in soil moisture can affect the volume and composition of nectar rewards (Zimmerman and Pyke 1988; Carroll *et al.* 2001; Nicolson *et al.* 2007; Burkle and Irwin 2009; Halpern *et al.* 2010; Waser and Price 2016), pollen (Waser and Price 2016), as well as the emission and composition of floral volatiles (Burkle and Runyon 2016), all of which may influence the ability of plants to attract pollinators.

Such water-mediated changes in floral attractants may go on to impact pollination through several mechanisms. First, pollinator visitation rates may be impacted by changes in floral display, including plant height and floral abundance. Tall plants and flowers at the tops of inflorescences can receive more pollinator visits, as well as larger and more genetically diverse pollen loads, than their shorter counterparts (Lortie and Aarssen 1999; Carromero and Hamrick 2005). As taller inflorescences also tend to have more flowers than shorter stems (Lortie and Aarssen 1999), pollinator preferences for taller plants may be due, in part, to attraction to plants with more flowers and larger floral displays (Eckhart 1991; Conner and Rush 1996; Galen 2000; Thomson 2001; Hegland and Totland 2005; Goulson 2010). Second, variation in corolla size can affect both pollinator visitation rate (Inouye 1980; Bell 1985; Galen and Newport 1987; Eckhart 1991; Suzuki 1994; Conner and Rush 1996; Galen 2000; Thomson 2001) and pollinator effectiveness (Galen and Newport 1987; Young and Stanton 1990; Campbell *et al.* 1991). Third, changes in nectar volume (Inouye 1978; Pleasants 1981; Wright 1988; Real and Rathcke 1991; Mitchell 1993; Nicolson *et al.* 2007; Waser and Price 2016) and composition (Pleasants 1981; Nicolson *et al.* 2007; Hoover *et al.* 2012) can impact visitation rate as pollinators seek the most rewarding options. While many studies have examined either how water availability influences floral traits or how floral traits influence pollination, evaluating both phenomena in situ provides valuable insight into how climate driven variation influences species interactions.

We investigated the extent to which variation in soil moisture impacts the capacity of plants to attract pollinators and produce seeds. We manipulated water availability during two growing seasons and measured effects on vegetative and floral traits, nectar rewards, pollinator visitation, and plant reproduction. We predicted that plant growth and performance would be influenced by differences in water availability, such that water limitation would (1) reduce floral display (i.e., ramet height and floral abundance), corolla size, and nectar rewards. We also hypothesized that floral responses to reduced soil moisture would (2) negatively impact pollinator visitation rates, which in turn would be (3) associated with a decline in seed set and seed mass.

MATERIALS AND METHODS

Fieldwork was conducted during the growing seasons of 2013 and 2014 in two subalpine meadows (38°59'48.5"N 107°00'57.5"W, 2,992 m and 39°00'20.3"N 107°01'53.5"W, 3,143 m) located 5.5 and 7.2 km from the Rocky Mountain Biological Laboratory (RMBL) in Gothic, Gunnison County, Colorado, USA. Both field sites have similar plant communities, dominated in mid-summer by large populations of the focal species, *Mertensia ciliata* (James ex Torr.) G. Don (Boraginaceae), as well as *Delphinium barbeyi* Huth. and *Veratrum californicum* Durand. Between 1973-2006; mean spring (April-June) temperatures at RMBL have increased by 2.0 °C, and the average date of spring snowmelt has advanced by nearly two weeks (Miller-Rushing and Inouye 2009). These trends of warmer temperatures and earlier snowmelt, which result in an earlier, longer dry season prior to mid-summer thundershowers (Blankinship *et al.* 2014), are expected to continue in the southern Rocky Mountains for the next century (Pederson *et al.* 2011).

Mertensia ciliata, the tall-fringed bluebell, is an herbaceous perennial found primarily along streams and wet meadows in subalpine and lower alpine zones of the Rocky and Sierra Nevada Mountains. Plants are rhizomatous and form compact clones of a few to several hundred flowering ramets (Pelton 1961). The pendant, tubular flowers are typically open for six days and can produce a maximum of four one-seeded nutlets. Stigmas are receptive throughout flowering, but pollen is usually removed within 24-48 hours of anther dehiscence by medium and long-tongued bumblebees, including *Bombus balteatus* (Dahlbom), *B. bifarius* (Cresson), *B. flavifrons* (Cresson), and *B. frigidus* (Smith) (Pelton 1961; Geber 1985; Suzuki 1994). Field insect exclusion and hand pollination experiments indicate that, despite being self-compatible, seed set is dependent on insect pollination (Geber 1985).

In the subalpine meadows around RMBL, *M. ciliata* blooms from late June through late July. Since 1973 *M. ciliata* has become less common at lower elevations (≤ 2,900 m), and observed declines in peak floral abundance correlate significantly with earlier snowmelt timing (Miller-Rushing and Inouye 2009). These patterns suggest that changes in snowmelt timing may be affecting persistence of *M. ciliata* populations.

EXPERIMENTAL DESIGN

To test the hypothesis that plant responses to differences in soil moisture impacts their capacity to attract pollinators and produce seeds, we manipulated water availability in two naturally occurring *M. ciliata* populations using a randomized block design. Within each population (hereafter site), dense patches of *M. ciliata* were marked with flags and designated as blocks. Within each block, three 1 m^2 treatment plots, separated from the others by 1 m, were marked with flags and randomly assigned to a precipitation treatment: water reduction, water addition, or ambient. To minimize the flow of resources with ramets outside of the plots, we cut 15 cm into the soil around each plot, bisecting the rhizomatous root system. In 2013; we had five blocks per site ($N_{2013} = 30$) and in 2014 we established new blocks, seven at one site and three at the other ($N_{2014} = 30$).

Precipitation manipulations were maintained through the growing seasons of 2013 (June 18 – August 6) and 2014 (June 21 – August 8) and discontinued at the end of flowering. Water addition treatments received twice the historic average rainfall during July from 1990 to 2009; which amounted to an additional 3.5L of water every 2 d to each 1 m^2 plot (Campbell and Wendlandt 2013). We watered plots manually with watering cans slowly and evenly to avoid pooling, in the mid to late afternoon to coincide with the timing of July thundershowers.

Water reduction plots were covered with 1.1 m^2 rainout shelters designed to intercept 50% of incoming summer precipitation (Yahdjian and Sala 2002). The larger area covered by the rainout shelters allowed us to minimize edge effects on plants within the 1 m^2 treatment plots under the shelters. Shelters were built from 1” PVC pipe and slats of U-shaped clear polycarbonate roofing (Palram Americas, Inc., Kutztown, Pennsylvania, USA) that formed a sloping partial roof covering 50% of the top area. The mean height of the shelters was 1.2 m, which was selected to exceed height of the plants in the study area. Slats deposited water into a trough at the down-slope end of the roof, which extended beyond the width of the shelter and shunted the water away from all treatment plots. The open-sided shelters allowed free access to the plants below by pollinators, herbivores, and scientists. Control treatment plots received only natural rainfall. To minimize the visual impact of our research near the Gunnison County road, we spray-painted the rainout shelter frames green and did not install infrastructure control shelters over the water addition and control treatments. To assess the impact of the rainout shelters on light levels, we affixed Hobo light data loggers (Onset Computer Corporation, Inc., Bourne, Massachusetts, USA) to 30 cm tall stakes and placed them in the center of two plots with rainout shelters and two plots without rainout shelters (total of eight plots, four per site). During the daylight hours of 0700 to 1700 hours, the plots with rainout shelters averaged 5.7% less light (35006 vs. 37138 lux) than the other plots, but this difference was not statistically significant (ANOVA, $F_{1,6} = 0.31$, $P = 0.6$).

Throughout the experiment, we measured soil moisture as volumetric water content (VWC) every other day using a 12 cm Campbell Scientific "HydroSense" probe inserted into the center of each plot (always before applying water). Control plots received average precipitation that was slightly higher than the historical average over the past 15 years (88 vs. 84 mm). The treatments led to 9.4%, 11.7%, and 15.4% average VWC in the soil for dry, control, and wet plots, creating a gradient in soil moisture. Average summer (June-July) VWC values for plots were analyzed with ANOVA with the factors of treatment and block. Although we found significant differences among the treatments in both years ($F_{2,48}= 16.55$, $P < 0.0001$), due to large within treatment variation among blocks (39% variance within treatments) we treated soil moisture as a continuous rather than discrete variable in our analyses. We tracked soil VWC of a subset of plots at a finer temporal scale as well (Figure 1.1).

VEGETATIVE AND FLORAL TRAIT MEASUREMENTS

We marked a randomized subset of ten ramets per plot, for which we measured vegetative traits, corolla size, and flower abundance. Vegetative traits included total leaf count and ramet height as measured from the base to the tip of the most terminal leaves. Vegetative traits were measured on July 16, 2013 and July 9 and 12, 2014; during peak flowering. For 3.7 ± 0.1 (Mean ± SEM) flowers per ramet on July 11-15, 2013 and July 2-29, 2014; we measured corolla width at the opening of the tube and corolla length from the base of the calyx to a randomly chosen corolla lobe. At the end of each flowering season, we determined the total number of flowers produced by each marked ramet by counting all reproductive structures (buds, aborted buds, flowers, fruits, aborted fruits).

During the second summer, we also counted the total number of ramets per plot, the number that flowered, and total floral abundance at the end of the season. For an additional five randomly selected ramets per plot, in 2014; we measured production of nectar rewards (i.e., nectar volume and concentration). To do this, we excluded pollinators from newly opened flowers using sheer organza draw-string pouches (Uline, Pleasant Prairie, WI, USA). After 48 hours, we measured nectar volume using 5μl microcapillary tubes (Kearns and Inouye 1993) and percent sugar concentration using a handheld nectar refractometer (Bellingham + Stanley Ltd., Basingstoke, Hants, UK). Nectar measurements were based on an average of 3.2 ± 0.2 flowers per ramet collected between July 7-29, 2014.

We analyzed the effects of experimental variation in soil moisture on vegetative and floral traits using the plot mean of each trait, calculated from the individual ramet means when appropriate, as the response in linear models with main effects of mean summer VWC and site as predictors. For those traits with two years of data, we included main effects of year, as well as mean summer VWC × year and site × year interactions as predictors. When interactions were not significant, we reran the models without the interaction to simplify interpretation. In these and all other analyses, mean summer VWC × year was not significant and so was excluded. To see if the observed differences in corolla size were explained by a general increase in ramet size, we performed an additional analysis with mean ramet height added as a model covariate.

Residuals of vegetative, corolla, and nectar traits were approximately normally distributed (Shapiro-Wilk, $P > 0.05$). We removed one outlier from our analysis of peak floral abundance that fell 30 standard deviations away from the mean, a significant deviation (Grubbs test, $P = 0.0001$, R package *outliers*, Lukasz 2011). After removing this outlier, the residuals of all floral abundance measurements were also normally distributed (Shapiro-Wilk, $P > 0.05$). All analyses were conducted using R (R Core Team 2015). For models including interaction terms we calculated type III sums of squares using the *car* package in R (Fox and Weisberg 2011) and with a contrast setting that sets the row sum to zero. For models without interaction terms, we report type II analyses. Block was not found to be a significant predictor in any of our analyses and so was removed as a factor for simplicity.

POLLINATOR VISITATION MEASUREMENTS

To test the hypothesis that floral responses to differences in water availability impacts pollinator visitation, we conducted pollinator observations at the whole plot level for a total of 93.5 hours (18.5 hours in 2013 and 75 hours in 2014). In 2014; we also directly tested the hypothesis that water-mediated changes in corolla size reduces pollinator visitation rates by tracking pollinator visitation to the ten marked ramets in each treatment plot for which we had specific data on corolla traits. We tracked pollinator identity and number of flowers visited during 30-minute observation periods between the hours of 9:00 and 16:00, when weather conditions allowed pollinator activity, between July 9-10, 2013 and July 2-31, 2014. Observations in 2013 occurred during peak flowering, while those in 2014 covered the whole flowering period for most of the plants in both communities. We observed all plots within a block on the same day, and all blocks before repeating the cycle. The order of observations was randomized among blocks and among treatment plots within blocks. Visitors were determined to be pollinators if they crawled inside the flower corolla. Of the 303 pollinators observed, two were flies, eight were solitary bees and the rest were bumblebees. Excluding non-bumblebee visitors from analyses did not alter results, therefore we report results including all visitors. Open flowers within plots and on marked ramets were counted at the beginning of each observation period. We calculated pollinator visitation rate at the whole plot level (N = 60) and for individually marked ramets (N = 224) as (total number of flowers visited / number of flowers available per hour of observations), averaged across the flowering season.

Pollinator responses to experimental variation in soil moisture were tested in a model using mean pollinator visitation rate per plot as the response. Predictors included mean summer VWC, site, year, site × year interaction, as well as a quadratic term for mean summer VWC, to test for a curvilinear relationship between visitation and soil moisture. We assessed whether changes in nectar availability influenced mean pollinator visitation rate per plot in a model with linear and quadratic terms for mean summer VWC, site, mean nectar volume, and site × nectar volume interaction as predictors (2014 data only, N=30). We tested whether pollinators respond to water-mediated changes in corolla size using mean pollinator visitation rate to individual ramets as the response variable. For each trait (corolla length or corolla width), we first tested whether selection varied by site, using a model with linear and quadratic terms for the trait, site, and the site by linear and quadratic terms as predictors. As we detected significant site by trait interactions, we ran final models separately by site, with linear and quadratic terms for the trait as predictors.

The residuals of these pollination analyses were not normally distributed (Shapiro-Wilk, $P \leq 0.05$), likely due to a high proportion of observation periods with zero pollinator visits. For this reason, we employed a zero-inflated mixture model with the package *pscl* in R (Jackman 2008), which modeled the pollinator visitation rate with a Poisson distribution and the excess zeroes with a binomial distribution and logit link. In each case, the zero-inflated model provided a better fit than a GLM with Poisson distribution, as judged by a lower value for Akaike Information Criterion (AIC).

To quantify responses of pollinator visitation to individual traits in a way comparable to other studies, we used phenotypic selection analysis with pollinator visitation rate to individual ramets as the fitness measure (Lande and Arnold 1983). Pollinator visitation rate for each ramet was divided by the mean to estimate an index of relative fitness. For each corolla trait and site, we calculated standardized directional (S') and quadratic (γ) selection differentials from linear regression models of relative fitness on standardized trait values (mean of 0, variance of 1). To estimate the strength of stabilizing selection, we calculated quadratic selection differentials (γ) by including both trait and trait^2 as predictors in the regression model, and doubling the quadratic regression coefficients (Stinchcombe *et al.* 2008). We also tested whether selection varied with water availability by examining relative fitness as a function of the standardized trait value, water treatment and the trait × water treatment interaction. In cases where we found a significant interaction, we estimated directional (S') and quadratic (γ) selection differentials separately for each corolla trait and water treatment (i.e., water reduction, control, and water addition).

SEED SET MEASUREMENTS

We counted the total number of nutlets produced per flower (measured as described by Forrest and Thomson 2010). Because *M. ciliata* can produce a maximum of four nutlets per flower, we calculated seed set as (100% × number of mature nutlets) / (4 × number of flowers). Mature nutlets from each marked ramet were collected in coin envelopes and transported to the University of California, Irvine where they were weighed. We calculated mean seed mass for a ramet as (mass of collected nutlets / number of collected nutlets). We analyzed the effects of experimental variation in soil moisture on mean seed set and mean seed mass for each plot as we did with vegetative and floral traits. Neither fitness component showed a significant quadratic relationship with soil moisture, so we removed that term from both analyses.

We used a GLM model with Poisson distribution for the seed set analysis, as the residuals were not normally distributed (Shapiro-Wilk, $P \leq 0.05$) and it provided a better fit than other models as judged by a lower AIC value. We used a normal distribution for seed mass (Shapiro-Wilk, $P > 0.05$). To assess whether differences in seed set are associated with pollinator visitation rate, we performed an additional analysis including linear and quadratic terms for mean pollinator visitation rate at the plot level as a factor in the model along with mean summer VWC, site, and year. We also estimated directional and stabilizing selection on corolla width and length using phenotypic selection analysis, as described above, except with seed set as the fitness measure.

RESULTS

EFFECTS ON VEGETATIVE AND FLORAL TRAITS

Prediction 1: Water-limitation will reduce floral display, corolla size, and nectar rewards. As predicted, corolla width increased with mean soil moisture (June-July) ($F_{1, 54}$ = 37.09, P < 0.0001, Figure 1.2a), as did corolla length ($F_{1, 55}$ = 16.45 in a model with non-significant site × year interaction removed, P = 0.0002, Figure 1.2b). When we added mean ramet height to these models, corolla width and length still increased significantly with soil moisture, indicating the responses of floral morphology were not due simply to an increase in plant size. Nectar volume also increased with soil moisture ($F_{1, 27}$ = 5.61, P = 0.025, Figure 1.2c), although nectar concentration did not ($F_{1, 27}$ = 0.23, P = 0.63). We saw the same patterns for floral traits when looking at June and July soil moistures separately, except for nectar volume which responded significantly to water in July ($F_{1, 27}$ = 8.96, P = 0.006) but not June ($F_{1, 27}$ = 0.02, P = 0.9).

Most measures of floral abundance that we tracked, including floral abundance of flowering plants and proportion of flowering plants per plot, were not influenced by changes in water availability ($P > 0.05$, Table 1.1). Peak floral abundance varied with soil moisture, but the pattern differed significantly between the two sites ($F_{1, 25}$ = 4.71, P = 0.04). At the higher elevation site, peak floral abundance increased with soil moisture, whereas it decreased with soil moisture at the lower elevation site (Figure 1.3a). Leaf count did not vary with changes in water availability ($F_{1, 52}$ = 0.04 in a model with non-significant site × year interaction removed, $P = 0.8$), but ramet height increased with soil moisture ($F_{1, 52} = 4.78$, $P = 0.03$, Figure 1.3b).

EFFECTS ON POLLINATOR VISITATION

Prediction 2: Floral responses to reduced soil moisture will negatively impact pollinator visitation rates. The probability of a plot receiving no pollinator visits in an hour of observations did not vary significantly with any predictor (zero-inflation part of the model, Table 1.2). Among those plots that received visits, pollinator visitation rates were highest for plots with intermediate soil moistures (Figure 1.4a), yielding a quadratic as well as a linear effect for the count part of the model (Table 1.2). There was also a significant site × year interaction, with higher mean visitation rates in 2013 than 2014; and a slightly bigger difference at the low site (count part of the model, Table 1.2). Pollinator visitation at the plot level in 2014 was not influenced by changes in nectar volume ($Z_{\text{zero-inflation model}} = -0.66$, $Z_{\text{count model}} = 1.71$, DF = 1, 28, $P = 0.09$).

At the individual ramet level, the likelihood that a pollinator would visit a plant did not vary with corolla width or length (zero-inflation part of the model, Table 1.3). When pollinators did visit, however, plants at the low site with intermediate corolla sizes and plants at the high site with intermediate corolla lengths received more visits per flower than those with smaller or larger corollas (Figure 1.5a,b,d), yielding a quadratic as well as a linear effect for the count part of the model (Table 1.3). The standardized selection differentials and the quadratic selection differentials indicate that corolla length, and width at the low site, is subject to pollinator-mediated positive directional selection for larger corollas, as well as stabilizing selection for intermediate corollas (Figure 1.5a,b,d). At the high site, however, we found evidence for weak disruptive selection on corolla width, where narrower and wider flowers received more visits per flower than those with intermediate corolla widths (Figure 1.5c, Table 1.3). Selection via pollinator visits differed among water treatments, judging by a significant trait × water treatment interaction term in the zero-inflation models ($P \leq 0.05$). Selection via pollinator visits shifted from strongly directional for large flowers under water reduction to stabilizing selection under water addition (Table 1.4).

EFFECTS ON SEED SET

Prediction 3: Reduced pollinator visitation associated with drought-induced changes in floral traits will negatively impact seed set and seed mass. At the plot level, mean seed set increased linearly with soil moisture (likelihood ratio $\chi^2_{1, 56} = 14.46$ in Poisson model with non-significant site × year interaction removed, $P = 0.0001$, Figure 1.4b), but mean seed mass did not ($F_{1, 55} = 3.6$, $P = 0.06$). When mean pollinator visitation rate was added to this model, we found seed set increased with pollinator visitation, but only up to a point (Linear: $\chi^2_{1, 54} = 16.63$, $P < 0.0001$; Quadratic: $\chi^2_{1, 54} = 11.17$, $P = 0.0008$, Figure 1.6). Soil moisture retained an effect in the model as well. Thus, seed set responded both to pollinator visitation and through other mechanisms to soil moisture. In 2014; the standardized selection differentials for corolla width and corolla length (Table 1.5) indicated positive directional selection for both traits via seed set at the low site, but no significant selection at the high site. We also tested for stabilizing selection on corolla size through seed set, but it was not significant ($P > 0.05$). When we tested for an interaction between corolla traits and water treatment, we found no evidence that selection based on seed set varied with water treatment ($P > 0.05$).

DISCUSSION

EFFECTS OF VARIATION IN SOIL MOISTURE ON PLANT-POLLINATOR INTERACTIONS

By following responses to experimental variation in soil moisture over two years under natural field conditions, we tested the hypothesis that impacts of climate on plant-pollinator interactions operate through changes in water availability. We found strong evidence for non-linear effects of soil moisture on pollinator visitation, largely mediated through changes in corolla size and floral display size. Although pollination peaked at intermediate soil moistures and corolla lengths, seed set continued to increase with additional moisture, even as visitation declined.

EFFECTS ON VEGETATIVE AND FLORAL TRAITS

Both corolla size and nectar production were strongly influenced by changes in soil moisture. Phenotypic plasticity in these traits, such as we observed (Figure 1.2), likely represents a trade-off between dealing with environmental stress and pollinator preference (Strauss and Whittall 2006). In sub-alpine and alpine systems, where growing seasons are short, having the ability to shift resources away from floral attractants may be adaptive for perennial species that have multiple opportunities to reproduce and are presented with inter-annual climate variability (Stinson 2004). For example, drought-stressed *Polemonium viscosum* that diverted resources from leaves to larger flowers experienced lower fitness, both in terms of seed set and post-reproductive survival the following year, than plants that did not produce large flowers during drought (Galen 2000). The strength of this trade-off between environment and pollinator preference, however, likely varies among different plant-pollinator systems (Thomson 2001; Hegland and Totland 2005) and among populations within plant-pollinator systems (Eckhart 1991; Caruso 2006).

