American Journal of Botany

RESEARCH ARTICL

Pollinator visitation rate and effectiveness vary with flowering phenology

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Manuscript received 18 September 2019; revision accepted 30 December 2019.

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Citation: Gallagher, M. K. and D. R. Campbell. 2020. Pollinator visitation rate and effectiveness vary with flowering phenology. *American Journal of Botany* 107(3): 445–455.

doi:10.1002/ajb2.1439

PREMISE: Flowering time may influence pollination success and seed set through a variety of mechanisms, including seasonal changes in total pollinator visitation or the composition and effectiveness of pollinator visitors.

METHODS: We investigated mechanisms by which changes in flowering phenology influence pollination and reproductive success of *Mertensia ciliata* (Boraginaceae). We manipulated flowering onset of potted plants and assessed the frequency and composition of pollinator visitors, as well as seed set. We tested whether floral visitors differed in their effectiveness as pollinators by measuring pollen receipt and seed set resulting from single visits to virgin flowers.

RESULTS: Despite a five-fold decrease in pollinator visitation over four weeks, we detected no significant difference in seed set among plants blooming at different times. On a per-visit basis, each bumblebee transferred more conspecific pollen than did a solitary bee or a fly. The proportion of visits by bumblebees increased over the season, countering the decrease in visitation rate so that flowering time had little net effect on seed set.

CONCLUSIONS: This work illustrates the need to consider pollinator effectiveness, along with changes in pollinator visitation and species composition to understand the mechanisms by which phenology affects levels of pollination.

KEY WORDS *Bombus*; *Boraginaceae*; *Mertensia*; mutualism; phenology manipulation; pollinator effectiveness; pollinator visitation; species interactions.

The seasonal timing of life-history events is a critical determinant of fitness (Rathcke and Lacey, 1985; Ims, 1990). In plants, differences in the timing of flowering (i.e., flowering phenology) directly affect reproduction and survival by exposing individuals to favorable or unfavorable abiotic conditions (Evans, 1989; Elzinga et al., 2007). In temperate zones, flowering early increases the risk of exposure to late spring storms or frost events (Inouye, 2008; Thomson, 2010), while flowering late can affect resource availability for ripening fruits and seeds (Schemske et al., 1978; Kudo, 2008). The time of flowering can also impact reproduction and survival by altering biotic interactions, such as releasing plants from antagonists or altering the overlap with pollinators (Waser, 1978; Campbell, 1985; Brody, 1997; Lay et al., 2011; Parsche et al., 2011; Rafferty and Ives, 2011; Ehrlén et al., 2015; Gezon et al., 2016). These effects can influence the evolution of flowering time (Munguía-Rosas et al., 2011), and understanding them has taken on additional urgency with global climate change (Fitter and Fitter, 2002; Parmesan and Yohe, 2003; Bartomeus et al., 2011; Thackeray et al., 2016). Species-to-species variation in responses to climate sometimes disrupts interspecies phenological synchrony (Ovaskainen et al., 2013; CaraDonna et al., 2014; Thackeray et al., 2016) and can have cascading effects on other trophic levels (Post et al., 2008; Both et al., 2009; Lany et al., 2015; Mortensen et al., 2016).

Predicting the reproductive consequences of phenology for animal-pollinated plants requires a mechanistic understanding of how differences in flowering phenology affect pollination success. Pollination success requires the receipt of adequate compatible pollen to the stigma, which is influenced by the frequency of pollinator visits and effectiveness of those pollinators in depositing 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). Theoretically, both visitation and pollinator effectiveness could change seasonally because of variation in pollinator abundances or variation in flowering by other plant species,

which might compete for or facilitate pollination (Waser, 1978; Rathcke, 1983; Campbell, 1985; Mitchell et al., 2009; Lázaro and Totland, 2010). Indeed, there is experimental evidence that changes in flowering onset can affect seed set through changes in pollinator visitation rates (Parsche et al., 2011; Gezon et al., 2016). In at least one case, seed set was also altered through changes in pollinator effectiveness associated with compositional changes in pollinator taxa and changes in effectiveness within pollinator taxa over time (Rafferty and Ives, 2012). Although that study was able to predict seasonal changes in seed set from data on effectiveness, it did so for flowers constrained to receive only one pollinator visit. In studies of effectiveness, pollen receipt on stigmas and seed set are not necessarily linearly related to number of visits allowed to the flower (Campbell et al., 1994; Delgado-Carrillo et al., 2018), so it remains unclear how well such predictions will work for flowers receiving natural levels of visitation.