In contrast to corolla size and nectar volume, changes in soil moisture during the growing season had little effect on most measures of floral display. Only ramet height and peak floral abundance at the high site increased with water (Figure 1.3). If *M. ciliata* preforms buds or uses stored resources to jump-start spring growth, as is common among alpine and subalpine plant species (Billings 1974), the impact of changes in water availability on floral abundance may only become evident over multiple years.

EFFECTS ON POLLINATOR VISITATION

We found evidence of both directional and stabilizing selection on corolla size based on pollinator visitation rate, except for corolla width at the high site, which experienced disruptive selection. Detecting directional selection for larger flowers is fairly common (reviewed in Harder and Johnson 2009), perhaps because larger flowers are more conspicuous and often provide larger rewards to pollinators (Hegland and Totland 2005). Our estimates of directional selection (S' = 0.03 to 0.47) were similar to the strength of directional selection seen in other studies of flower size (Harder and Johnson 2009). Reports of stabilizing and disruptive selection are less common, in part because they are less often tested for. In *Mertensia ciliata*, stabilizing selection on corolla length may arise in part from the behavior of specific bumblebee species. Both nectar- and pollen-foraging worker bumblebees can assort themselves among plant species by body size (Peat *et al.* 2005; Corbet and Huang 2014). The range of potential bumblebee species and castes that can access rewards in flowers may be restricted when corollas are too long (Inouye 1980; Suzuki 1994). But, visitation rates by longer-tongued bees may also decline when corollas are too short (Peat *et al.* 2005), resulting in stabilizing selection with a broad fitness peak. The switch from directional selection for larger flowers under water reduction to stabilizing selection under water addition may simply reflect an overall optimal size for foraging by the bumblebee species that visit *M. ciliata.* It is also theoretically possible that the relationship of pollinator visitation to flower size resulted in part from the influence of volatiles or other unmeasured traits.

While pollinators showed a consistent preference for intermediate corolla lengths, pollinators responded differently to changes in corolla width at the two sites. At the low site, pollinator visitation peaked with intermediate corolla widths, but at the high site pollinators exhibited disruptive selection on corolla width, with wider and narrower flowers receiving more visits than flowers of intermediate widths. As with corolla length, bumblebees may preferentially visit flowers with corolla widths that match to their body size, as these may be more accessible (Suzuki 1994) and allow for a faster handling time (Inouye 1980; Peat et al. 2005). It is unclear, however, why pollinators would show a preference for intermediate flowers at the low site and not at the high site. Not only do the two sites share similar plant communities, but the *M. ciliata* populations at both sites were visited by the same assemblage of pollinator species, largely dominated by *Bombus flavifrons* and *B. frigidus*. Even though the same species are present at both sites, it is conceivable that there were differences in the size distribution of visitors to *M. ciliata*.

In addition to trait-mediated pollinator responses to individual ramets, changes in community-level interactions in and around the plots may also explain why pollinator visitation peaked at intermediate water levels. At the community-level, pollinator visitation may vary according to the spatial distribution of conspecifics, as well as the spatial distribution of and rewards offered by other co-flowering species (Goulson 2010). Moreover, as floral abundance increases, at either the plant or community-level, the proportion of open flowers that are visited per plant may decline (Geber 1985; Harder and Barrett 1995). If co-flowering species abundance was higher in blocks with high soil moisture, and those species compete for pollinator visits, some of the decline in pollinator visitation at high soil moistures may have been due to potentially larger floral displays or rewards from co-flowering species in and around the plots.

Larger nectar rewards from *M. ciliata* flowers, however, did not influence pollinator visitation. There are at least two possible reasons why pollinators did not respond to increased nectar availability. First, pollinators may use more obvious visual cues, such as flower size, as a proxy for the size of the floral reward (Hegland and Totland 2005). Second, while pollinators certainly visit *M. ciliata* to consume nectar, pollen may be the primary resource that attracts some pollinators to *M. ciliata* flowers. If water availability affects pollen quantity or quality, as has been suggested in other studies (Turner 1993; Waser and Price 2016), then this might influence the behavior of pollen-collecting visitors, including bumblebees (Harder 1990).

This experiment showed that changes in water availability can impact plant-pollinator interactions, and that the effects of water on pollinator visitation can be non-linear, but one important caveat is in order. Our data showed that pollinators preferred intermediate length flowers when a range of sizes were available, however, we cannot know whether this preference would be maintained if an entire population were to undergo a drought or receive abundant rain. It is possible that if all plants in the population experienced a drought and produced short flowers, pollinators would avoid *M. ciliata* altogether. But it is also possible that under such conditions, pollinators would visit *M. ciliata* despite the smaller flower size when not given a choice.

EFFECTS ON SEED SET

Seed set increased linearly with the higher water availability expected in later snowmelt years (Figure 1.4b), and that increase is likely explained both by pollinator visitation and by other mechanisms. Indeed, in a model including both terms, water and pollinator visitation rate independently influenced seed set. Pollinator visitation, however, declined at high soil moistures (Figure 1.4a), while seed set continued to increase with the additional resources. This result suggests that, while some minimum level of pollinator visitation is necessary for *M. ciliata* to set a full complement of seed (Geber 1985, Figure 1.6), seed set may not have been pollinator limited at most soil moisture levels. In attempts to estimate pollen limitation with pollen supplementation experiments, hand-pollinated plants made fewer seeds than insect-pollinated plants (Gallagher, unpublished data). This result is not easily interpreted as it can be due to a variety of mechanisms including crowding of pollen tubes, stigma damage, or missing peak receptivity (Young and Young 1992).

The observed increase in seed set may be due to a general expansion in carbohydrate availability for growth and easier acquisition of nutrients when water is abundant, which is also apparent from the increase in ramet height at higher soil moistures (Figure 1.3b). Since seed set increased linearly with water availability, as did corolla size, the result was net directional selection on corolla size when measured by seed set at the low site.

CONCLUSIONS AND LESSONS FOR STUDIES OF CLIMATE CHANGE

We found that changes in water availability during the growing season can affect the ability of plants to attract pollinators, as well as their seed production. While the water manipulation methods we used do not capture the effect of altered snowmelt on growing season length, they do capture the changes in soil moisture that accompanies altered snowmelt. In these subalpine communities, accelerated timing of snowmelt with climate change is predicted to increase the overall length of the growing season and the length of the dry period experienced prior to the onset of mid-summer thunderstorms (Pederson *et al.* 2011), with effects on soil moisture that can last for months (Blankinship *et al.* 2014).

Pollinator visitation to *Mertensia ciliata* peaked at intermediate soil moistures, which suggests that it may be important to consider whether changes in the variance, as well as mean, water availability over multiple years impacts plant-pollinator interactions. Around RMBL, the average snowmelt date is < 1% earlier than it was a decade ago, but year-to-year variation in snowmelt date has increased by 20% (Barr 2016). These swings in snowmelt date translate into large swings in soil moisture during the growing seasons (Blankinship *et al.* 2014) and may have important implications for plant-pollinator interactions.

We had expected that increasing water would lead to increased floral display, corolla size and nectar rewards, and these in turn would translate into increased pollinator visitation and seed set. But that is not what we found. Pollinator visitation declined both when water was limited *and* when water was super abundant, but seed set increased monotonically with additional water. This decoupling of pollinator visitation from seed set at higher soil moistures suggests that for *M. ciliata*, seed set responds to other factors, in addition to some minimum level of pollinator visitation necessary to achieve seed set. Understanding the demographic consequences of altered precipitation patterns associated with anthropogenic climate change, therefore, will require determining how those factors that influence seed set respond to changes in water availability over time.

In our system, the non-linear effects of water on pollinator visitation were mediated by responses of bumblebees to differences in corolla length. We may expect to see similar non-linear effects of water on pollinator visitation in systems where flower size has high phenotypic plasticity and where pollinators are known to respond to differences in flower size, as in bee, hummingbird, hawkmoth, and butterfly pollinated species (e.g., Campbell *et al.* 1991; Eckhart 1991; Thomson 2001). As the frequency of extreme summer climates increases (Pederson *et al.* 2011), plants that experience selection for intermediate floral traits may be between the proverbial rock and a hard place. If both too little and too much water can drive plants to produce floral displays that are less attractive or less accessible to their current pollinators, over time the accumulation of low-pollination years may affect the persistence of these populations. Testing the net effect that increased interannual variation in water availability has on plant-pollinator interactions and, potentially, on plant fitness, will be an important area of future research.

Shifts in abiotic conditions due to climate change may have important consequences for plant-pollinator mutualisms. Much of the focus in pollination biology as it relates to climate change has been on documenting patterns of phenological variation and modeling the potential consequences of changes in temporal overlap between flowering and pollinator activity (reviewed in Forrest 2015). This study demonstrates that, even without changes in phenology, climate change may affect plant-pollinator interactions through responses of floral traits to changes in water availability. Only by considering and comparing each of these potential climate impacts can we predict the overall consequences of global climate change.

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Table 1.1 Effects of water availability on floral abundance measurements. Measurements include (a) peak floral abundance, determined to be the maximum number of flowers counted in one census (N = 30), (b) mean floral abundance per ramet calculated from marked ramets that did flower (N = 60), (c) floral abundance per plot calculated as (total number of flowers per plot) / (total number of flowering ramets per plot), and (d) flowering ramets per plot calculated as (total number of flowering ramets per plot) / (total number of ramets per plot). For floral abundance measures (c) and (d), N = 28 because cows sat on two plots. We used linear model analyses to test the effects of experimental variation in soil moisture on each floral abundance variable. Model predictors included main effects of mean summer VWC and site, and year when appropriate, as well as interactions between site × soil moisture, year × soil moisture, and site × year. Non-significant interactions were excluded.

(a) Peak floral abundance per plot

Variable	DF	F value	*P*
Soil moisture	1, 25	2.42	0.3
Site	1, 25	1.19	0.3
Site × Soil moisture	1, 25	4.71	0.04

(b) Floral abundance per ramet

Variable	DF	F value	*P*
Soil moisture	1, 56	0.02	0.9
Site	1, 56	68.78	< 0.0001
Year	1, 56	2.75	0.1

(c) Floral abundance per plot

Variable	DF	F value	*P*
Soil moisture	1, 25	0.02	0.9
Site	1, 25	22.69	< 0.0001

(d) Flowering ramets per plot

Variable	DF	F value	*P*
Soil moisture	1, 25	0.16	0.7
Site	1, 25	0.75	0.4

Table 1.2 Results of zero-inflation model analysis testing effects of experimental variation in soil moisture on *Mertensia ciliata* pollinator visitation rate per plot.

Count model (Poisson with log link)			
Variable	Estimates	Z value	*P*
Soil moisture	0.18	3.76	0.0001
Soil moisture (quadratic)	- 0.01	- 3.26	0.001
Site	- 0.09	- 2.78	0.005
Year	0.37	9.71	< 0.0001
Site × Year	- 0.07	- 2.04	0.04
Zero-Inflation model (binomial)			
Variable	Estimates	Z value	*P*
Soil moisture	- 0.61	- 1.33	0.18
Soil moisture (quadratic)	0.03	1.29	0.19
Site	- 4.07	- 0.005	0.99
Year	4.72	0.005	0.99
Site × Year	3.55	0.004	0.99

Mean pollinator visitation rate per plot, calculated as (total number of flowers visited / number of flowers available per hour of observations) averaged for each flowering season, was used as the response in model selection with linear and quadratic terms for mean summer volumetric water content (VWC), site, year, and site × year interaction as predictors (N = 60).

Table 1.3 Results for zero-inflation model analysis testing responses of pollinator visitation to differences in *Mertensia ciliata* corolla width and length at two sites.

Site	Variable	Count portion of model (Poisson with log link)			Zero-Inflation portion (binomial with logit link)		
		Estimate	Z value	P	Estimate	Z value	P
High	Corolla width	- 2.27	- 4.27	< 0.0001	1.51	0.42	0.67
High	Corolla width (quadratic)	0.18	3.95	< 0.0001	- 0.18	- 0.59	0.56
Low	Corolla width	1.10	3.83	< 0.0001	- 1.32	- 0.58	0.56
Low	Corolla width (quadratic)	- 0.09	- 3.71	< 0.0001	0.08	0.38	0.70
Site	Variable	Count portion of model (Poisson with log link)			Zero-Inflation portion (binomial with logit link)		
		Estimate	Z value	P	Estimate	Z value	P
High	Corolla length	3.51	5.13	< 0.0001	1.49	0.45	0.65
High	Corolla length (quadratic)	- 0.13	- 5.44	< 0.0001	- 0.06	- 0.55	0.59
Low	Corolla length	6.85	8.28	< 0.0001	- 2.6	- 0.77	0.44
Low	Corolla length (quadratic)	- 0.22	- 8.26	< 0.0001	0.08	0.66	0.51

Mean pollinator visitation rate to individual ramets, calculated as (total number of flowers visited) / (number of flowers available per hour of observations) averaged across the flowering season, was used as the response, with either corolla width or corolla length, as well as the quadratic term for that corolla trait as predictors ($N_{high} = 86$, $N_{low} = 138$). Estimates based on multiplying visitation rate by 100 and converting it to an integer.

Table 1.4 Standardized directional (S') and quadratic (γ) selection differentials of pollinator visitation on corolla traits. Corolla width and length, estimated separately for each corolla trait and water treatment (i.e., water reduction, control, and water addition) from linear regression models of relative fitness on standardized linear and quadratic corolla trait values. Pollinator visitation rate for each ramet was divided by the mean to estimate an index of relative fitness. Significance estimates based on zero-inflation models ($N_{\text{water reduction}} = 73$, $N_{\text{control}} = 72$, $N_{\text{water addition}} = 79$).

	Corolla width		Corolla length	
	S'	γ	S'	γ
Water reduction	0.46*	0.32	0.79	0.57
Control	0.21	- 0.11	0.48	- 0.53
Water addition	0.18**	- 0.06*	- 0.20	- 0.19*

Significance codes: P ≤ 0.01 **, P ≤ 0.05 *

Table 1.5 Standardized directional (S') selection differentials on *Mertensia ciliata* corolla width and length, estimated separately for each site, using relative seed set as the measure of fitness and standardized linear and quadratic corolla trait values in the model.

	Corolla width			Corolla length		
	S'	χ^2	*P*	S'	χ^2	*P*
High site	0.11	1.65	0.20	0.07	1.31	0.25
Low site	0.15	5.63	0.02	0.13	4.16	0.04

Significance estimates based on likelihood ratio test for Poisson models (N_{high} = 86, N_{low} = 138).

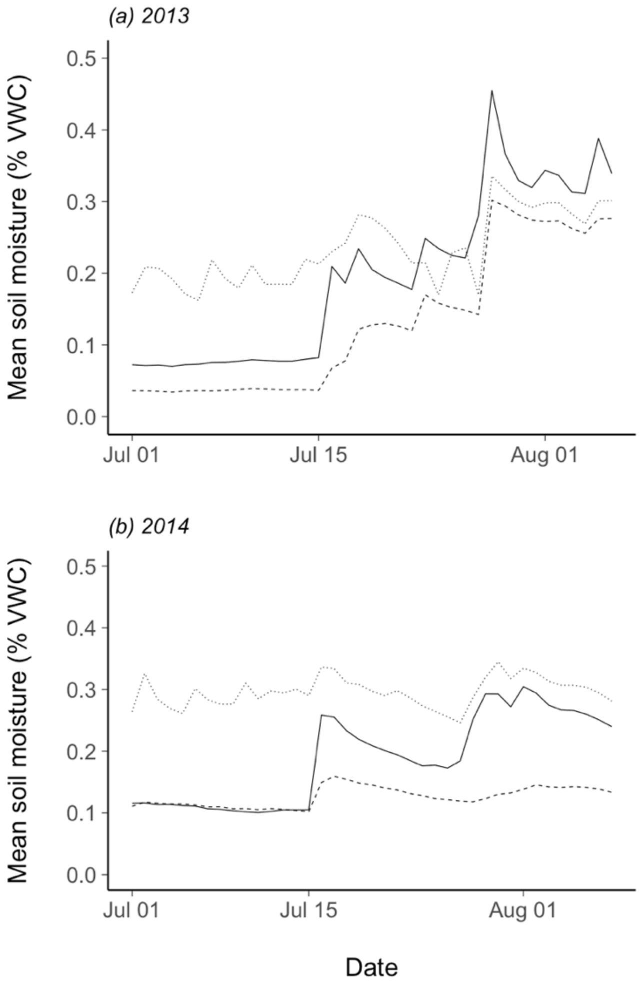


Figure 1.1 Mean daily maximum soil volumetric moisture content for (a) 2013 and (b) 2014. Measured by Hobo soil moisture smart sensors (Onset Computer Corporation, Inc., Bourne, Massachusetts, USA) buried at a depth of 15 cm in the center of two replicates of each treatment (one at each site). For one block per site, the three probes were connected to a HOBO data logger (Onset Computer Corporation, Inc., Bourne, Massachusetts, USA), which recorded the soil volumetric moisture content of the water addition (dotted line), ambient (solid line) and water reduction (dashed line) plots every hour.

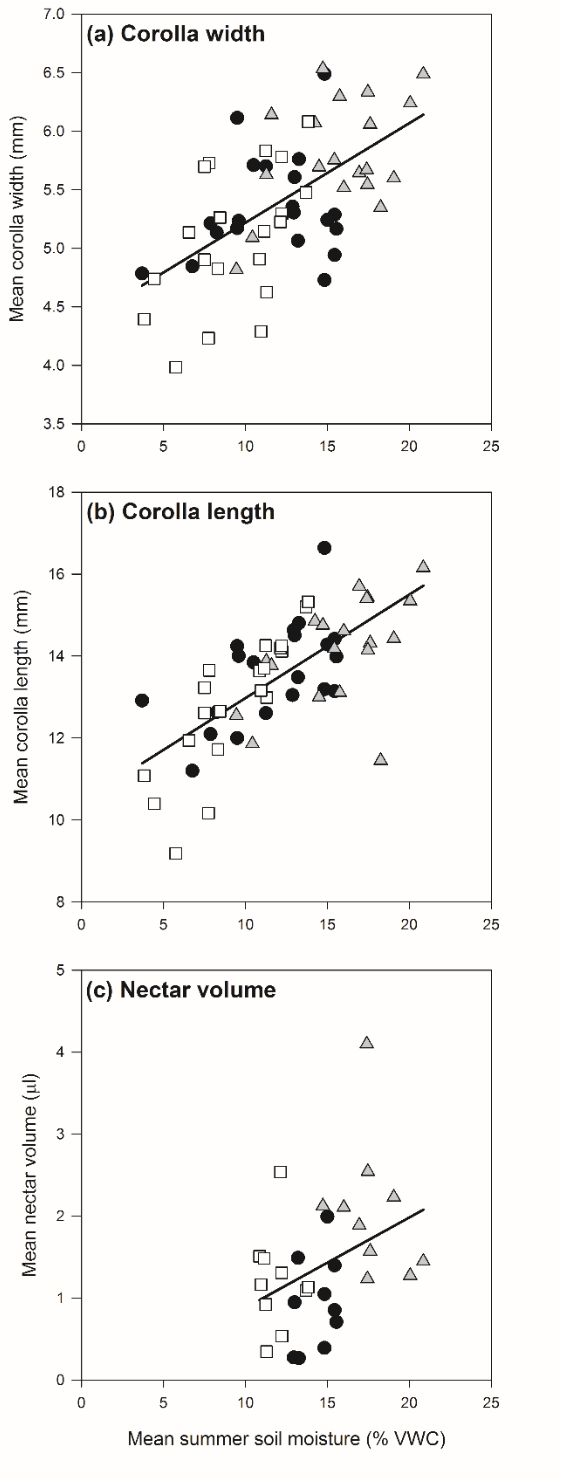
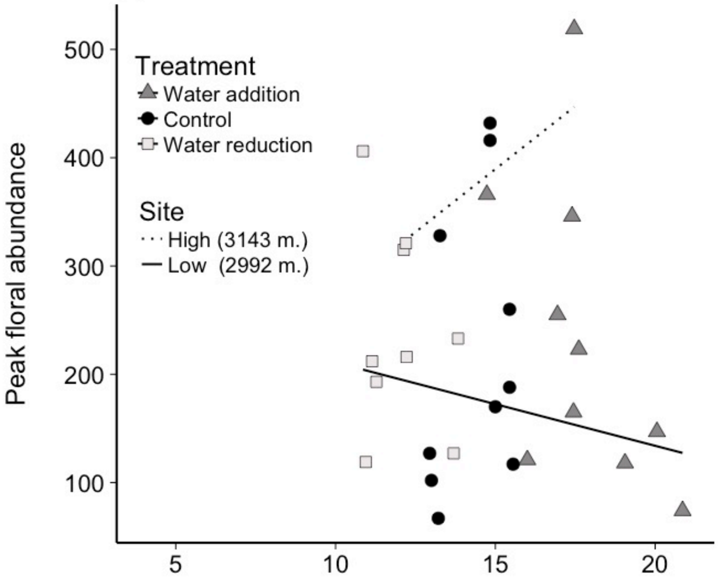


Figure 1.2 Effects of experimental variation in soil moisture on (a) corolla width, (b) corolla length, and (c) nectar volume of *Mertensia ciliata*. Corolla traits were measured in both 2013 and 2014 (N = 60), whereas nectar measurements were only collected in 2014 (N = 30). White square: Water reduction. Black circle: Control. Gray triangle: Water addition.