We explored the mechanisms by which changes in one aspect of phenology, the onset of flowering, influences pollination and reproductive success for plants receiving natural levels of visitation. First, we tested the hypothesis that time of flowering onset influences the total number of pollinator visits. Second, we tested the hypothesis that time of flowering onset changes the quality of pollinator visits by altering the frequencies of visits made by pollinators that differ in effectiveness. To test these hypotheses, we conducted two experiments. Controlling the onset of flowering allowed us to determine effects on total pollinator visits and relative frequencies of visits by different species, while eliminating any correlations that might exist in the natural population between flowering time and other plant traits. 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 seed set. Our study is among the first to investigate the role that pollinator visitation rate, pollinator community assembly, and pollinator effectiveness play in determining how flowering time affects 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 in subalpine and lower alpine meadows in the Rocky and Sierra Nevada mountains. Plants form compact clones of a few-to-several hundred stems (Pelton, 1961). The pendant flowers of *M. ciliata* are tubular, expanding to a wider, lobed mouth, and are borne in dense clusters on cymes along leafy stems. The number of flowers per ramet produced over a season averaged 22 (range = 0–70) in our study area in 2015 (also see Fig. 1A). Flowers open successively on cymes and 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 flowers exposed to open pollination set more than 12 times as many seeds as bagged flowers (Geber, 1985). Because about half of pollen received is outcross pollen, and outcross pollen also leads to higher seed set than self-pollen (Geber, 1985), the majority of seed production is due to deposition of outcross pollen by pollinators.