(a) Peak floral abundance



(b) Ramet height

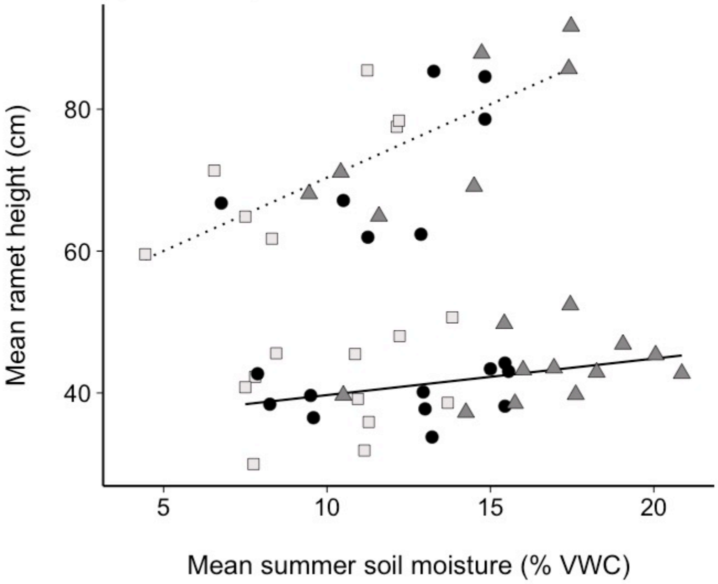


Figure 1.3 Effects of variation in soil moisture on *Mertensia ciliata* (a) peak floral abundance and (b) ramet height at the high elevation site (3,143 m.) and low elevation site (2,992 m.). Ramet height was measured in both 2013 and 2014 (N = 60), whereas peak floral abundance was only collected in 2014 (N = 30).

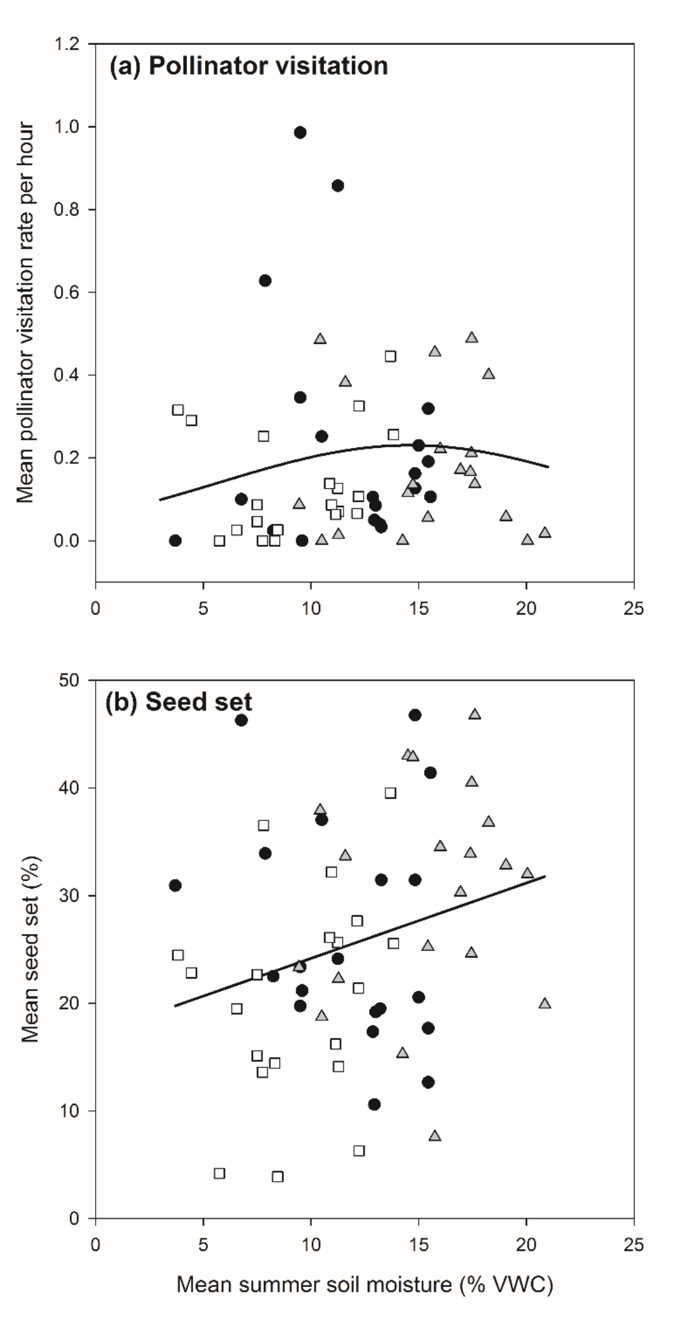


Figure 1.4 Effects of experimental variation in soil moisture on *Mertensia ciliata* (a) pollinator visitation rate per plot, calculated as (total number of flowers visited / number of flowers available per hour of observations) averaged across the flowering season and (b) mean seed set per plot, calculated as (100% × number of mature nutlets) / (4 × number of flowers) (N = 60). Fit line for pollinator visitation was plotted with coefficients from the count portion of the zero-inflated Poisson model from Table 1.2, using the inverse log link to obtain expected values and shows the relationship with water only. White square: Water reduction. Black circle: Control. Gray triangle: Water addition.

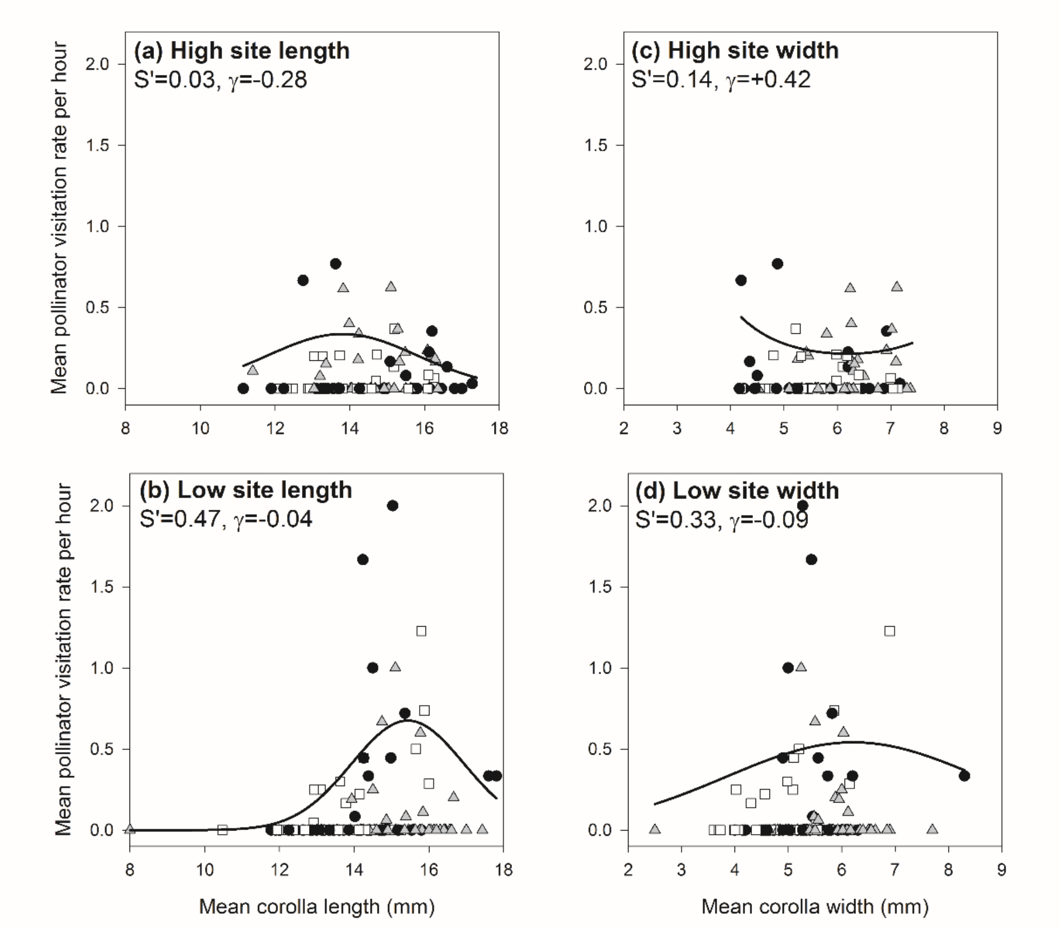


Figure 1.5 Effects of variation in flower size in *Mertensia ciliata* on pollinator visitation rate of individual ramets, calculated as (total number of flowers visited / number of flowers available per hour of observations) averaged across the flowering season (N = 224). (a) corolla length at high site; (b) corolla length at low site; (c) corolla width at high site; (d) corolla width at low site. White square: Water reduction. Black circle: Control. Gray triangle: Water addition. Functions plotted are the fits from the count portion of the zero-inflated Poisson model, using the inverse log link to obtain expected values. In all cases both the linear and quadratic terms were significantly different from zero in the count portion of the model (all $P < 0.0001$), but not in the zero-inflation portion (all $P > 0.4$). S' = standardized linear selection differential. γ = standardized quadratic selection differential.

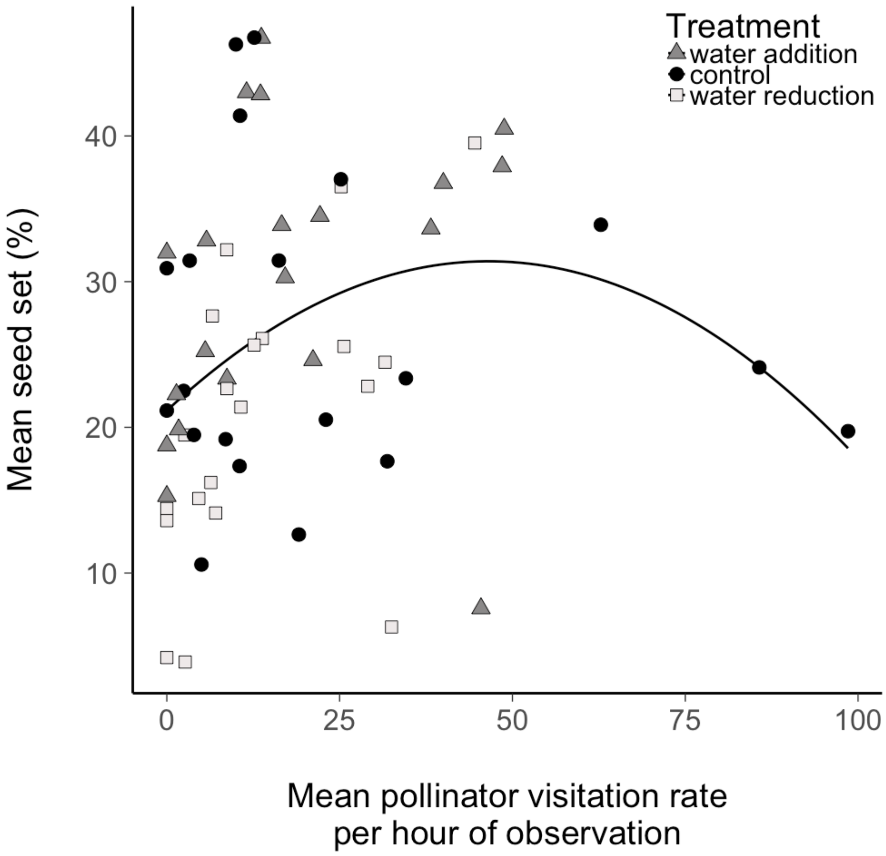


Figure 1.6 Effects of pollinator visitation rate per plot on mean seed set of *Mertensia ciliata* (N = 60). Mean pollinator visitation rate was calculated per plot as (total number of flowers visited / number of flowers available per hour of observations) averaged across the flowering season. Mean seed set per plot was calculated as (100% × number of mature nutlets) / (4 × number of flowers).

CHAPTER 2: FLOWERING PHENOLOGY INFLUENCES POLLINATION BY ALTERING VISITATION RATE AND POLLINATOR EFFECTIVENESS

INTRODUCTION

The seasonal timing of life-history events is a critical determinant of fitness for organisms (Rathcke and Lacey 1985; Ims 1990). Among plants, differences in the timing of flowering (i.e., flowering phenology) can directly affect reproduction and survival by exposing individuals to unfavorable abiotic conditions (Evans, Smith and Gendron 1989; Elzinga et al. 2007). In temperate zones, flowering early may increase the risk of exposure to late spring storms or frost events (Inouye 2008), while flowering late may increase the risk of reduced resource availability for ripening fruits and seeds (Schemske et al. 1978; Kudo, Ida and Tani 2008; Pau et al. 2011).

Variation in flowering phenology can also impact plant reproduction and survival by altering the temporal overlap with interacting species (Waser 1978; Augspurger 1981; Campbell 1985; Ims 1990; Ågren and Willson 1992; Brody 1997; Kudo et al. 2004; Elzinga et al. 2007). Changes in phenological events, including flowering time and pollinator emergence, are among the most widely reported consequences of climate change (Fitter and Fitter 2002; Parmesan and Yohe 2003; Walther 2003; Bartomeus et al. 2011). Differences in the strength or direction of phenological responses to climate change among individual species can disrupt interspecies phenological synchrony (Ovaskainen et al. 2013). Changes in the temporal overlap among interacting species can affect plant reproductive success by altering the overlap with herbivores or seed predators and releasing plants from direct antagonists (Brody 1997; Pilson 2000; Lay, Linhart and Diggle 2011; Parsche, Fründ and Tscharntke 2011; Ehrlén, Raabova and Dahlgren 2015) and by altering the overlap with pollinators and impacting pollination success (Kudo et al. 2004; Memmott et al. 2007; Hegland et al. 2009; Thomson 2010; Parsche et al. 2011; Rafferty and Ives 2011; Gezon, Inouye and Irwin 2016), all of which could have cascading effects on other trophic levels (Post et al. 2008; Both et al. 2009; Lany et al. 2015; Mortensen et al. 2016).

To predict how climate change may affect the reproductive success of zoophilous plants, it is important to develop a mechanistic understanding of how differences in flowering phenology affects pollination success through changes in plant-pollinator interactions. Pollination success requires the receipt of adequate compatible pollen to the stigma, which is influenced by the frequency of pollinator visits and the effectiveness of those pollinators in depositing a sufficient quantity of quality pollen (Ne'eman et al. 2010). Pollinator effectiveness, calculated as the amount of conspecific pollen transferred, or seeds produced, after a single visit to a virgin flower (Ne'eman et al. 2010), can vary widely among pollinator visitors (Motten et al. 1981; Sahli and Conner 2007). While pollination success is often strongly correlated with visitation frequency (Vázquez, Morris and Jordano 2005; Sahli and Conner 2006; Sahli and Conner 2007), pollinator taxa that visit with equal frequency may not contribute equally to a plants reproductive success due to differences in per-visit effectiveness (Bischoff et al. 2013).

Experimental studies show that changes in flowering onset can affect seed set, both through changes in pollinator visitation rates (Parsche et al. 2011; Gezon et al. 2016) and through changes in pollinator effectiveness associated with compositional changes in pollinator taxa or changes in effectiveness within pollinator taxa over time (Rafferty and Ives 2012). The mechanisms through which phenological shifts affect seed set are expected to act through changes in pollen receipt, but as yet, the extent to which each mechanism actually does affect pollen receipt remains unexplored.

Changes in plant-pollinator interactions due to phenological shifts may affect pollination success through differences in the quantity and quality of pollen receipt. Changes in pollinator visitation rates may alter the quantity of pollen receipt, while changes in pollinator type can alter both pollen quantity and quality (i.e., amount of heterospecific pollen or self-pollen) via differences in pollinator effectiveness or changes in pollinator fidelity (Rathcke 1983; Herrera 1987; Mitchell et al. 2009; Ne'eman et al. 2010). Because plants in the same community may not respond in the same way to environmental changes (CaraDonna, Iler and Inouye 2014), species-level phenological shifts in plant communities may also impact the composition and relative abundances of hetero- and conspecific co-flowering plants. Changes in floral neighborhood can affect conspecific pollen deposition by altering pollinator visitation rates (Pleasants 1980; Brody 1997; Lázaro, Lundgren and Totland 2009; Lázaro and Totland 2010), as well as heterospecific pollen deposition through changes in pollinator fidelity (Waser 1978; Campbell and Motten 1985). These changes in floral neighborhood can also influence the types of pollinators that visit a plant (Moeller 2005; Mitchell et al. 2009), which in turn may affect pollination success through shifts in pollinator assemblages towards less effective pollinators (Bruckman and Campbell 2014). Additionally, differential genotypic responses to the environment may alter the degree of synchrony among conspecific plants within communities, potentially leading to mate-limitation (Schmitt 1983; Kunin 1993) and lower quantities of compatible pollen deposition early or late in the flowering season. Changes in pollinator effectiveness in depositing adequate compatible pollen is likely to be an important mechanism affecting reproductive success across the flowering season (Rafferty and Ives 2012). To interpret how a change in pollinator visitation and species composition influences pollination success, therefore, it is necessary to know the effectiveness of each type of pollinator on a per-visit basis, both in terms of conspecific and heterospecific pollen deposition as well as seeds produced. To date, however, no study has experimentally tested the extent to which changes in plant-pollinator interactions due to phenological shifts affects both pollen receipt and seed set.

We investigated two mechanisms by which changes in flowering phenology influence pollination and reproductive success. First, we tested the hypothesis that phenological shifts change the total number of pollinator visits. Second, we tested the hypothesis that phenological shifts change the quality of pollinator visits by altering the frequencies of visits made by pollinators of different single-visit effectiveness. To test these hypotheses, we conducted two experiments. A manipulation of flowering phenology allowed us to determine impacts on total pollinator visits and relative frequencies of visits by different species. We also measured single-visit pollinator effectiveness, in terms of both pollen receipt and seed set. Finally, we examined whether the combination of visitation pattern and pollinator effectiveness explained phenological patterns in relative pollen receipt and seed set. To our knowledge, ours is the first study to investigate the role that pollinator visitation rate, pollinator community assembly, and pollinator effectiveness play in determining how phenological shifts affects both pollen receipt and seed set.

MATERIALS AND METHODS

STUDY SYSTEM

Mertensia ciliata (James ex Torr.) G. Don (Boraginaceae), the tall-fringed bluebell, is a rhizomatous, herbaceous perennial found primarily along streams and wet meadows in subalpine and lower alpine zones of the Rocky and Sierra Nevada Mountains. Plants form compact clones of a few to several hundred flowering stems, which can reach over 1 meter in height (Pelton 1961). The pendant flowers of *M. ciliata* are tubular, expanding to a wider, lobed mouth, and are borne in dense clusters of cymes along leafy stems. Flowers are typically open for six days and can produce a maximum of four one-seeded nutlets (hereafter seeds). Stigmas are receptive throughout flowering, but pollen is usually gone within 24-48 hours of anther dehiscence (Pelton 1961). *Mertensia ciliata* is self-compatible, but insect exclusion and hand pollination experiments indicate that seed set is dependent on insect pollination (Geber 1985).

Mertensia ciliata is primarily pollinated by medium and long-tongued bumblebees, including *Bombus balteatus* (Dahlbom), *B. bifarius* (Cresson), *B. flavifrons* (Cresson), and *B. frigidus* (Smith) (Geber 1985, Suzuki 1994, Gallagher and Campbell 2017). Other floral visitors that may contribute to *M. cilata* pollination include flies (Bombyliidae, Muscoidea, and Syrphidae) and solitary bees (Colletidae: *Colletes p. paniscus Vier.* and Megachilidae: *Osmia spp.*) (Pelton 1961; Gallagher and Campbell 2017).

FIELD SITES

Fieldwork was primarily conducted in a subalpine meadow along Rustler's Gulch in Gunnison National Forest (38°59'32.68'' N, 107°00'23.16'' W; 3,009 m.a.s.l.) located 4.3 km from the Rocky Mountain Biological Laboratory (RMBL) in Gothic, Gunnison County, Colorado, USA. To increase the sample size of the pollinator effectiveness study, additional fieldwork was conducted at Schofield Pass (39°00'54.98'' N, 107° 2'49.40'' W; 3,263 m.a.s.l.), located 7.9 km from RMBL. *Mertensia ciliata* is abundant at both sites, with peak flowering occurring approximately two weeks later at Schofield Pass than at Rustler's Gulch (Gallagher, unpublished data).

At RMBL (2,900 m. a.s.l.), *M. ciliata* typically flowers from late June to mid-July, although since 1973, the average date of first bloom (DFB) has advanced by more than a week (Miller-Rushing and Inouye 2009). While phenological responses in the plant communities surrounding RMBL differ among species (CaraDonna et al. 2014), shifts in peak *M. ciliata* flowering correlate significantly with earlier snowmelt timing (0.42 d earlier for each day earlier that snow melted) (Miller-Rushing and Inouye 2009). In warmer years, these phenological shifts are producing a longer mid-season decline in floral abundance (Aldridge et al. 2011), which may negatively impact pollinator abundance and pollination success of summer-blooming plants like *M. ciliata*.

PHENOLOGY MANIPULATION EXPERIMENT

To test the hypothesis that differences in the timing of flowering alters the visitation rate and the types of potential pollinator visitors, resulting in lower pollination success and seed set, we manipulated flowering phenology of potted *M. ciliata* plants. Sixty plants were collected from a large *M. ciliata* population in Rustlers Gulch in 2012 and overwintered in the ground at RMBL. In 2015, we induced the plants to flower at different times using natural variation in temperature and light found along an elevation gradient in the East River Valley. To inhibit flowering, potted plants were moved to Schofield Pass in early June, where they were placed in a shaded snow bank under a mesh shade-shelter. Each week, 10 randomly selected plants were moved back to RMBL, where the higher light and warmer temperatures at low elevation induced them to flower.

Each week, ten flowering plants were moved from RMBL to a meadow near the original source population in Rustlers Gulch. Plants were arranged 30 cm apart into two randomized arrays of five plants, with 2 m between arrays. To create distinct experimental populations, arrays were located 50 meters away from unmanipulated *M. ciliata* populations. A total of forty plants flowered and were included in the experiment, for a total of four phenology treatment groups spanning four weeks (June 23 - July 20).

Plants in each phenology group were left open to pollination for one week. During that time, we conducted pollinator observations and tracked pollinator identity and the number of flowers visited during multiple 30-minute observation periods between the hours of 9:00 and 16:00. At the beginning of each observation period, we counted the number of open flowers per potted plant. Visitors were counted as pollinators if they crawled inside the flower corolla. The order of observations was randomized between the two arrays for a given week. We completed 15 hours of pollinator observations per phenology group, except in week four because of lower flower abundance.

The floral abundance among arrays during the first three weeks ranged from 12 to 86, with a mean of 78.27 ± 12 (Mean ± SEM) flowers per array (Figure 2.1). The floral abundance for week four plants, however, was significantly lower than for the other three weeks ($F_{3,39} = 10.15$, $P < 0.0001$), and we observed zero pollinators during our first 15 hours of observations that week. To compensate for the difference in floral display, in week four we increased the number of available flowers by adding cut stems in plant picks to each pot and completed an additional 15 hours of observations. We combined the data from both rounds of observations in our analyses of pollinator visitation rate and pollinator type for week four.

At the end of each week, individual flowers in each phenology group were labeled and bagged with fine mesh jewelry bags (Uline, Pleasant Prairie, WI, USA) to prevent further pollination and loss of seeds. To measure deposition of hetero- and conspecific pollen load, stigmas were collected after the corollas fell from the flowers and stigma squashes were made with fuchsin gel (Kearns and Inouye 1993). For an average of 5.2 ± 0.7 flowers per plant we counted the number of conspecific and heterospecific pollen grains using a compound microscope at 200×.

To standardize conditions after pollination exposure, all plants remained in the field until seeds were collected. We counted the total number of seeds produced per flower for all labeled flowers (measured as described by Forrest and Thomson 2010). We calculated the average seeds per flower for each potted plant as (number of mature seeds / number of flowers). Mature seeds from tagged flowers were collected in coin envelopes and transported to the University of California, Irvine to be weighed. We calculated mean seed mass for each plant as (mass of collected seeds / number of collected seeds).

Because the goal of this experiment was to track how floral timing may impact pollination and seed set, we monitored soil moisture and tracked floral traits that are known to vary with water availability (e.g., corolla size and nectar rewards, see Gallagher and Campbell 2017). We measured soil moisture as volumetric water content (VWC) every week using a 12 cm Campbell Scientific "HydroSense" probe inserted into the center of each pot (always before applying water). We tested for differences in the average summer (June-July) VWC values of the pots in an ANOVA with phenology week as the predictor. Average soil moisture in the pots was 25.4 ± 0.7 % and did not differ significantly among phenology groups ($F_{3,36} = 0.79$, $P = 0.5$). For an average of 4.3 ± 0.3 flowers per plant, we measured corolla width at the opening of the tube and corolla length from the base of the calyx to a randomly chosen corolla lobe. For plants with flowers remaining at the end of each week, we measured nectar volume and percent sugar concentration 48 hours after plants were bagged (N = 20). For an average of 4.3 ± 0.3 flowers per plant, we measured nectar volume using 5μl microcapillary tubes (Kearns and Inouye 1993) and percent sugar concentration using a handheld nectar refractometer (Bellingham + Stanley Ltd., Basingstoke, Hants, UK). For each floral trait, we tested whether the mean trait value of each plant (response) differed among phenology weeks (predictor) using ANOVAs. Corolla length, nectar volume, and sugar concentration did not differ among phenology weeks ($P > 0.05$), but corollas in the last week were 1.2 ± 0.1 mm narrower than flowers in previous weeks ($F_{3, 36} = 5.72$, $P = 0.002$).

Data analysis – All analyses were conducted using R (R Core Team 2016). For models including interaction terms we calculated type III sums of squares using the *car* package in R (Fox and Weisberg 2011) and a contrast setting that sets the row sum to zero. When interactions were not significant, we reran the models without the interaction to simplify interpretation. For models without interaction terms, we report type II analyses. In the event of significant main effects, Tukey's post hoc tests were performed using the *multcomp* package (Hothorn, Bretz and Westfall 2008).

We tested the extent to which experimental variation in flowering time affects pollination and plant fitness with the following response variables: pollinator visitation rate, percent of visitors that were bumblebees (i.e., pollinator type), conspecific and heterospecific pollen receipt, seed set and seed mass. For each response variable, we first looked at the effect of phenology week alone and then at a second model with main and interactive effects of phenology week and one or more additional predictor variables (Table 2.1). Because corolla width differed significantly among phenology treatment weeks, and variation in corolla size can affect both pollinator visitation rates and the assemblage of pollinator visitors (Inouye 1980; Galen and Newport 1987; Eckhart 1991; Suzuki 1994; Peat et al. 2005; Galen 2000; Thomson 2001; Gallagher and Campbell 2017), we ran a second set of analyses on pollinator visitation rate and pollinator type including main and interactive effects of phenology week and mean corolla width in the models (Table 2.1). We tested the hypothesis that pollen receipt is positively correlated with pollinator visitation rate (Engel and Irwin 2003; Sahli and Conner 2006; Sahli and Conner 2007) in a second set of analyses of conspecific and heterospecific pollen receipt that included main and interactive effects of phenology week and mean visitation rate (Table 2.1). To test the relationship between pollen receipt and seed set, we ran a second analysis including main and interactive effects of phenology week with both conspecific and heterospecific pollen receipt in the model. Conspecific and heterospecific pollen receipt were not significantly correlated ($r = 0.31$, $n = 36$).

We calculated mean pollinator visitation rate per plant as (total number of flowers visited) / (number of flowers available per hour of observations) averaged across the phenology week. Initially we used a generalized linear model (GLM) with Poisson distribution for the analyses of pollinator visitation rate, however the residual deviances of both models greatly exceeded the residual degrees of freedom, indicating over-dispersion; therefore, we repeated the analyses assuming a quasi-Poisson error distribution. We calculated mean percent of visitors that were bumblebees as (100% × number of flower visitors that were bumblebees) / (total number of flower visitors per hour of observation) averaged across the phenology week. We used a quasi-Poisson family GLM for the analyses of pollinator type because of over-dispersion in the data.

We analyzed the effects of experimental variation in flowering phenology on conspecific and heterospecific pollen receipt using the per plant mean of each trait, calculated from the mean number of *M. ciliata* or non-*M. ciliata* pollen grains received per stigma per plant, as the response in models with normally distributed residuals. Both conspecific and heterospecific pollen receipt were log transformed to meet the assumption of normally distributed residuals. To test whether differences in flowering time affect seed set and seed mass, we used the per plant mean of each trait as the responses in linear models. Residuals of seed set and seed mass were approximately normally distributed (Shapiro-Wilk, $P > 0.05$).

NATURAL PHENOLOGY TRANSECTS

To assess the extent to which the phenology and seed set of manipulated plants differed from unmanipulated plants, we tracked flowering phenology and seed set of 50 naturally occurring *M. ciliata* ramets from the source population at Rustler's Gulch. Along each of five transects, we randomly selected and tagged ten ramets at 1 meter intervals. Each week, we counted and marked the calyx of all open flowers on the tagged plants with a paint pen. We used a different color each week to mark the open flowers, which allowed us to track the seeds produced per week, calculated as (number of mature seeds per plant / number of flowers per plant).

Changes in floral neighborhood can influence the frequency and the taxonomical composition of pollinator visitors that plants receive (Schmitt 1983; Feinsinger 1987; Lázaro et al. 2009; Bruckman and Campbell 2014). To test the extent to which the floral neighborhood diversity and abundance differed over the course of the phenology manipulation experiment, each week, we also tracked the flowering phenology and floral abundance of all co-flowering species along two 50 × 1 meter transects bisecting the experimental arrays.

Data analysis – We tested whether seed set of unmanipulated *M. ciliata* plants differed across the flowering season. Week six was omitted from this analysis due to a low sample size. We calculated the mean seed set of unmanipulated plants from the average number of seeds produced per week by ten plants in each of five transects, and used that as the response in a linear model with phenology week and transect as predictors. Residuals were approximately normally distributed (Shapiro-Wilk, $P > 0.05$).

We determined that 11 of the 19 species that we tracked along the co-flowering species transects are likely to share pollinators with *M. ciliata* (Pleasants 1980; Pyke 1982; Forrest, Inouye and Thomson 2010; Gallagher, personal obs.). We then tested whether the floral abundance across all 11 species that share pollinators with *M. ciliata* differed among phenology weeks using a GLM model with Poisson distribution that included treatment week and species ID as predictors.

SINGLE-VISIT POLLINATOR EFFECTIVENESS

To estimate the single-visit pollinator effectiveness of different insect visitors, in 2016 we measured pollen receipt and seed set resulting from single visits to virgin *M. ciliata* flowers in the wild populations at Rustler's Gulch (June 27 – July 12) and Schofield Pass (July 12 – 29). Cymes on individual ramets were bagged with fine mesh jewelry bags while all flowers were in the bud phase to provide a supply of virgin flowers. For each single-visit ramet, two additional ramets in the same clone were bagged to serve as controls. Each group of three ramets, including the single-visit ramet and two control ramets, belonged to the same *M. ciliata* clone, and no clone was used for more than one group of three. The first control group remained bagged throughout the experiment to serve as a control for self-pollination. Non-production of seeds by plants in this bagged control group would indicate that despite being self-compatible, *M. ciliata* flowers are not self-pollinating and therefore require insect pollination. Cymes in the second control group (hereafter open control) were made available to pollinators during observation periods but not observed to be visited, thus serving as a control for missed visits by observers.

Bags were removed from flowering cymes on single-visit and open control ramets during observation periods. We recorded the number of flowers available on single-visit and open control ramets during each observation period. Once a single visit to a single flower was received on the single-visit ramet, the visited flower was marked on the calyx with permanent marker and both the open control and single-visit ramet were re-bagged to prevent further pollination and loss of seeds. For each visitor, we recorded the pollinator identity and number of flowers visited. Only insects that crawled inside of a corolla were counted as visitors. For each single-visit ramet, we also measured corolla width and length of marked flower, or if marked corollas were withered or had fallen off, we calculated mean corolla width and length from up to five randomly selected flowers on the same ramet. In a few cases, more than one flower was visited on the single-visit ramet; when this happened, we marked the calyx of each visited flower, and used mean trait values (e.g., pollen receipt, seed set, etc.) for these ramets in our analyses. When a single visit was observed to an open control ramet, we re-designated that ramet as a single-visit ramet and marked the visited flower(s).

We monitored 235 marked flowers from 95 ramets that received single visits, as well as 65 open control and 74 bagged control ramets. The most common pollinators, bumblebees (*Bombus spp.)* and flies (Muscoidea, hereafter flies), accounted for 97.5% of visits, with solitary bees (*Osmia spp.* 2.1%) and a syrphid fly (Syrphidae 0.4%) making up the rest. We excluded the syrphid fly from our analyses. To measure conspecific and heterospecific pollen receipt, we collected stigmas from marked flowers on single-visit ramets, as well as one randomly selected flower from each open control ramet. Stigmas were collected after the corollas fell from the flowers and stigma squashes were made with fuchsin gel (Kearns and Inouye 1993). For an average of 8.25 ± 1.6 flowers per visitor/control type we counted the number of conspecific and heterospecific pollen grains using a compound microscope at 200×.

For each single-visit ramet, we counted the total number of seeds produced per marked flower and, when a ramet had more than one marked flower, calculated mean seed set of marked flowers as the (number of mature seeds / number of marked flowers). For each open and bagged control ramet, we calculated mean seed set per flower as (number of mature seeds / number of bagged flowers). Seed set was measured as described by Forrest and Thomson (2010). Mature seeds were collected in coin envelopes and transported to the University of California, Irvine to be weighed. We calculated mean seed mass per flower as (mass of collected seeds / number of collected seeds). Ramets that failed to set seed because of herbivory or accidental damage were excluded from analyses.

Data analysis – We assessed whether floral visitors differed in their effectiveness as pollinators with the following response variables: conspecific pollen receipt, heterospecific pollen receipt, seed set, and seed mass. We analyzed whether pollen receipt from single visits to virgin flowers differed among pollinator types (i.e., bumblebee, solitary bee, fly, and open control) using number of *M. ciliata* or non-*M. ciliata* pollen grains received per stigma per ramet as the response in linear models. Both conspecific and heterospecific pollen receipt were log transformed to meet the assumption of normally distributed residuals. We tested whether seed set from single pollinator visits to virgin flowers differed among pollinator types, using GLM with a quasi-Poisson error distribution, to account for over-dispersion in the data. We included visitor identity and site as predictors in this analysis, where visitor identity included bumblebee, solitary bee, and fly visits, as well as open and bagged controls.

Because the per-visit rate of pollen transfer is often positively correlated with the closeness of the morphological 'fit between flower depth and the length of mouthparts (Nilsson 1988; Campbell, Waser and Price 1996; Johnson and Steiner 1997), we hypothesized that corolla size might affect pollinator effectiveness. We performed two additional analyses to test whether conspecific pollen receipt and seed set from single pollinator visits were influenced by differences in corolla size. These models included mean corolla length, mean corolla width, site, and visitor identity (excluding controls) as predictors.

We used the generalized linear hypothesis test function in the *multcomp* package in R (Hothorn et al. 2008) to test *a priori* contrasts. First, we tested for differences in seed set of insect-pollinated flowers and control flowers. Because *M. ciliata* is a primarily outcrossing species (Geber 1985), we expected that insect-pollinated flowers would produce significantly more seeds than control flowers. Second, we tested for differences in seed set among flowers visited by different insect types, including bumblebee vs. solitary bee and the average for bees vs. flies. *Mertensia ciliata* is traditionally described as a bumblebee pollinated species (Pelton 1961; Geber 1985; Suzuki 1994; Gallagher and Campbell 2017), and therefore we expected single bumblebee visits would produce more seeds than visits by solitary bees. Because self-pollinated *M. ciliata* flowers exhibit inbreeding depression in the form of lower seed set and seed mass (Geber 1985), we hypothesized that bee-pollinated flowers (bumblebees, solitary bees, and combination) would produce more seeds than flowers visited by flies. Bees foraging for pollen and nectar are likely to move more out-cross pollen among plants than flies, which stay for extended periods in the same flower or among flowers on the same plant (Forrest et. al 2011; Forrest and Thomson 2011; Bischoff et. al 2013; Gallagher personal obs.). Third, we tested for differences in seed set between open-control and bagged-control flowers.

As a measure of how much a single visit by each pollinator contributes to the reproductive success of the plant relative to open-pollinated plants, we used the expression proposed by Spears (1983):

$$\frac{P_i - Z}{U - Z}$$

To calculate the required parameters, we used the mean number of seeds set / flower of the following populations: single-visit ramets receiving a single visit from species i (P_i), bagged control ramets receiving no visits (Z), and ramets from adjacent natural phenology transects that were open to unrestrained visitation (U).

We tested whether seed mass from single pollinator visits to virgin flowers differed among pollinator types in linear model analysis with visitor identity and site as predictors. Residuals of seed mass were approximately normally distributed (Shapiro-Wilk, $P > 0.05$).

EXPECTED VERSUS OBSERVED POLLINATION SUCCESS

Because higher visitation rates by less effective pollinators may result in the same reproductive output as less frequent visits by more effective pollinators, we estimated the expected rate of successful pollination, both through conspecific pollen receipt and seed set. For this analysis, we summed the product of each pollinator taxon's effectiveness and the mean visit rate of each pollinator taxon for each week. To compare the expected pollination success with observed values from both our phenology manipulation experiment and natural phenology transects, we converted each value to a relative value by dividing the value for a particular week by the highest value observed for any week.

RESULTS

PHENOLOGY MANIPULATION EXPERIMENT

We observed 78 floral visitors to experimental plants during 75 hours of pollinator observations. The pollinator visitation rate varied significantly among phenology weeks ($\chi^2_{3,\,34} = 13.48$, $P = 0.004$). Visitation rate was over five-times higher in week one than in week four (Figure 2.2a). When we included corolla width in the model, we detected a significant interaction between phenology week and corolla width ($\chi^2_{3,\,32} = 40.1$, $P < 0.0001$). During week one, pollinator visitation rates increased with wider corollas, but during weeks three and four pollinator visitation rates decreased with wider corollas. Over time, the composition of pollinators shifted from a diverse array of solitary bees, flies, and bumblebee queens to 100% worker bumblebees by the final week of observations ($\chi^2_{3,\,29} = 7.9$, $P = 0.04$, Figure 2.2b). The percent of pollinators that were bumblebees was not significantly influenced by corolla width ($\chi^2_{1,\,28} = 1.22$, $P = 0.3$).

Conspecific pollen receipt did not differ significantly with phenology week ($F_{3, 31}$ = 2.2, P = 0.1, Figure 2.3a) or with pollinator visitation rate ($F_{1, 31}$ = 0.44, P = 0.5). The amount of conspecific pollen receipt varied widely within and among plants (Figure 2.3a), particularly in the week of June 30th. Heterospecific pollen receipt was higher in week one than in subsequent weeks ($F_{3, 31}$ = 3.96, P = 0.02, Figure 2.3b). There was no significant effect of pollinator visitation rate on heterospecific pollen receipt ($F_{1, 31}$ = 0.01, P = 0.9).

Although seed set of experimental plants did not vary among phenology weeks ($F_{3, 36}$ = 1.01, P = 0.4, Figure 2.4a), the seeds of plants open during week one weighed 0.86 ± 0.3 mg more than those in the last week ($F_{3, 34}$ = 2.9, P = 0.049, Figure 2.5). Neither conspecific nor heterospecific pollen receipt significantly influenced seed set of experimental plants (Conspecific pollen: $F_{1, 30}$ = 0.1, P = 0.8, Heterospecific pollen: $F_{1, 30}$ = 0.12, P = 0.7).

NATURAL PHENOLOGY

In the study area, *M. ciliata* along the natural phenology transects began flowering one week before the first phenology treatment group, and nearly 10 days earlier than the average start date in the area (Miller-Rushing and Inouye 2009). Peak floral abundance was measured on July 9th (during phenology week three, July 7—13), which is about average in the area (July 11 ± 2d between 1973–2006; Miller-Rushing and Inouye 2009). Flowering along the transects extended one week beyond the last phenology treatment group (Natural phenology: June 16th – July 27th, Figure 2.1). Seed set of unmanipulated plants varied significantly among phenology weeks ($F_{4, 14}$ = 10.89, P = 0.0003) but not among transects ($F_{4, 14}$ = 0.09, P = 0.98). Plants in the latter three weeks produced 1.13 ± 0.25 fewer seeds than those in the first two weeks (Figure 2.4b).

We tracked the phenology and floral abundance of 19 species, including *M. ciliata*, that flowered during the phenology manipulation experiment (Figure 2.6). Although species richness remained between 7–9 species each week, only two species, *Vicia americana* and *Potentilla spp*., had flowering phenologies that overlapped completely with that of *M. ciliata* (Figure 2.6, 2.7b). Among species that share pollinators with *M. ciliata,* however, total flower abundance differed significantly among weeks ($\chi^2_{4, 34} = 612.29$, $P < 0.0001$), with peak flowering around July 14th (Figure 2.7).

SINGLE-VISIT POLLINATOR EFFECTIVENESS

Both conspecific and heterospecific pollen receipt in single visits varied significantly across visitor type (Conspecific: Figure 2.8a, $F_{3, 29} = 4.14$, P = 0.01. Heterospecific: Figure 2.8b, $F_{3, 29} = 3.7$, P = 0.02). Pollinator identity was a significant predictor of seed set per flower ($\chi^2_{4, 230} = 169.67$, $P < 0.0001$, Figure 2.8c). Flowers of both bagged and open control ramets also produced seed and did not differ in amount (Z = 1.56, $P = 0.1$), but *a priori* contrasts indicate that seed set per flower was higher for insect-pollinated flowers than for control flowers (Z = 9.98, $P < 0.0001$). Moreover, while there were no significant differences in seeds per flower between bumblebees and solitary bees (Z = 1.47, $P = 0.14$), bee visits produced 63% more seeds per flower than fly visits (All bees, Z = 4.33, $P < 0.0001$). Relative to open-pollinated plants, a single bumblebee visit contributes 81.5% to the reproductive success of an average flower, whereas a single fly visit contributes only 36.3% (Spears measure). Solitary bees, however, may be even more effective pollinators than bumblebees, contributing 121.3% to the reproductive success of an average flower in a single visit (Spears measure, Solitary bees: N = 6). We found no effect of either corolla measurement on conspecific pollen receipt (Corolla width: $F_{1, 21} = 0.01$, $P = 0.9$, Corolla length: $F_{1,21} = 0.81$, $P = 0.3$) or seed set (Corolla width: $\chi^2_{1,72} = 0.28$, $P = 0.6$, Corolla length: $\chi^2_{1,72} = 0.14$, $P = 0.7$). There was no significant difference in seed mass among visitor or control groups (Figure 2.8d, $F_{3,61} = 1.43$, $P = 0.24$).

EXPECTED VERSUS OBSERVED POLLINATION SUCCESS

The relative rate of successful pollination, calculated from each pollinator taxon's effectiveness and per-week visit rate, did not accurately predict the observed levels of pollen receipt, but was useful in predicting relative seed set (Table 2.2). Over the four weeks, pollen receipt was expected to follow a curved relationship with a peak in the week of June 30th (Table 2.2), but the observed pollen receipt showed no significant change and actually had its lowest mean in the middle of the season (Figure 2.3a, Table 2.2). Both expected and observed mean seed set of experimental plants peaked in the week of June 30th and then declined, although not significantly so for the observed data (Figure 2.4a, Table 2.2). Seed set of unmanipulated plants peaked in the week of June 23rd and declined significantly for the rest of the season (Figure 2.4b, Table 2.2). Although expected and observed seed set followed similar trends, expected seeds per flower fell off more sharply for the last two weeks.

DISCUSSION

By manipulating the onset of flowering, we tested the hypothesis that differences in flowering phenology alters pollination and reproductive success through changes in the total pollinator visitation rate and the types of potential pollinators that visit. Over four weeks, we observed a five-fold decrease in pollinator visitation and a change in pollinator assemblage from a diverse array of solitary bees, flies, bumblebee queens to 100% worker bumblebees by the final week. Neither receipt of conspecific pollen on stigmas nor seed set varied significantly among treatment weeks. Those data are reconciled by the higher per-visit effectiveness of bumblebees. On a per-visit basis, each bumblebee transferred more conspecific pollen than did a solitary bee or a fly, leading to higher seed set in the case of the comparison with flies. Thus, while the total pollinator visitation rate declined over the season, the proportion of visits by more effective worker bumblebees increased, with the result that differences in flowering phenology had no significant effect on *M. ciliata's* reproductive fitness.

CONSEQUENCES OF PHENOLOGICAL SHIFTS

Although there was no difference in conspecific pollen receipt across the four weeks, heterospecific pollen receipt was higher in week one than in subsequent weeks. This difference in heterospecific pollen receipt suggests that pollinators early in the season may have lower floral fidelity than late-season worker bumblebees. This may be explained by a change in floral neighborhood over the course of the season (Figure 2.6). Differences in floral neighborhood abundance can affect both the quantity and the quality of floral visits that plants receive (Feinsinger 1987). Among plants that share pollinators with *M. ciliata*, floral abundance was lowest at the start of the season and peaked around July 14th (Figure 2.7a). Over that same period, the pollinator visitation rate, percent of visits by non-bumblebees, and amount of heterospecific pollen receipt declined (Figure 2.2, 2.3b), perhaps in part due to a change in how much pollinator visitors specialized on *M. ciliata* flowers over the course of the summer. Worker bumblebees tend to specialize more on a specific floral resource compared bumblebee queens (Heinrich 1976). This difference in pollinator behavior between bumblebee queens and workers, may account for the change in heterospecific pollen receipt over the course of the season as the proportion of visits by worker bumblebees increased.

The effectiveness study revealed that bumblebees deposited more heterospecific pollen than either flies or solitary bees, which suggests that the higher quantity of heterospecific pollen receipt early in the season is more likely the result of bumblebee visits than visits from other species. Because the amount of heterospecific pollen receipt declined after the first week, it seems likely that bumblebee visitors became more effective pollinators over time, perhaps because they specialized more on *M. ciliata* flowers as floral abundance increased. While we did not examine whether pollinator effectiveness changes over time, there is some evidence that it can. Rafferty and Ives (2012) found that pollinator effectiveness varied with the onset of flowering, both through changes in the taxonomic composition of pollinators and through changes in single-visit effectiveness within pollinator taxa. Moreover, these changes in effectiveness influenced seed set (Rafferty and Ives 2012). Although differences in heterospecific pollen deposition did not affect seed set in *Mertensia ciliata*, heterospecific pollen deposition impacts seed set in other species (Waser 1978; Bruckman and Campbell 2016), both by physically blocking the receipt of conspecific pollen and by chemically inhibiting conspecific pollen tube growth (Waser and Fugate 1986; Briggs et al. 2016). In other systems, therefore, changes in pollinator effectiveness through differences in heterospecific pollen deposition may be an important mechanism affecting reproductive success across the flowering season.

Deposition of conspecific pollen in a single visit could also, in theory, change across the flowering season. An increase in the per-visit effectiveness of bumblebees would explain why the amount of conspecific pollen receipt did not differ with phenology week (Figure

2.3a), despite a five-fold decrease in pollinator visitation rate (Figure 2.1a). Pollinator effectiveness is generally influenced both by behavioral aspects, such as visit duration and the probability of visiting conspecific flowers, and morphological features, such as body size and tongue length (Beattie 1971; Ivey, Martinez and Wyatt 2003; Sahli and Conner 2007; Theiss, Kephart and Ivey 2007). Although we were unable to test for differences in effectiveness between bumblebee workers and bumblebee queens in this study, it is possible that they would differ in their pollinator effectiveness, either because of a difference in the morphological match between *M. ciliata* flowers and bumblebees of different castes (Suzuki 2004) or because of behavioral differences during foraging bouts. If so, the increase in workers at the expense of queens over the season could have led to changes in per-visit pollinator effectiveness of bumblebees.

A change in the per-visit effectiveness of bumblebees over time is also one possible explanation for why the expected levels of conspecific pollen receipt and seed set dropped off more so than the observed values (Table 2.2). The expected and observed values may also differ because of high variance around the estimates, because single visits can give biased estimates of visit effectiveness for later visits, or because we underestimated the visit rate in week four. The differences between the expected and observed values may not be detectible when considering the error propagation in our estimates of visit rate and single-visit effectiveness. It is possible that if we had a larger sample size and less variance around our estimates we may have found a closer match between our predicted and observed values. There may also have been bias in the estimates if there was a non-linear relationship between the visit number and amount of pollen deposited. Such non-linear, saturating relationships have been reported (Campbell et al. 1994) but so have linear ones

(Galen and Stanton 1989). The visit rate in week four was extremely low and resulted in lower predictions for both pollen receipt and seed set than were observed (Table 2.2). Unless the temporal foraging patterns of pollinators changed over time, it seems unlikely that we underestimated the pollinator visitation rates in week four alone because we made observations for the same number of hours and during the same time periods for all four weeks. While it is possible that we underestimated the actual pollinator visitation rates, the discrepancy between the estimated and observed values could also stem from undetected changes in single-visit effectiveness within pollinator taxa over the course of the season.

Seed set in outcrossing plants may also vary over the course of the flowering season if pollinator effectiveness differs through changes in self pollen deposition. In our system, flies are likely depositing greater rates of self-pollen than either solitary bees or bumblebees because they spend time crawling from flower to flower or sitting in the same flower for long periods. A difference in self-pollen deposition may explain why the seed set resulting from solitary bees was twice that of flies (Figure 2.8a), when both flies and solitary bees deposited similar amounts of conspecific pollen per visit (Figure 2.8c). Measuring pollen tube growth would be one way to assess whether there were differences in the compatibility of pollen deposited by different pollinator taxa.

While the effectiveness, assemblage, and visitation rates of pollinators to *M. ciliata* differed with flowering phenology, the effects on pollen receipt and seed set were limited. *Mertensia ciliata* has only four ovules per flower. Results from the single-visit pollinator effectiveness study indicate that flowers likely require few visits to receive enough pollen to develop all four ovules. On average, however, less than half of the possible seeds per flower were produced, both for plants receiving a single visit (Fig 6c) and for open pollinated plants (Figure 2.4). Seed set in *M. ciliata* increases linearly with water availability (Gallagher and Campbell 2017), and it is possible that the discrepancy between ovule number and seed set may be due to resource limitation rather than to pollen limitation.

PHENOLOGICAL SHIFTS AND CLIMATE CHANGE

We observed that experimental shifts in flowering phenology altered pollinator visitation rates and pollinator community assemblage. But the potential impact that changes in pollination may have had on pollen receipt and seed set were negated by differences in pollinator effectiveness between early and late-season pollinator assemblages. We did not manipulate flowering phenology outside of the natural phenological range for our species. Phenological shifts outside of that window could influence pollen receipt and seed set in different ways than observed here. Differences in flowering time affected both the assemblage and visitation rates of pollinators to the spring-blooming, subalpine herb, *Claytonia lanceolata*, with the result that late-flowering plants experienced lower seed set (Gezon et al. 2016). In contrast, for the prairie wildflowers, *Tradescantia ohiensis* and *Asclepias incarnata,* shifts in flowering phenology altered pollinator effectiveness and visitation rates and resulted in higher seed set in late-flowering plants (Rafferty and Ives 2011; Rafferty and Ives 2012). In our system, multiple visits by less-effective pollinators early in the season resulted in similar plant reproductive success as fewer visits by more-effective pollinators late in the season. As flowering onset advances, the phenological overlap with spring-blooming flowers may increase, and plants may experience fewer visits by less-effective pollinators. If the overlap with spring-blooming flowers does not increase, *M. ciliata* may receive increased visitation by less-effective pollinators as flowering onset advances.

Effects of advanced flowering time on pollination may be exacerbated by changes in environment associated with phenological shifts. In temperate climates, spring flowering plants face trade-offs when flowering is advanced, between the potential reward of enhanced pollination services from early-emerging pollinators and increased risk of damaging spring frosts (Inouye 2008; Gezon et al. 2016). The potential trade-offs of flowering early may be different for summer-blooming plants. In the meadows around RMBL, *M. ciliata* is among the first summer blooming plants to flower (Pleasants 1980, Figure 2.6, 2.7), which means it is likely an important resource for insect pollinators in mid to late June. This may explain why *M. ciliata* in the first phenology treatment week attracted many more pollinators than plants in later phenology weeks (Figure 2.1). While early-flowering plants can reap the benefits of enhanced pollination services, there is also increased risk that low water availability before summer thundershowers may alter their ability to attract pollinators via changes in floral attractants (Gallagher and Campbell 2017). In this study, we maintained the same soil moisture across all treatment weeks, and except for corolla width, the floral traits measured remained consistent among treatments. The result of this change in corolla width, however, was that early-season visitors seemed to prefer plants with wider corollas, whereas late-season visitors preferred narrow corollas. In the southern Rocky Mountains, earlier, longer dry seasons prior to mid-summer thundershowers are expected to become more common over the next century, due to rising temperatures and decreased snow fall (Stewart et al. 2005; Overpeck and Udall 2010; Pederson et al. 2011; Blankinship et al. 2014). Plants that are water-limited are more likely to produce smaller flowers that are less attractive to early-season pollinators (Mal and Lovett-Doust 2005; Caruso 2006; Gallagher and Campbell 2017). Thus, climate change may constrain the success of early-flowering plants through pollinator responses to water-mediated changes in floral traits. The degree to which pollinator responses to changes in floral traits varies with changes in flowering phenology requires investigation.

CONCLUSIONS

In our system, early-flowering plants experienced high visitation rates from a diverse array of pollinator taxa, while late-flowering plants experienced low visitation rates from the more effective worker bumblebees. This compensatory effect underscores how important it is to assess pollinator effectiveness in addition to visitation rate. Pollinator effectiveness differed with flowering phenology, both in terms of the pollinator assemblage and the rates of compatible pollen deposition within pollinator taxa, with the result that there was no net difference in pollen receipt or seed set over time. Finally, differences in conspecific and heterospecific pollen deposition among pollinator taxa and phenology weeks, revealed some of the mechanisms by which changes in flowering phenology influence pollination and reproductive success.

Changes in the temporal overlap between plants and their pollinators associated with environmental change are widely expected to alter plant pollination and reproductive success (Memmott et al. 2007; Hegland et al. 2009; Parsche et al. 2011; Forrest 2015; Rafferty and Ives 2011; Rafferty and Ives 2012; Rafferty, CaraDonna and Bronstein 2015; Gezon et al. 2016). We showed that pollinator visitation and species composition can vary substantially with flowering phenology, even within the natural phenological range of the species. To interpret those results, we found it is necessary to know the effectiveness of each type of pollinator on a per-visit basis, both in terms of conspecific and heterospecific pollen deposition as well as seeds produced. The ultimate consequences of phenological shifts for plant reproductive success will depend on several other factors, including the extent to which plant responses to environmental changes influences pollination success and whether differences in pollination success among phenology weeks extend to affect seed germination and recruitment.

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Table 2.1 Final model components for analyses testing the extent to which experimental variation in flowering time affects pollination and plant fitness in *Mertensia ciliata.*

Response variable	Predictor variable(s) of model analyses
Pollinator visitation rate	Phenology week
	Phenology week + Corolla width + Phenology week × Corolla width
Pollinator type⚹	Phenology week
	Phenology week + Corolla width *
Conspecific pollen receipt	Phenology week
	Phenology week + Pollinator visitation rate *
Heterospecific pollen receipt	Phenology week
	Phenology week + Pollinator visitation rate *
Seed set	Phenology week
	Phenology week + Conspecific pollen receipt + Heterospecific pollen receipt*
Seed mass	Phenology week
	Phenology week + Conspecific pollen receipt + Heterospecific pollen receipt*

* Non-significant interaction term(s) removed

⚹Percent of visitors that were bumblebees

Table 2.2 Relative expected and observed effects of variation in *Mertensia ciliata* flowering phenology on (a) conspecific pollen receipt and (b) seed set per flower. Observed seed set per flower calculated for both experimental and unmanipulated *M. ciliata* plants. Expected seed-set and pollen values are based on the summed product of each pollinator taxon's effectiveness and the mean visit rate of each pollinator taxon for each week. To compare the expected pollination and reproductive success with observed values, relative values were calculated by dividing the value for a particular week by the highest value observed for that week.

(a) Pollen receipt			
Week	Expected	Observed	
June 23	0.58	0.9	
June 30	1	0.74	
July 7	0.65	0.67	
July 14	0.21	1	

(b) Seeds per flower			
Week	Expected	Observed	
		Experimental	Unmanipulated
June 23	0.72	0.85	1
June 30	1	1	0.62
July 7	0.3	0.91	0.61
July 14	0.11	0.69	0.39

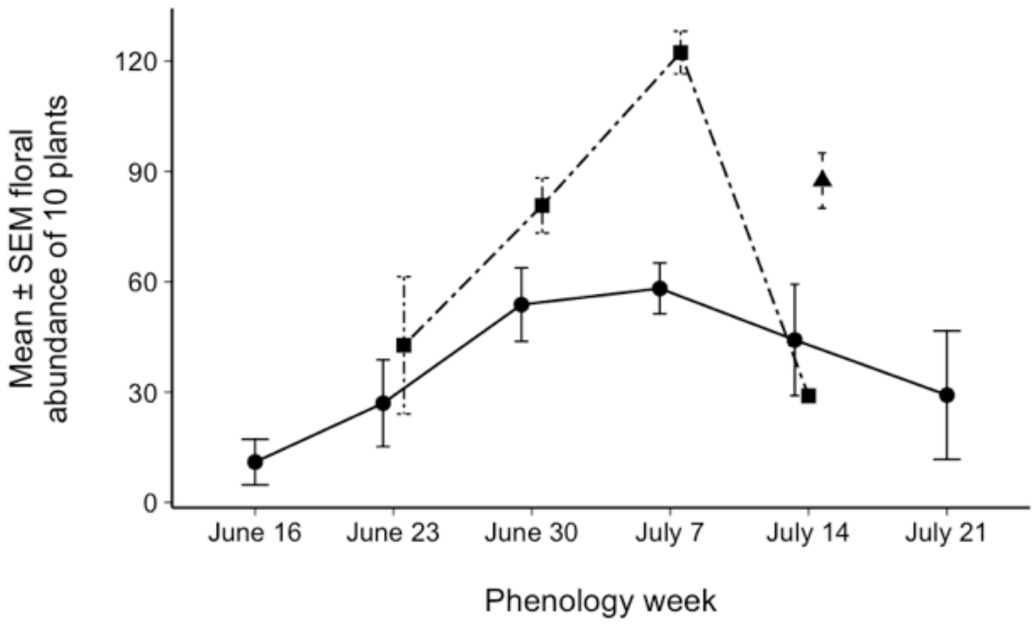
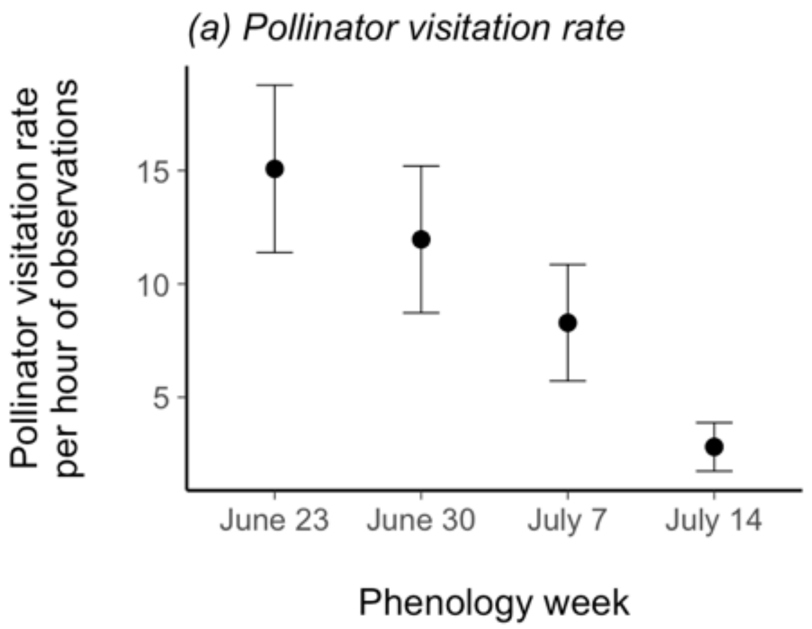


Figure 2.1 Mean ± SEM for floral abundance of ten *Mertensia ciliata* plants per week during the summer of 2015; calculated for experimental plants (squares), experimental plants with additional flowers in floral picks (triangle), and unmanipulated plants (circles).


(a) Pollinator visitation rate

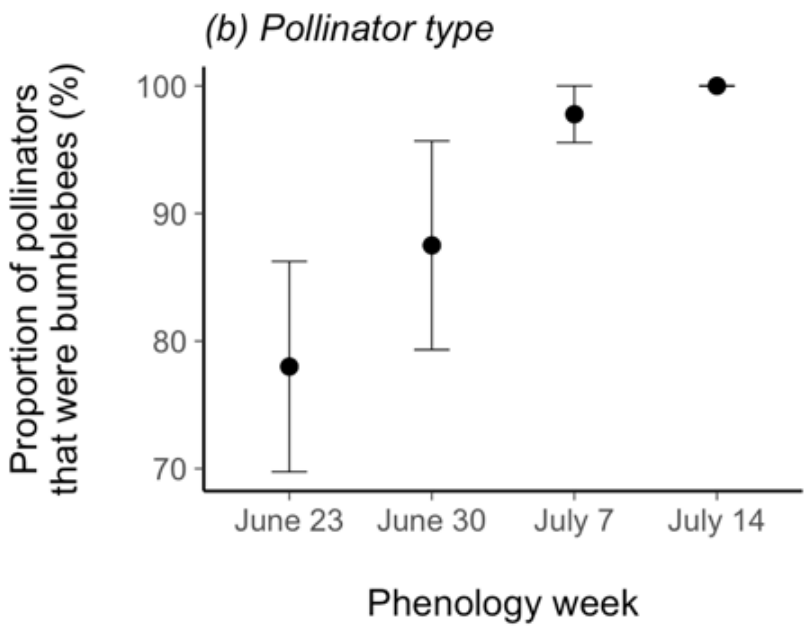
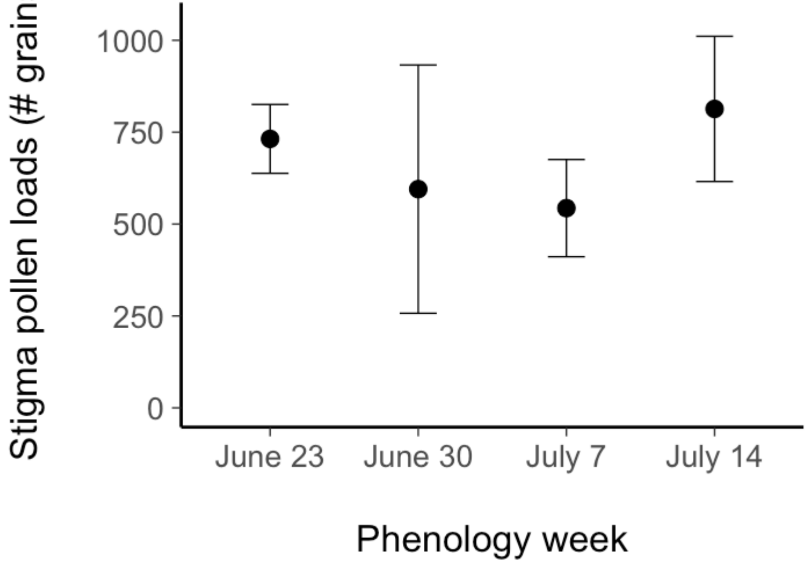
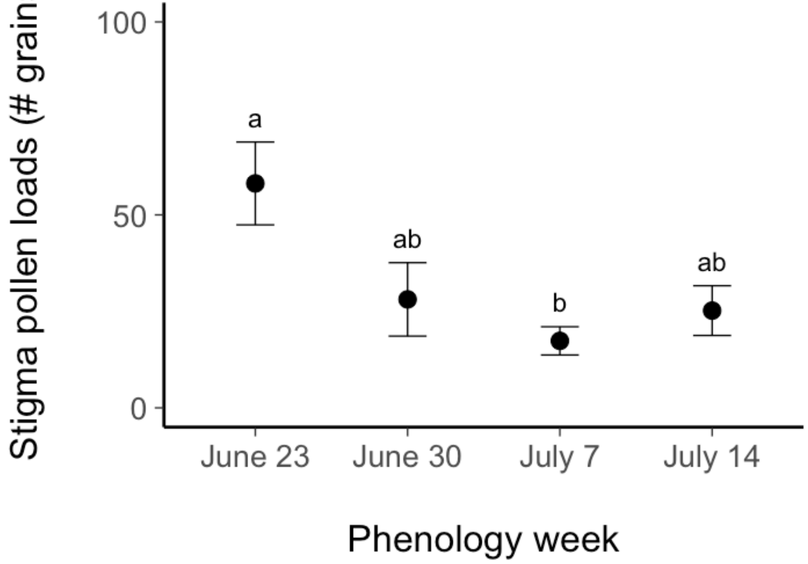

(b) Pollinator type

Figure 2.2 Effects of variation in *Mertensia ciliata* flowering phenology on (a) mean pollinator visitation rate per plant, calculated as (total number of flowers visited / number of flowers available per hour of observation) averaged across the phenology week, and (b) pollinator type, defined as the mean percent of visitors that were bumblebees per plant, and calculated as (100% × number of flower visitors that were bumblebees) / (total number of flower visitors) averaged across the phenology week. Mean ± SEM for each phenology week were calculated from averages of ten plants per week (N = 40).



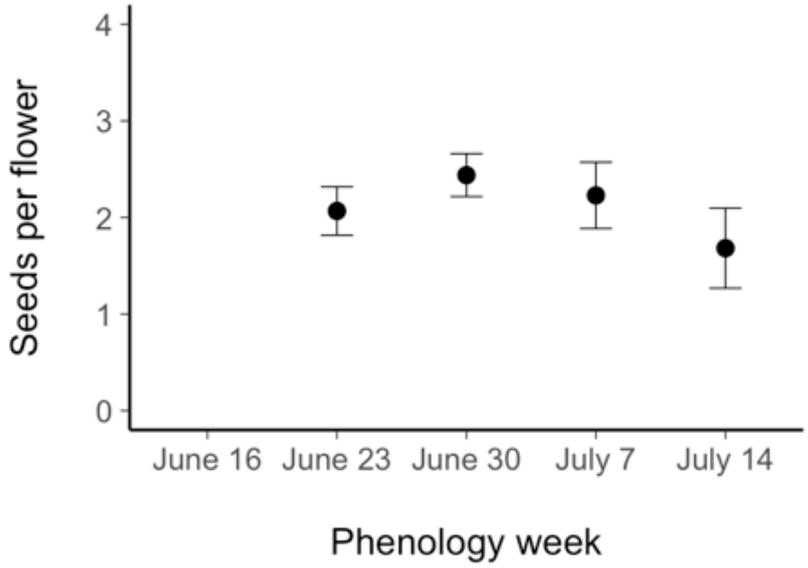
(a) Conspecific pollen



(b) Heterospecific pollen

Figure 2.3 Effects of variation in flowering phenology on pollen loads of (a) conspecific and (b) heterospecific pollen on *Mertensia ciliata* stigmas. Mean ± SEM were calculated from the average number of pollen grains deposited on 5.2 ± 0.7 stigmas per plant, for ≤ 10 plants per week (N = 36). Means with the same letter were not significantly different based on Tukey's HSD pairwise comparisons (P < 0 05).

(a) Experimental plants



(b) Unmanipulated plants

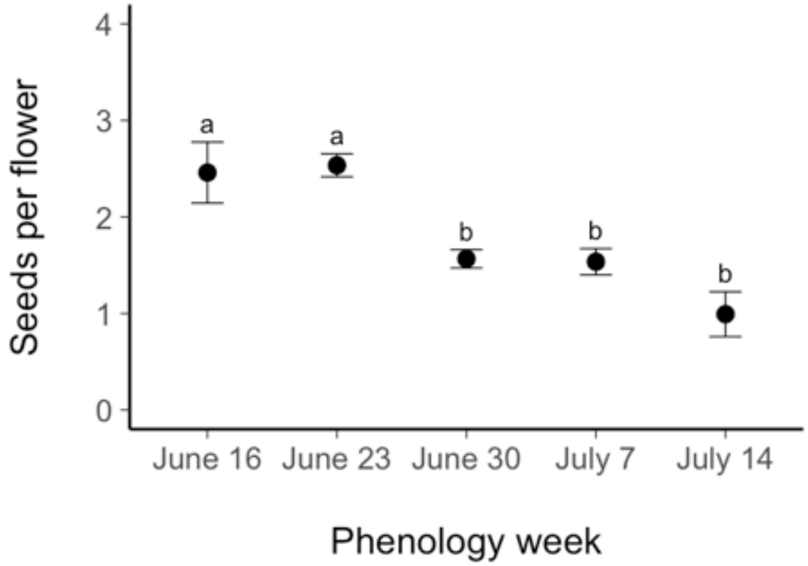


Figure 2.4 Effects of variation in *Mertensia ciliata* flowering phenology on seed set of (a) experimental plants and (b) unmanipulated plants, calculated as (number of mature seeds per plant / number of flowers per plant). Mean ± SEM of experimental plants were calculated from seed set of ten plants per week (N = 40). Mean ± SEM of unmanipulated plants were calculated from the average number of seeds produced per week by ten plants in each of five transects (N = 25). For unmanipulated plants, means with the same letter were not significantly different based on Tukey’s HSD pairwise comparisons (P < 0 05).

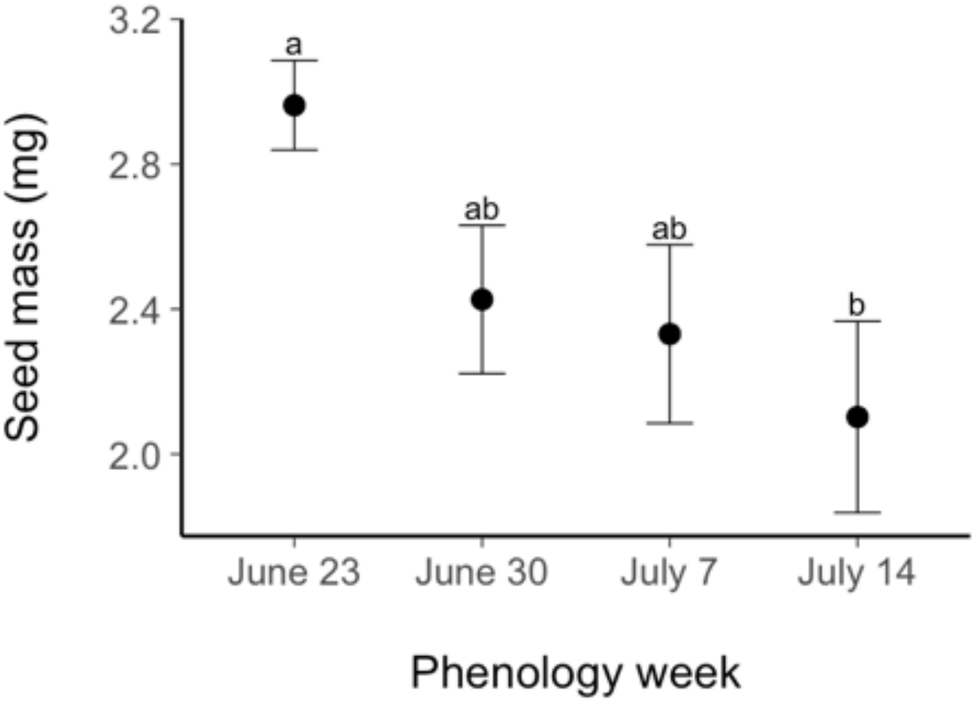


Figure 2.5 Effects of variation in *Mertensia ciliata* flowering phenology on mean seed mass, calculated for each plant as (mass of collected seeds / number of collected seeds). Mean ± SEM were calculated from seed set of ten plants per week (N = 40). Means with the same letter were not significantly different based on Tukey's HSD pairwise comparisons (P < 0 05).

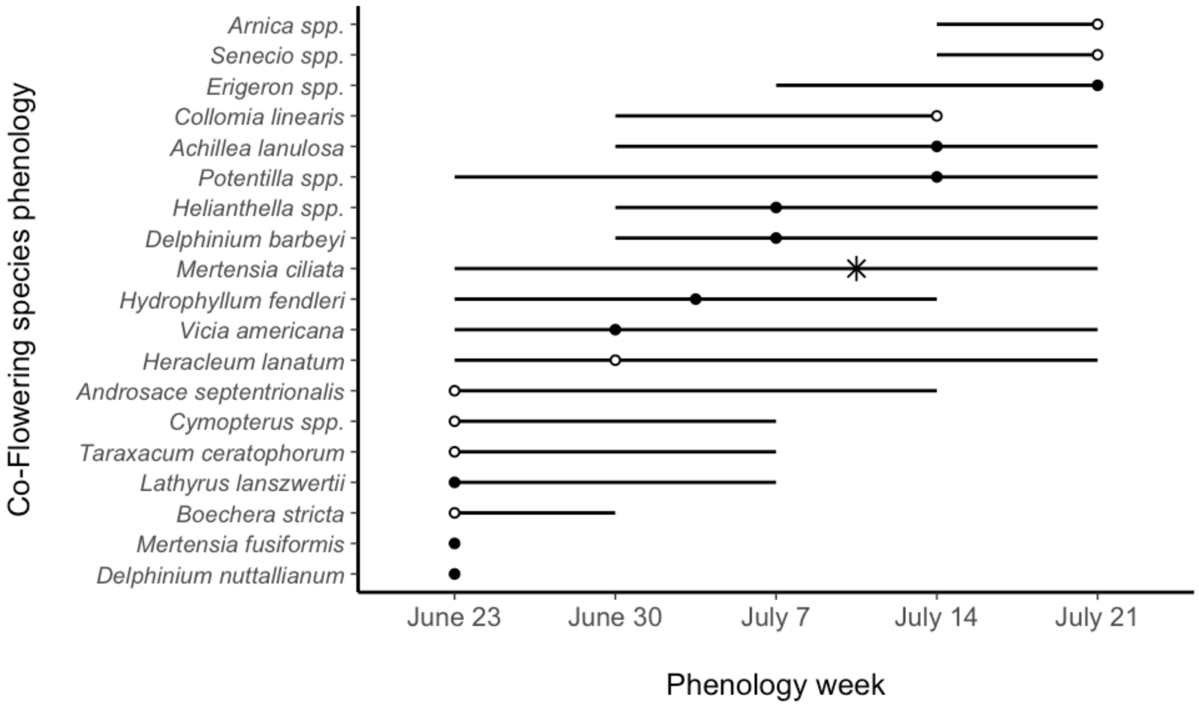
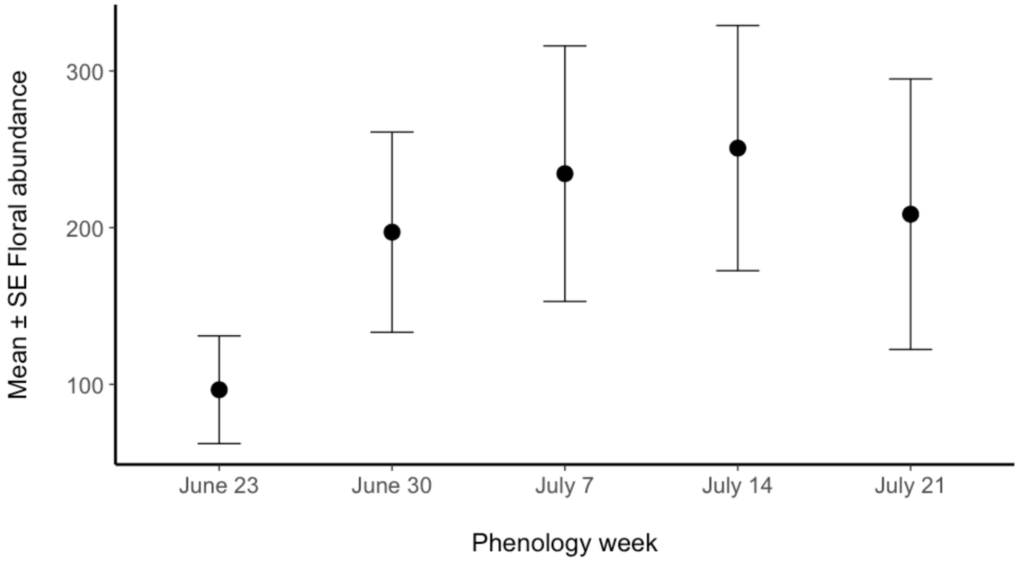


Figure 2.6 Flowering phenology of all co-flowering species along two 50 × 1 meter transects bisecting the experimental arrays. Points mark the week in which the peak number of flowers were observed for each species. In cases where there was no difference in floral abundance between two weeks, the peak is marked in the center between those weeks. Peak *Mertensia ciliata* abundance fell on July 9th and is marked with an asterisk. Peak floral abundance for species that share pollinators with *M. ciliata* are marked with closed circles. Open circles indicate peak flowering for species that are not known to share pollinators with *M. ciliata*.

(a) Total floral abundance



(b) Per species floral abundance

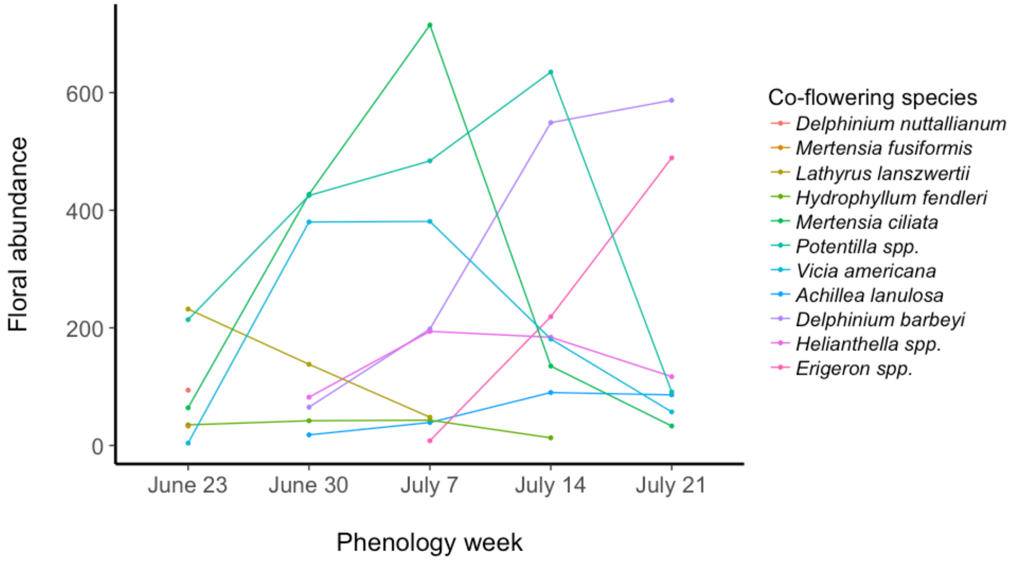


Figure 2.7 Weekly (a) Total and (b) per species floral abundance of co-flowering species found along two 50 × 1 meter transects bisecting the experimental arrays. Only species that share pollinators with *M. ciliata* are included.

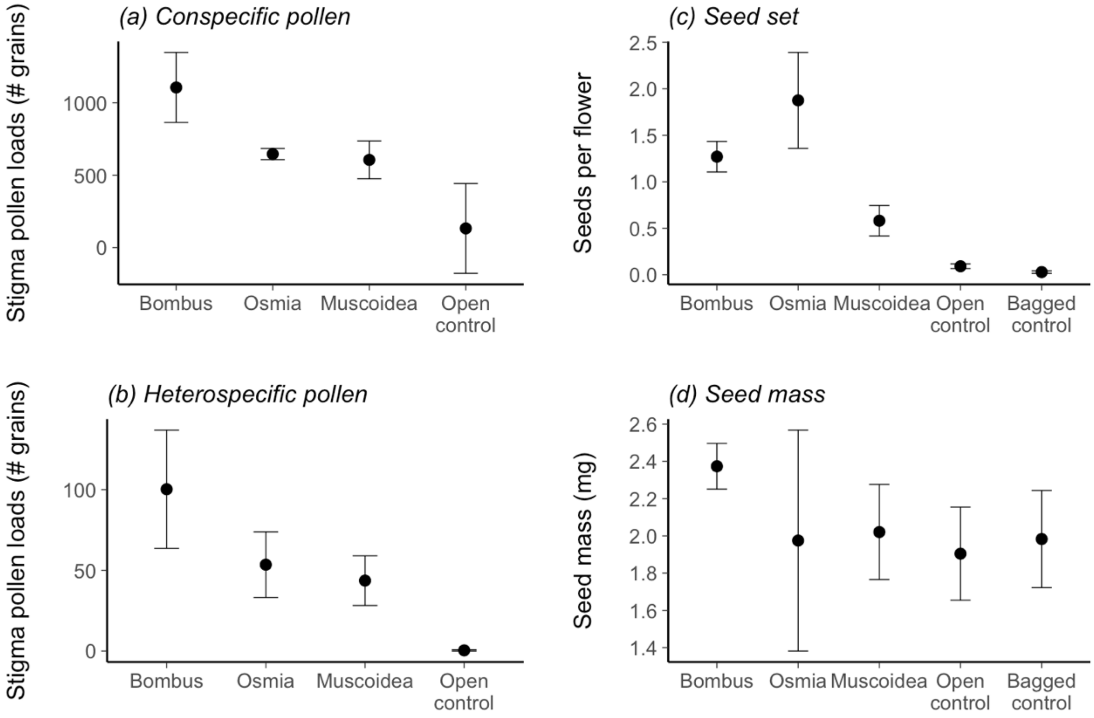


Figure 2.8 Single-visit pollinator effectiveness of different insect visitors on (a) conspecific and (b) heterospecific pollen receipt (N = 33), (c) seed set (N = 232), and (d) seed mass (N = 69) of *Mertensia ciliata* plants. Results from flowers on open and bagged control ramets included when available. Open control ramets were made available to pollinators during observation periods but not observed to be visited, thus serving as a control for missed visits by observers. Bagged control ramets remained bagged throughout the experiment to serve as control for self-pollination. Error bars indicate ± 1 standard error.

CHAPTER 3: EXPERIMENTAL ENVIRONMENTAL CHANGE ALTERS PLANT–POLLINATOR INTERACTIONS AND SEED SET

INTRODUCTION

Plant-pollinator mutualisms are vital to maintaining the functional integrity of most terrestrial ecosystems and to global crop production (Abrol 2012; Aizen et al. 2009). Anthropogenically-driven changes in climate, however, may disrupt these ecologically and economically important relationships (Memmot et al. 2007; Forrest and Miller-Rushing 2010; Cardinale et al. 2012; Jamieson et al. 2012; Winfree 2013). Rising temperatures and changing precipitation patterns have been linked to changes in the timing of key life history events (i.e., phenology) of many plant and pollinator species (Fitter and Fitter 2002; Walther 2003; Parmesan 2006; Marshall et al. 2008; Hegland et al. 2009; Bartomeus et al. 2011). Species-specific phenological shifts among mutualist partners can alter their interactions through changes in temporal overlap between flowering and pollinator activity (Cleland et al. 2007; Memmott et al. 2007; Hegland et al. 2009; Forrest et al. 2010; Aldridge et al. 2011; Bartomeus et al. 2011; Forrest and Thomson 2011). Altered precipitation patterns are also expected to have direct physiological effects on plants and insect pollinators, which can lead to changes in plant-pollinator interactions that are mediated by plant responses to changes in water availability (Gallagher and Campbell 2017). Both changes in phenology and water availability are critical co-occurring responses to environmental change that can alter plant-pollinator interactions.

Independent phenological responses to climate between plants and their pollinators may affect plant reproductive success by altering both the pollinator visitation rate and community of potential pollinators that visit plants (Parsche et al. 2011; Rafferty and Ives

2011; Rafferty and Ives 2012; Gezon et al. 2016). Reduced pollinator visitation rates are predicted to result in insufficient pollen deposition. Changes in the community of pollinators could either increase or decrease pollen quantity, via changes in pollinator effectiveness (Bruckman and Campbell 2014), and pollen quality, via changes in pollinator fidelity. Because plants in the same community may not respond in the same way to environmental changes (CaraDonna et al. 2014), species-level phenological shifts in plant communities may also impact the composition and relative abundances of hetero- and conspecific co-flowerings plants, potentially altering pollinator visitation rates (Lázaro et al. 2009; Lázaro and Totland 2010) and the types of pollinators that visit a plant (Moeller 2005; Mitchell et al. 2009).

Altered precipitation patterns associated with climate change can affect plant-pollinator interactions by altering the capacity of plants to produce a floral display that is attractive to pollinators (e.g., by changing flower number, size, or reward) (Herrera 1995; Galen 2000; Carroll *et al.* 2001; Mal and Lovett-Doust 2005; Caruso 2006; Strauss and Whittall 2006; Burkle and Irwin 2009; Gorden and Adler 2013). This may happen as a response to changes in water availability during the growing season, which can alter the capacity of plants to maintain turgor and transpiration, and also impact their ability to uptake nutrients by affecting plant-microbial interactions and mass flow of nutrients in the soil (Galen et al. 1999; Caruso et al. 2005; Burkle and Irwin 2009; Berdanier and Klein 2011; Barber and Soper Gorden 2014). Changes in water availability may also affect the ability of plants to attract pollinators through changes in the emission and composition of floral volatiles (Burkle and Runyon 2016) and the volume and composition of nectar and pollen rewards (Zimmerman and Pyke 1988; Carroll et al. 2001; Nicolson et al. 2007;

Burkle and Irwin 2009; Halpern et al. 2010; Waser and Price 2016). Floral responses to changes in water availability have been shown to impact pollinator visitation rate, sometimes in non-linear ways (Gallagher and Campbell 2017).

Changes in water availability and flowering phenology are often co-occurring responses to earlier, warmer springs and snowmelt. Thus, it is important to consider if the two processes have interactive effects on plant-pollinator mutualisms. An interaction may occur through a number of mechanisms that depend on the extent to which early and late-season pollinators respond to differences in water-mediated changes in floral traits, and the extent to which plant reproductive success depends on differences in pollen limitation or water resource availability. For example, if flower size and nectar amount have a greater influence on pollinators that dominate late in the season than they have on those that are more common early in the season, then there might be an interaction between flowering time and water availability for the dependent variable of pollinator visitation. If, however, the effects of these mechanisms are additive and drought-mediated changes in floral traits alter pollinator visitation equally through time, then there may be a general decline in pollination and seed set throughout the season when water is limited compared to when water is abundant. Because changes in water availability and flowering phenology are unlikely to occur in isolation, it is important to test the potential interactive effects that these two ecological responses may have on plant-pollinator mutualisms. Such interactions have not yet been investigated through experimental manipulation.

In this study, we asked (1) whether changes in water availability or differences in flowering phenology have larger effects on plant-pollinator interactions and plant reproductive success and (2) the extent to which changes in water availability interact in their effects on pollination and reproductive success with differences in flowering phenology. To answer these questions, we manipulated both flowering onset and soil moisture of the tall-fringed bluebell, *Mertensia ciliata* (Boraginaceae), in a factorial experiment and measured effects on floral traits, pollination, and female reproductive success (i.e., seed set and seed mass).

METHODS

STUDY SYSTEM

Fieldwork was conducted on *Mertensia ciliata* (James ex Torr.) G. Don (Boraginaceae) in a subalpine meadow along Rustler's Gulch in Gunnison National Forest (38°59'32.68'' N, 107°00'23.16'' W; 3,009 m.a.s.l.) located 4.3 km from the Rocky Mountain Biological Laboratory (RMBL) in Gothic, Gunnison County, Colorado, USA. Between 1973-2006, mean spring (April-June) temperatures at RMBL have increased by 2.0 °C, and the average date of spring snowmelt has advanced by nearly two weeks (Miller-Rushing and Inouye 2009). In many subalpine systems, both flowering phenology and summer water availability are largely driven by spring temperatures, snowpack depth, and snowmelt timing (Wielgolaski and Inouye 2013). In the southern Rocky Mountains, over the next century, temperatures are expected to continue to increase, while both winter snow fall and total precipitation are expected to decrease (Stewart et al. 2005; Overpeck and Udall 2010; Pederson et al. 2011), resulting in earlier snow melt timing (Saunders et al. 2008) and earlier, longer dry seasons prior to mid-summer thundershowers (Stewart et al. 2005).

Mertensia ciliata, the tall-fringed bluebell, is an herbaceous, rhizomatous perennial of the subalpine and lower alpine zones of the Rocky and Sierra Nevada Mountains. Plants form compact clones of a few to several hundred flowering ramets and are commonly found along streams and wet meadows (Pelton 1961). The flowers are pendant and tubular, expanding to a wider, lobed mouth, and are borne in dense clusters of cymes along leafy stems. Flowers are typically open for six days, with receptive stigmas throughout flowering and can produce a maximum of four one-seeded nutlets (hereafter seeds).

Pollen is usually removed within 24-48 hours of anther dehiscence by medium and long-tongued bumblebees, including *Bombus balteatus* (Dahlbom), *B. bifarius* (Cresson), *B. flavifrons* (Cresson), and *B. frigidus* (Smith) (Geber 1985; Suzuki 1994; Gallagher and Campbell 2017). *Mertensia ciliata* flowers are also visited by flies (Bombyliidae, Muscoidea, and Syrphidae) and solitary bees (Colletidae: *Colletes p. paniscus Vier.* and Megachilidae: *Osmia spp.*) (Pelton 1961; Gallagher and Campbell 2017). Although *M. ciliata* is self-compatible, seed set is dependent on insect pollination (Geber 1985).

Mertensia ciliata flowers from late June through late July in the subalpine meadows around RMBL. As with many species in this region, *M. ciliata*'s flowering phenology is strongly correlated with the timing of snow melt (Miller-Rushing and Inouye 2009; Wielgolaski and Inouye 2013). In fact, since 1973 the average date of first bloom (DFB) has advanced by more than a week (Miller-Rushing and Inouye 2009). Over the same 34-year period, *M. ciliata* has become less common at lower elevations (≥ 2,900 m a.s.l.) and observed declines in peak floral abundance (15 fewer flowers per decade) correlate with earlier snowmelt timing (1.6 fewer flowers per day earlier snowmelt) (Miller-Rushing and Inouye 2009). In the plant communities surrounding RMBL, phenological responses to warmer spring temperatures and early snowmelt are producing a longer mid-season decline in floral abundance (Aldridge et al. 2011), which may negatively impact pollinator abundance and pollination success of summer-blooming plants like *M. ciliata*. These patterns suggest that changes in spring temperatures and snowmelt timing may alter *M. ciliata* pollination and reproductive success through changes in water availability and flowering phenology.

Previous field experiments with *M. ciliata* revealed that both changes in water availability and changes in flowering phenology can independently affect pollination success in this species. Floral responses to experimental changes in water availability altered pollinator visitation rates, but the effects were non-linear, with visitation peaking at intermediate water levels (Gallagher and Campbell 2017). The timing of flowering phenology also affected the pollination of plants, such that early-flowering plants receive a higher frequency and diversity of pollinator visitors than late-flowering plants. But those pollinators that visited late-flowering plants were more effective pollinators than their early-season counterparts (Gallagher and Campbell, *unpublished data*).

EXPERIMENTAL DESIGN

To test the hypothesis that plant responses to differences in water availability interact in their effects on pollination with those caused by differences in flowering phenology, we manipulated both flowering onset and water availability of potted *M. ciliata* plants. Between 2013 and 2015, 120 plants were collected from a large *M. ciliata* population in Rustlers Gulch and overwintered in the ground at RMBL. In 2016, potted plants were randomly assigned to one of three precipitation treatments, dry, average, and wet. We induced the plants to flower at different times using natural variation in temperature and light found along an elevation gradient in the East River Valley. To inhibit flowering, potted plants were moved to Schofield Pass (39°00'54.98'' N, 107° 2'49.40'' W; 3,263 m.a.s.l.) in early June, where they were placed in a shaded snow bank under a mesh shade-shelter.

Each week, 30 randomly selected plants, 10 per water treatment, were moved back to RMBL, where the higher light and warmer temperatures at low elevation induced them to flower.

Each week, ten flowering plants in each water treatment (30 total) were moved from RMBL to a meadow near the original source population in Rustlers Gulch. Plants were arranged 30 cm apart into five randomized arrays of six plants, with 2 m between arrays. Each array included two plants of each water treatment. In week four, 12 plants stopped flowering mid-week and therefore we re-randomized the plants that had flowers remaining into three new arrays with six plants each. Where appropriate, we calculated the mean values per array of week four plants before and after the plants were rearranged, and then averaged those two values for each plant. To create distinct experimental populations, arrays were located 50 meters away from unmanipulated *M. ciliata* populations. A total of 114 plants flowered and were included in the experiment, for a total of four phenology treatment groups spanning four weeks (June 20 — July 17).

The precipitation manipulations were maintained through the growing season (June 10 – August 1) and discontinued once seeds were collected. We watered pots manually with watering cans slowly and evenly to avoid pooling, in the mid to late afternoon to coincide with the timing of July thundershowers. Throughout the experiment, we measured soil moisture as volumetric water content (VWC) every third day using a 12 cm Campbell Scientific "HydroSense" probe inserted into the center of each pot (always before applying water). We used these VWC measurements to maintain soil moistures within the pots at levels that correspond with VWC levels measured in a previous water manipulation experiment where *M. ciliata* plants within naturally occurring populations received either

50% reduction in precipitation, twice the historic average rainfall during July from 1990 to 2009, or ambient conditions (Campbell and Wendlandt 2013, Gallagher and Campbell 2017). In the end, we maintained the desired soil moisture levels by watering wet pots daily, control pots every other day, and dry pots every third day. Average VWC values for plots were analyzed with a linear mixed model with the main and interactive effects of water treatment and phenology week as fixed effects, and array nested in phenology week as a random effect. The precipitation treatments led to 10.4 ± 0.4%, 12.7 ± 0.4%, and 17.5 ± 0.6% average VWC in the soil for dry, control, and wet pots respectively (Mean ± SEM), creating a gradient in soil moisture that was significant, but did not vary significantly among phenology weeks (Water: χ^2_2= 106.3, $P < 0.0001$, Phenology: χ^2_3= 5.2, $P = 0.2$, Water ×Phenology: χ^2_6= 1.44, $P = 0.96$, Figure 3.1a).

Plants in each phenology group were left open to pollination for one week. During that time, we conducted pollinator observations and tracked pollinator identity and the number of flowers visited during multiple 30-minute observation periods between the hours of 9:00 and 16:00. At the beginning of each observation period, we counted the number of open flowers per potted plant. We calculated mean pollinator visitation rate per plant as (total number of flowers visited) / (number of flowers available per hour of observations) averaged across the phenology week. Visitors were counted as pollinators if they crawled inside the flower corolla. We completed 25 hours of pollinator observations per phenology group, for each round of observations to the five arrays we randomized the order of observations among arrays.

During 100 hours of pollinator observations to six plants at a time, we observed 340 floral visitors to experimental plants. The most common pollinators, bumblebees (*Bombus* *spp.)* and solitary bees (*Osmia spp.)*, accounted for 92.6% of those visits, with flies (Muscoidea and Syrphidae 7.1%) and a moth (0.3%) making up the rest. We excluded the moth from our analyses. For a metric of pollinator type, for each potted plant we calculated mean percent of visitors that were bumblebees as (100% × number of visitors that were bumblebees) / (total number of flower visitors per hour of observation) averaged across the phenology week.

For each phenology week, we measured floral traits, including total abundance of flowers open during the phenology week, corolla size, and nectar volume and sugar concentration. We measured corolla width at the opening of the tube and corolla length from the base of the calyx to a randomly chosen corolla lobe for an average of 4.4 ± 0.4 flowers per plant. At the end of each week, individual flowers in each phenology group were labeled and bagged with fine mesh jewelry bags (Uline, Pleasant Prairie, WI, USA) to prevent further pollination and loss of seeds, and to provide a count of the total number of flowers open during that phenology week. For plants with flowers remaining at the end of each week, we measured nectar volume and percent sugar concentration 48 hours after plants were bagged (N = 71). For an average of 2.6 ± 0.2 flowers per plant, we measured nectar volume using 5μl microcapillary tubes (Kearns and Inouye 1993) and percent sugar concentration using a handheld nectar refractometer (Bellingham + Stanley Ltd., Basingstoke, Hants, UK). No flowers remained to for nectar measurements in week four, therefore we only include nectar data from phenology weeks 1-3 in our analyses. For each floral trait, we calculated the mean trait value of each potted plant, to be used as the response variables in our analyses.

All plants remained in the field until seeds were collected to standardize conditions after pollination exposure. We counted the total number of seeds produced per marked flower (as described by Forrest and Thomson 2010). We calculated the average seeds per flower for each potted plant as (number of mature seeds / number of flowers). Mature seeds from tagged flowers were collected in coin envelopes and transported to the University of California, Irvine to be weighed. We calculated mean seed mass for each plant as (mass of collected seeds / number of collected seeds).

STATISTICAL METHODS

We tested whether experimental changes in flowering phenology and soil moisture had interactive effects on floral traits, pollinator visitation and percent of visitors that were bumblebees (i.e., pollinator type), and seed set. For each response variable, we tested whether the effects of flowering timing varied with changes in water availability using a model with main and interactive effects of water treatment and phenology week as fixed effects, and array nested in phenology week as a random effect.

Residuals of the analyses for flowers per array, corolla width and length, nectar concentration, seed set, and seed mass were all approximately normally distributed, and we used linear mixed model (LMM) analyses to test whether changes in those traits resulting from differences in water availability differed among phenology weeks. We used generalized linear mixed models (GLMM) with a Poisson distribution (log link) to test for main and interactive effects of phenology week and water treatment on floral abundance per plant and nectar volume.

We first attempted to test the effects of phenology week and water treatment on pollinator visitation using zero-inflated and hurdle models due to excess zeroes, but the models failed to converge. We therefore ran separate analyses of the probability that a plant would receive a visit and the visitation rate to plants that received at least one visit. These analyses were performed using Proc Glimmix in SAS (v 9.3; SAS Institute, Inc., Cary, NC, USA). We tested whether the likelihood of receiving a pollinator visit differed among treatments in a GLMM with a binomial distribution and logit link. For plants receiving at least one visit, we tested the effects of water treatment and phenology week on pollinator visitation rate using a log-normal distribution, which provided a better fit than a Poisson, negative binomial, or normal distribution, based on Aikake's information criterion (AIC).

The GLMM model analyses on pollinator type also failed to converge. Consequently, we used the mean percent of visitors that were bumblebees for the two plants in each array that belonged to the same water treatment, as the response variable in our analyses. This averaging eliminated the need to designate array nested in phenology week as a random effect, and allowed us to test the effects of phenology week and water treatment on pollinator type using a model with normally distributed residuals, which provided the best fit as judged by lower AIC.

The floral abundance among arrays during the first three weeks ranged from 15 to 71, with a mean of 44.26 ± 1.9 (Mean ± SEM) flowers per array (Figure 3.1b). The floral abundance per array for week four plants, however, averaged 15 ± 1.3 flowers and was significantly lower than in the previous three weeks ($\chi^2_3 = 29.47$, $P < 0.0001$). We assessed whether these differences in floral abundance influenced pollination by repeating the analyses on pollination with floral abundance per array added to the models. For the GLMM and LMM analyses, probability of receiving a visit and visitation rate to plants receiving at least one visit, floral abundance per array was included as a fixed effect along with main and interactive effects of phenology and water treatment, and array nested in phenology week as a random effect. For the analysis of pollinator type, floral abundance per array was included as a main effect in model with main and interactive effects of phenology and water treatment.

Seed set is expected to be positively correlated with both pollinator visitation rate (Engel and Irwin 2003; Sahli and Conner 2006; Sahli and Conner 2007) and soil moisture (Burkle and Irwin 2009; Berdanier and Klein 2011). To test whether seed set increased with pollinator visitation rate, and whether the influence of pollinator visitation rate on seed set differed among water treatments, we performed a second analysis on seed set, adding mean pollinator visitation rate and the interaction between mean pollinator visitation rate and water treatment to the model. Because the interaction between mean pollinator visitation rate and water treatment was not significant, we reran the model without the interaction to simplify interpretation.

Analyses were conducted using R (R Core Team 2016), except where noted otherwise. We used the *lme4* package in R (Bates et al. 2015) for all GLMM and LMM analyses, and tested the models with type III Wald likelihood ratio tests using the *car* package in R (Fox and Weisberg 2011). We used Proc Glimmix in SAS for our analyses of pollinator visitation (v 9.3; SAS Institute, Inc., Cary, NC, USA). In the event of significant effects, we performed Tukey post hoc comparisons using the *lsmeans* package (Lenth 2016).

RESULTS

EFFECTS ON FLORAL TRAITS

Plants in water addition and control treatments produced flowers that were wider and longer, with more nectar than those in drought treatments (Tukey pairwise comparison, P

< 0.05, Figure 3.2a, b, c). Week four plants also produced flowers that were 1.24 ± 0.01 mm shorter than those in week two (Tukey pairwise comparison, $P < 0.01$), but there were no significant differences in corolla width or nectar volume among phenology weeks, and no significant interaction between the two treatments (Table 3.1, Figure 3.2a, b, c). Neither water nor phenology treatments had significant main or interactive effect on nectar concentration (Table 3.1, Figure 3.2d).

The number of flowers plants produced differed significantly among phenology weeks (Table 3.1). Floral abundance peaked in week two, with plants producing an average of 52% more flowers than those in other weeks. Week four plants produced significantly fewer flowers than those in the other phenology weeks (Tukey pairwise comparisons, $P < 0.001$, Figure 3.3). The effect of water treatment differed significantly among phenology weeks (Table 3.1), such that control plants made 65% more flowers than drought plants in week one, but water addition plants made 33% more than controls and drought plants in week three.

EFFECTS ON POLLINATOR VISITATION

The probability that a plant received a pollinator visit differed among phenology weeks and water treatments, but the effects were additive (Table 3.2, Figure 3.4a). Between week two and week four, the likelihood of receiving a visit decreased by an average of 61% among plants in all water treatments (Figure 3.4a). Across all weeks, plants in the water addition treatments were 11% more likely to receive a visit than plants in the other water treatments (Figure 3.4a). The probability that a plant received a pollinator visit also increased with the number of flowers in the array ($F_{1,86} = 5.8$, $P = 0.02$), but in this model, the effects of phenology week and water treatment were no longer significant ($P > 0.7$).

Moreover, the decline in the probability of receiving a visit after week two (Figure 3.4a) coincided with the decline in floral abundance in the arrays (Figure 3.1b). These data suggest that pollinators were attracted to arrays based on the overall floral abundance, which differed among phenology weeks ($\chi^2{}_3 = 29.47$, $P < 0.0001$).

Once a pollinator visited a plant in the arrays, however, floral abundance no longer had a significant influence on pollinator visitation rates ($F_{1,52} = 0.96$, $P = 0.3$). Among those plants that received visits, both the timing of flowering and water treatment had significant, additive effects on pollinator visitation rates (Table 3.2, Figure 3.4b). Visitation rates decreased by an average of 77% after the first phenology week for plants in all water treatments (Tukey pairwise comparison, $P < 0.001$), and remained consistently low until the final week, when drought treatment plants alone experienced a non-significant resurgence in pollinator visits (Table 3.2). On average, plants in the water addition treatment were visited at a 40% higher rate than those in the control treatment in all four weeks (Tukey pairwise comparison, $P < 0.003$). Visitation to drought plants had higher variance, which meant that differences in visitation between drought and water addition plants could only be detected in week three (Tukey pairwise comparison, $P < 0.006$).

Water availability and flowering time had a significant interactive effect on the types of pollinator visitors (Table 3.1). Over the four phenology weeks, the composition of pollinator visitors generally shifted from a diverse array of solitary bees, flies and bumblebees to 100% bumblebees among all water treatments, except for drought plants which received a resurgence of visits by flies and solitary bees in week four (Table 3.1, Figure 3.4c). The percent of pollinator visitors that were bumblebees was not significantly influenced by the number of flowers available in the array ($\chi^2 = 1.01$, $P = 0.3$).

EFFECTS ON SEED SET

Flowering phenology influenced *Mertensia ciliata* reproductive success through changes in both the number of seeds produced per flower as well as seed mass (Table 3.1). Over four weeks, seed set decreased by an average of 15% (Figure 3.5a). While this decline in seed set coincided with the decline in pollinator visitation rate (Figure 3.4b), the relationship was not significant ($\chi^2 = 3.14$, $P = 0.08$). Seeds from week four plants were 0.54 ± 0.09 mg heavier those produced by plants in the previous two weeks (Tukey pairwise comparisons, $P < 0.05$, Figure 3.5b). Water availability, had a significant, positive effect on both metrics of *Mertensia ciliata* reproductive success (Table 3.1). Plants in the water addition treatments produced an average of 40% more seeds per flower, which were an average of 0.74 ± 0.02 mg heavier, than seeds produced by drought plants (Tukey pairwise comparisons, $P < 0.001$, Figure 3.5). There was no significant interaction between the two treatments for either seed set or seed mass (Table 3.1).

DISCUSSION

Both flowering phenology and water availability influenced pollinator visitation and seed production, but the strength of those effects differed among response variables. Increased water availability had positive effects on both the likelihood and frequency of pollinator visits, while the effect of water treatments on pollinator taxonomic assemblage differed among phenology weeks. The positive effects of water on pollination, however, were largely outweighed by the general decline in visits across the season for all water treatment groups. While seed set declined over the four phenology weeks, both seed set and seed mass showed a strong, positive relationship with water availability across all phenology weeks. These results indicate that changes in water availability can have a much larger effect on plant reproductive success than shifts in flowering phenology. Moreover, our results reveal that changes in water availability interacted with phenological shifts in their effects on pollinator taxonomic composition, but not in their effects on seed set.

EFFECTS ON POLLINATION

Although we successfully manipulated flowering onset, there were differences in floral abundance per plant among phenology weeks. Over the four weeks, floral abundance had a slight positive skew, with peak flowering occurring during week two and a low-abundance tail in week four (Figure 3.1). The effect of water treatment on floral abundance per plant differed among phenology weeks, but there was no discernable pattern, making it unlikely that water availability played a significant role (Figure 3.3). Like many alpine and subalpine plant species (Billings 1974), *M. ciliata* may preform buds or use stored resources to jump-start spring growth. It may be that by delaying the onset of flowering, the plants were forced to use those stored resources or abort buds to survive in the prolonged shade and cold. If this is the case, the decline in floral abundance after week two may have been an artifact of our experimental treatments and may have affected the ability of plants to attract pollinators to the arrays.

The likelihood that a plant would receive a pollinator visit declined over the course of the four phenology weeks, and was influenced by differences in the number of flowers available per array. The positive relationship between the density and diversity of floral resources and flower visitor activity has been well established (Rathcke 1983; Laverty 1992; Johnson et al. 2003; Ghazoul 2006; Feldman 2008; Hegland and Boeke 2006). The decline in visits to the arrays in the last two weeks may have been driven by unintended treatment effects on floral abundance. But, the decline in visits also coincides with an increase in floral abundance and diversity throughout the plant community (Gallagher and Campbell, *unpublished data*). *Mertensia ciliata* is a summer-blooming subalpine perennial. In our study site, however, the onset of flowering in this species begins about a week earlier than most other summer-blooming species (Gallagher and Campbell, *unpublished data*). It is possible that *M. ciliata* receives more frequent pollinator visits early in the season, as we observed (Figure 3.4a, b), because it is one of the few floral resources available at that time. As floral abundance increases in the entire community, pollinators may become more selective when visiting different plant species (Schmitt 1983; Lázaro et al. 2013), which may account not only for the decline in the number of plants that received visits (Figure 3.4a), but also the change in visitation rates and assemblages of pollinators once they were in the arrays (Figure 3.4b, c).

Among plants that received visits, pollinator visitation rates also declined over the course of the flowering season. This change in visit rate, however, was not influenced by the number of flowers in the array, but was generally positively influenced by water availability (Figure 3.4b). This suggests that once pollinators have selected a patch to visit, they may make choices among available flowers based on differences in floral attractants, rather than overall abundance. The finding that water-mediated changes in floral traits can influence pollinator visitation has been previously found in this system (Gallagher and Campbell 2017). Pollinator responses to water-mediated changes in floral attractants are likely to differ depending on the magnitude and the timing of the change in water availability. Both corolla width and nectar production differed among water treatments but not phenology weeks. Under natural conditions, mean floral trait values within *M. ciliata* populations are likely to vary as soil water availability changes over the growing season. In years with early snowmelt, not only would *M. ciliata* plants flower earlier (Miller-Rushing and Inouye 2009), but those early-flowering plants may be more likely to have shorter, smaller corollas with less nectar due to lower water availability in the soil prior to mid-summer thundershowers (Blankinship et al. 2014). Phenotypic plasticity in floral traits associated with differences in water availability has been documented in many flowering plant species (Zimmerman and Pyke 1988; Galen 2000, Carroll et al. 2001; Mal and Lovett-Doust 2005; Caruso 2006; Strauss and Whittall 2006; Nicolson et al. 2007; Burkle and Irwin 2009; Halpern et al. 2010; Waser and Price 2016; Gallagher and Campbell 2017). When snowmelt is early, many plant species within the community may generate smaller, less rewarding flowers. In our study, pollinators had the option to visit flowers of other species that were not water-limited. But if an entire region were to undergo a drought or receive abundant rain, floral traits throughout the community may be affected, which may drive pollinators to make different choices about which plant species to visit.

Our results suggest that a generalist plant, like *M. ciliata*, may be able to attract pollinators of different taxa or castes, both when flowers are small or large. We detected a significant interaction between water availability and phenology on the assemblage of pollinator visitors, with drought plants experiencing a resurgence of visitation in the last week by attracting smaller insects (Figure 3.4c). This result suggests that the effects that changes in flowering phenology have on pollinator assemblage are more likely to vary with changes in water availability for generalist plant species that have a wide variety of potential pollinators. Plants that are serviced by very few pollinator species are expected to be under stronger selection to maintain phenological synchrony with their pollinators

(Rafferty et al. 2015) and should also experience more consistent pollinator-mediated selection on floral traits regardless of water availability.

EFFECTS ON SEED SET

Both seed set and seed mass were positively affected by increased water availability and differed among phenology weeks, with fewer, heavier seeds in week four compared to all other weeks (Figure 4a). This decline in seed set over the four weeks was not explained by the coincident decrease in pollinator visitation rate (Figure 3a, b). In fact, water addition plants made significantly more seeds than drought plants, even when drought plants received more visits than water addition plants. Examinations of pollen receipt from single visits to virgin flowers revealed that flowers likely require very few visits to receive enough pollen to develop all four ovules in each *M. ciliata* flower (Gallagher and Campbell, *Chapter 2*). In a previous study of this system, seed set depended on pollinator visitation only when those rates were low, with seed set leveling off strongly at higher visitation levels (Gallagher and Campbell 2017). Plants in the current study experienced higher pollinator visitation rates than those in our previous study, which may explain why we did not detect an effect of pollinator visitation on seed set. We maintained similar soil moisture levels across treatments in both experiments, which resulted in similar effects on floral attractants. The differences in pollinator visitation rates among years, therefore, can likely be attributed to natural variation in pollinator availability across years and sites.

The decrease in seed set over the four weeks may be explained by differences in pollinator effectiveness, that is the capacity of different pollinators to deposit sufficient, compatible pollen on the stigmas of flowers (Ne'eman et al. 2010). Single-visit pollinator effectiveness studies revealed differences in the amount of pollen deposited and seed set among the various pollinator taxa that visit *M. ciliata.* Bumblebee and solitary bee visitors contribute more per visit to the reproductive success of plants, than do flies (Gallagher and Campbell, Chapter 2). In week one, however, the proportion of total visits by bumblebees, solitary bees, and flies by water addition and drought plants were nearly equal (Figure 3c), but again the seed set of water addition plants was significantly greater. This pattern, further supports the hypothesis that water availability, and not pollinator visitation, has a larger effect on seed set in *M. ciliata*.

It is possible that changes in water availability may have an outsized effect on seed set in this system because plants are not highly pollen-limited. In systems where plants are more pollen-limited, changes in pollinator visitation and pollinator effectiveness can have significant effects on seed set. For example, experimental shifts in flowering phenology of the spring-blooming, subalpine herb, *Claytonia lanceolata*, altered both the assemblage and visitation rates of pollinators with the result that late-flowering plants experienced higher levels of pollen-limitation and lower seed set (Gezon et al. 2016). Thus, the potential for changes in flowering phenology to affect seed set may increase in systems that are more pollen-limited.

Considering climate change

Under global climate change, years with warmer springs and early snowmelt are expected to become more frequent (Pederson et al. 2011) and in subalpine meadow communities, plants are predicted to experience conditions similar to those in the week one, drought treatment combination. Compared to the ‘average’ conditions of week two, control treatment plants, plants in the ‘climate change’ conditions of the week one, drought plants were more likely to receive a pollinator visit, were visited more frequently, and were visited by a greater diversity of pollinator taxa. Despite this increased pollination, neither seed set nor seed mass of 'climate change' plants were greater than that of plants under 'average year' conditions. These data suggest, that for a summer-blooming, generalist perennial, like *M. ciliata*, there may be a significant benefit to flowering early in terms of pollination success, but an increased risk that reproductive success may be affected by changes in water availability, depending on the severity of the drought. Spring-blooming plants have been found to face a similar trade-off when flowering early, between increased pollinator visitation and an increased risk of exposure to late spring storms or frost events (Inouye 2008; Gezon et al. 2016). For both spring and summer-blooming subalpine plants, phenological shifts and changes in the abiotic environment (i.e., water availability or frost events) influenced pollination and reproductive success. Phenological shifts had a greater effect on pollinator visitation rates and pollinator taxonomic composition than did changes in water availability for the summer-blooming *M. ciliata*. But in both cases, effects of phenological shifts on seed set were outweighed by changes in the abiotic environment.

CONCLUSIONS

Climate change may affect plant-pollinator interactions through a variety of mechanisms, including changes in precipitation patterns, temperature, CO_2 levels, and phenology (Hoover et al. 2012; Gornish and Tylianakis 2013; Forrest 2015). Here we considered the simultaneous effects that two of these, potentially co-occurring mechanisms have on the pollination and seed set of an alpine wildflower. We found that changes in water availability interacted with differences in flowering time in their effects on pollinator taxonomic composition, but their effects on pollinator visitation and seed set were additive. Moreover, in our system, the strength of these two mechanisms differed, with phenological shifts having a greater effect on the likelihood and frequency of pollinator visits and water availability having a greater effect on seed set and seed mass. To adequately assess the potential reproductive consequences of climate change, this study illustrates the necessity of examining the relative strength and potential interactive effects of co-occurring mechanisms.

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Table 3.1 Results from analyses testing the effects of experimental variation in phenology and water treatments on floral traits, percent of visitors that were bumblebees (i.e., pollinator type), seed set, and seed mass. Linear mixed models and generalized linear mixed models included main and interactive effects of water treatment and phenology week as fixed effects, and array nested in phenology week as a random effect. Mean percent bumblebee visitors was calculated for the two plants in each array that belonged to the same water treatment, instead of each separate plant, and therefore was analyzed with a linear model including main and interactive effects of water treatment and phenology week as predictors. Models were tested with type III Wald likelihood ratio tests in R, df are given in parentheses following the test statistic (χ^2).

	Phenology Treatment χ^2 (3)		Water Treatment χ^2 (2)		Phenology x Water χ^2 (6)	
Corolla width	3.85		78.69	***	11.84	.
Corolla length	19.01	***	36.84	***	4.55	
Nectar volume ‡	4.35		41.93	***	4.48	
Nectar concentration	3.74		2.60		1.83	
Floral abundance per plant ‡	51.03	***	2.92		119.48	***
Pollinator type	21.76	***	3.02		12.64	*
Seed set	16.11	**	11.31	**	1.87	
Seed mass	18.17	***	18.84	***	1.37	

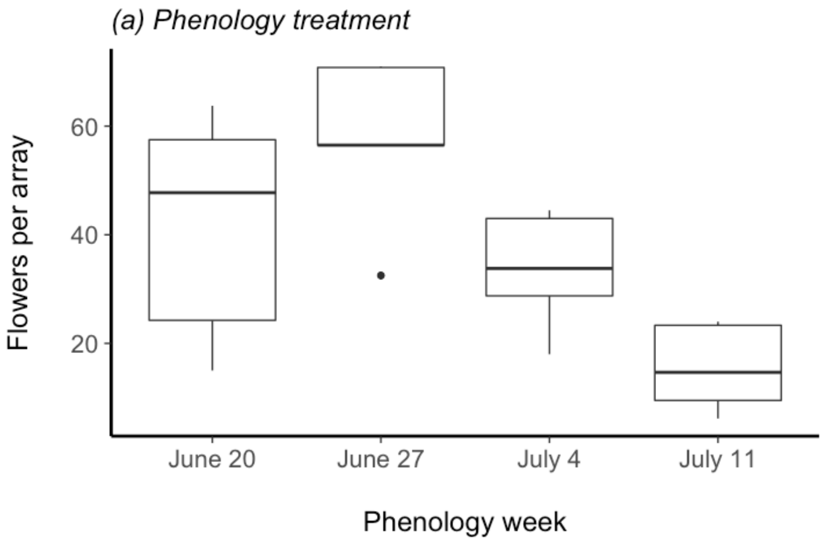
Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
‡ GLMM Poisson distribution

Table 3.2 Results from analyses testing the effects of experimental variation in phenology and water treatments on the likelihood that a plant received a pollinator visit and the pollinator visitation rate to plants that received at least one visit, calculated as (total number of flowers visited / number of flowers available per hour of observation) averaged across the phenology week. Models included main and interactive effects of water treatment and phenology week as fixed effects, and array nested in phenology week as a random effect. Models were tested with Wald type III F ratio tests using Proc Glimmix in SAS.

	Phenology Treatment			Water Treatment			Phenology x Water		
	F	df	*P*	F	df	*P*	F	df	*P*
Likelihood of pollinator visit ‡	63.9	3, 16	<0.001	95.1	2, 87	<0.001	0.5	5, 87	0.8
Pollinator visitation rate ⋆	24.8	3, 15	<0.001	6.1	2, 52	0.004	1.9	6, 52	0.1

‡ Proc Glimmix binomial distribution

⋆ Proc Glimmix log normal distribution



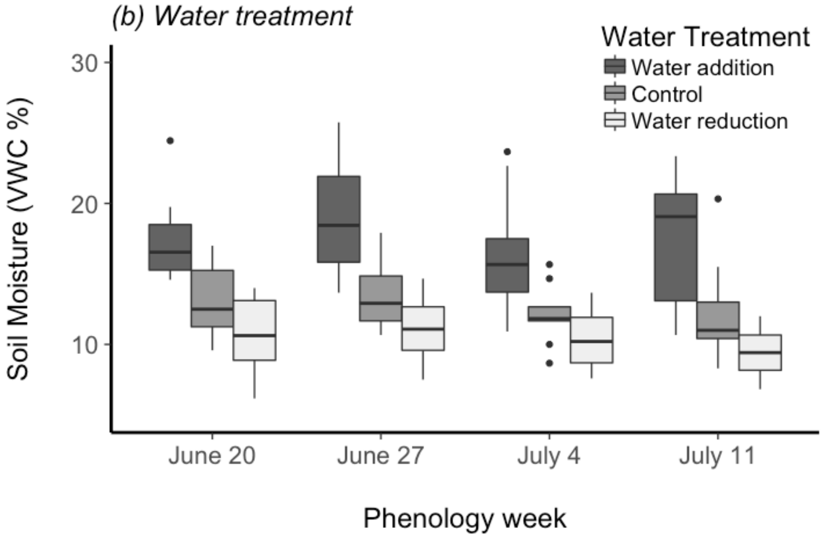


Figure 3.1 Mean (a) soil moisture of three water treatments and (b) floral abundance per array of Mertensia ciliata plants across four phenology weeks (N = 114). Soil moisture measured as volumetric water content (VWC). The center of the boxplot represents the median value, the edges of the box indicate the 25th and 75th percentiles, the whiskers represent the 5th and 95th percentiles of the distributions, and the points indicate outlaying values.

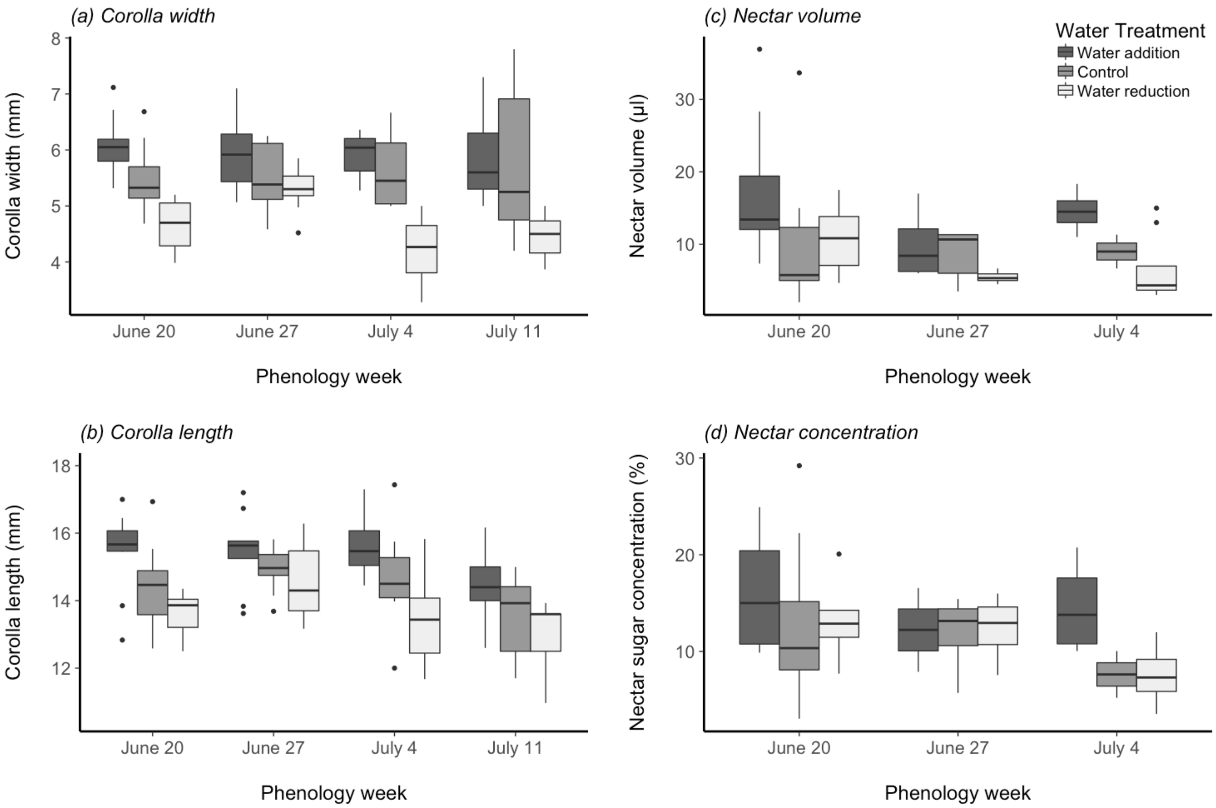


Figure 3.2 Effects of experimental variation in soil moisture and flowering phenology on (a) corolla width, (b) corolla length, (c) nectar volume and (d) nectar sugar concentration of *Mertensia ciliata.* Corolla traits include all four phenology weeks (N = 106), whereas nectar measurements were only collected for three phenology weeks (N = 63). Boxplots follow the same conventions as in Figure 3.1.

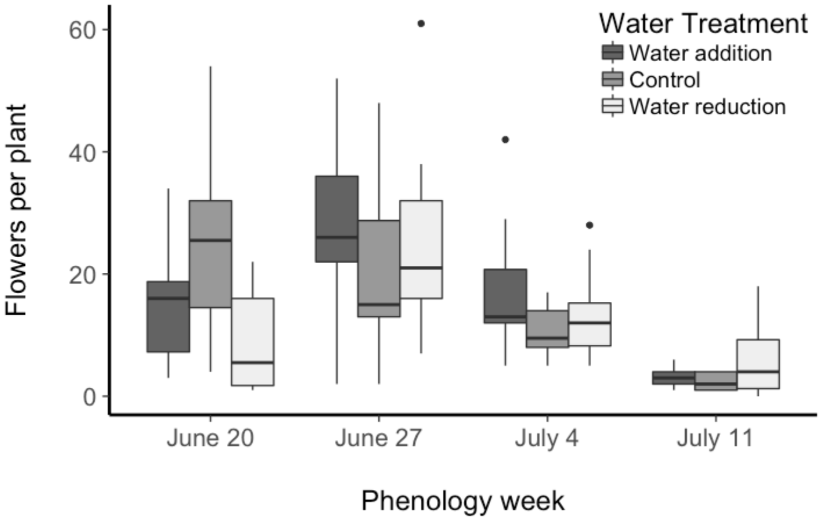
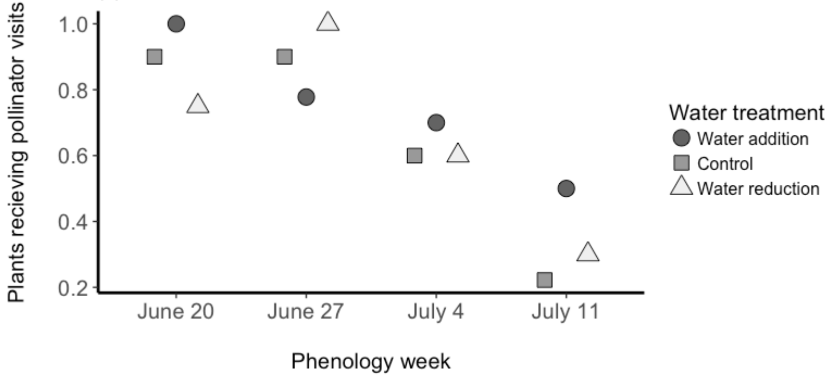
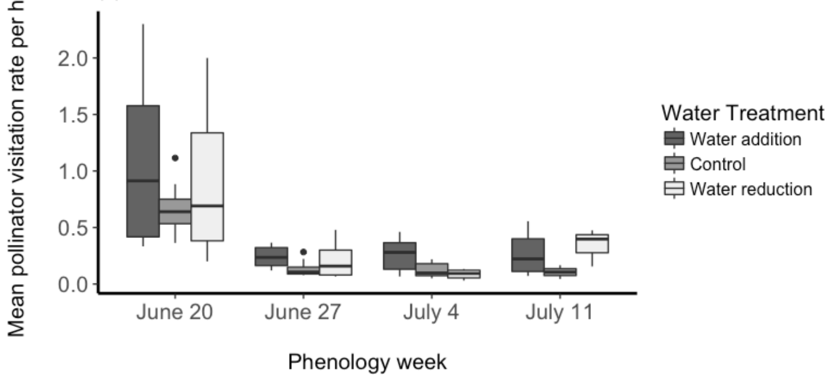


Figure 3.3 Effects of experimental variation in soil moisture and flowering phenology on *Mertensia ciliata* floral abundance per plant (N = 114). Boxplots follow the same conventions as in Figure 3.1.


(a) Likelihood of pollinator visit


(b) Pollinator visitation rate

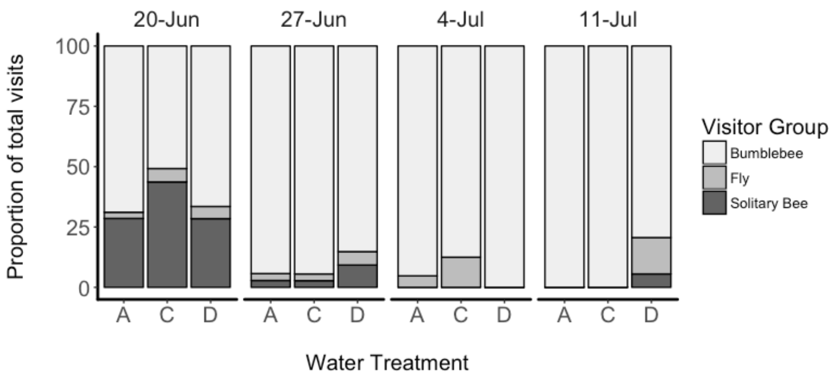
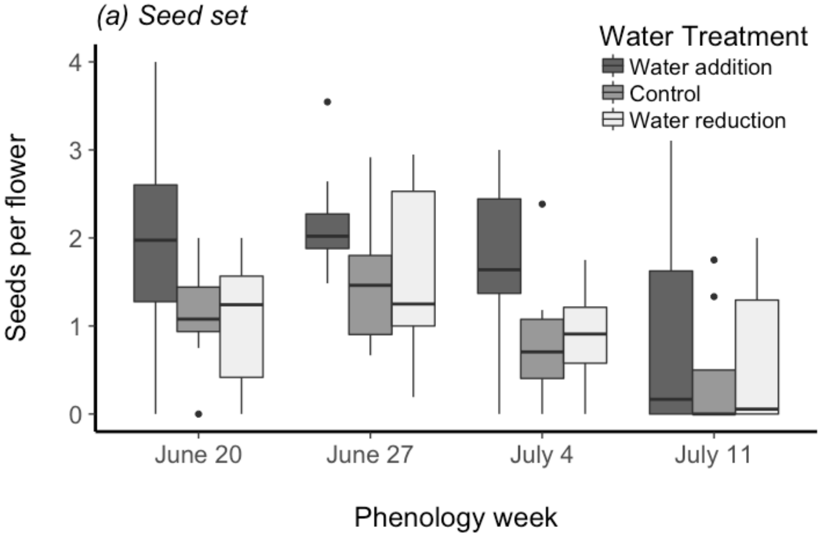

(c) Pollinator type

Figure 3.4 Effects of experimental variation in soil moisture and flowering phenology on (a) the likelihood that a plant received a pollinator visit, (b) the mean pollinator visitation rate per plant among plants that received at least one visit, calculated as (total number of flowers visited / number of flowers available per hour of observation) averaged across the phenology week, and (c) the percent of total pollinator visits that were comprised of bumblebees, flies, and solitary bees, (N = 114). Boxplots follow the same conventions as in Figure 3.1.

(a) Seed set



(b) Seed mass

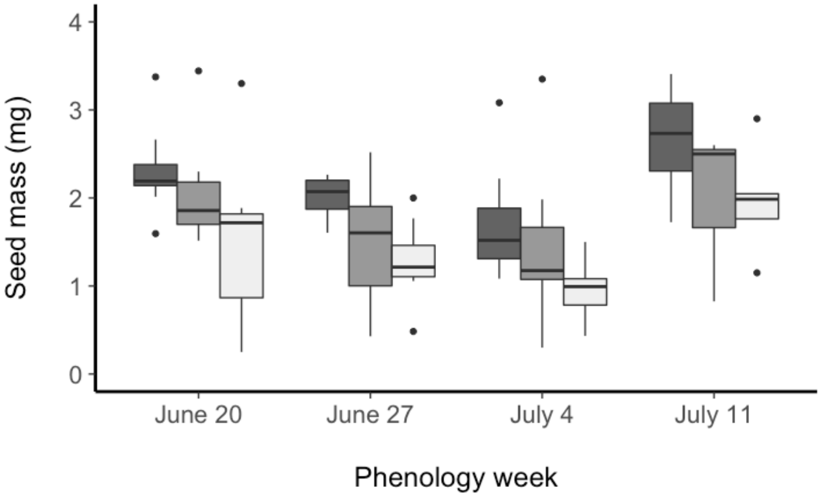


Figure 3.5 Effects of experimental variation in soil moisture and flowering phenology on (a) seeds set per flower, calculated as (number of mature seeds / number of flowers), and (b) seed mass, calculated as (mass of collected seeds / number of collected seeds) (N = 114). Boxplots follow the same conventions as in Figure 3.1.