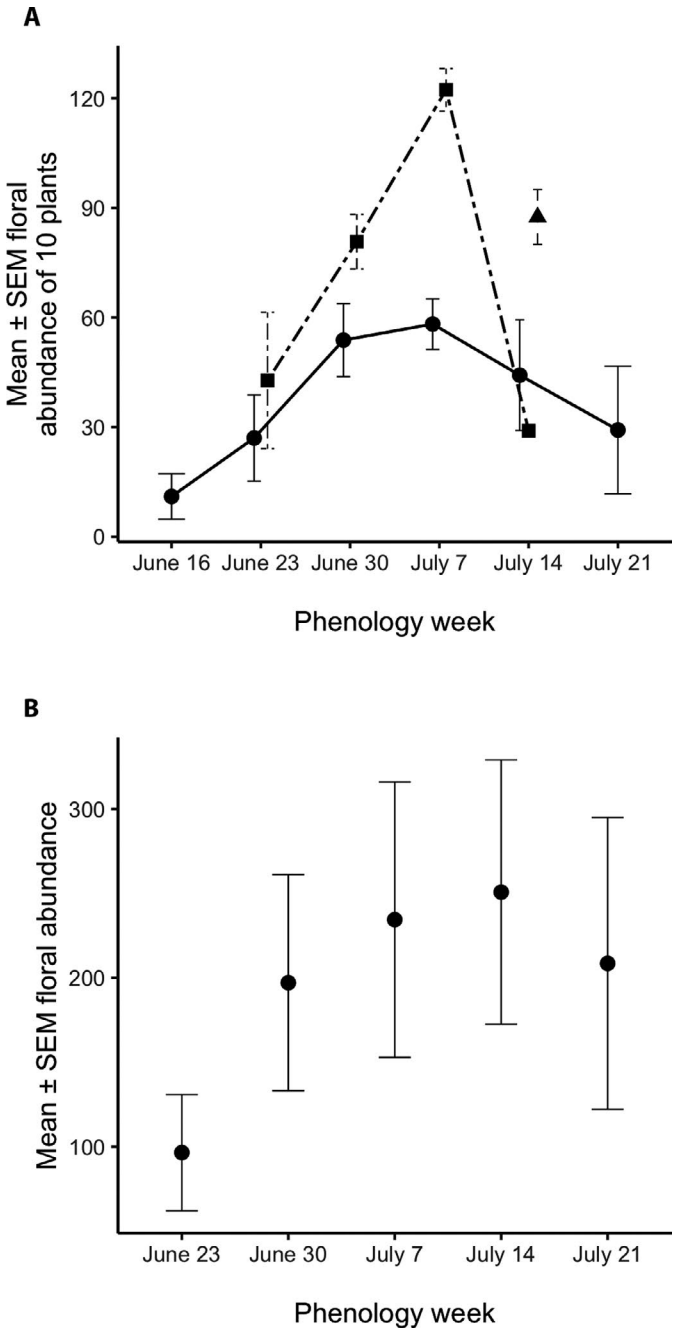

FIGURE 1. (A) Floral abundance of 10 *Mertensia ciliata* plants per week during the summer of 2015; calculated for manipulated plants (squares), manipulated plants with additional flowers in floral picks (triangle), and unmanipulated ramets (circles) (see Appendix S2). (B) Weekly floral abundance of co-flowering species found along two 50 × 1 m^2 transects bisecting flowering onset manipulation experimental arrays (see Appendix S3). Only species that potentially share pollinators with *Mertensia ciliata* are included.

Mertensia ciliata is primarily pollinated by bumblebees, including *Bombus balteatus* (Dahlbom), *B. bifarius* (Cresson), *B. flavifrons* (Cresson), and *B. frigidus* (Smith) (Geber, 1985; Suzuki, 1994; Gallagher and Campbell, 2017). Other 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 conducted in a subalpine meadow along Rustler 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 north of the Rocky Mountain Biological Laboratory (RMBL) in Gothic, 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 north of RMBL. *Mertensia ciliata* is abundant at both sites, with peak flowering occurring approximately two weeks later at Schofield Pass than at Rustler Gulch (Gallagher, unpublished data).

At RMBL (2900 m a.s.l.), *M. ciliata* typically flowers from late June to mid-July, although since 1973, the average date of first bloom has advanced by more than a week, in accord with earlier snowmelt in the spring (Miller-Rushing and Inouye, 2009). In warmer years, phenological shifts produce a longer midseason decline in floral abundance between spring and summer-blooming species (Aldridge et al., 2011), which may negatively affect pollinator abundance and pollination success of summer-blooming plants like *M. ciliata*.

Experiment controlling onset of flowering

To test the hypothesis that flowering time affects pollination success and seed set through the mechanism of seasonal changes in pollinator visitors, we controlled the flowering onset of wild-collected, potted *M. ciliata* plants. After overwintering under snow, randomly chosen subsets of plants were induced to flower at controlled times, placed in the field during their first week of flowering, exposed to pollinators for one week, and subsequently bagged until seed set was collected.

Sixty plants were collected from Rustler Gulch in 2012. These plants were used for a pilot study in 2013, but otherwise remained in trenches and overwintered in the ground under snow at RMBL until their use for this experiment (Appendix S1). In 2015, we induced plants to flower at different times. To inhibit flowering for all plants, potted plants were moved to Schofield Pass in early June and placed in a shaded snowbank under a shade shelter. Each week, 10 randomly selected plants were moved to lower elevation at RMBL, where higher light and warmer temperatures induced them to flower at the experimentally chosen time. This experimental manipulation disassociated any natural correlations between flowering time and other traits or environmental conditions that would have existed in the natural population.

Upon flowering, 10 plants per week were moved from RMBL to a meadow near the original source population. Plants were arranged 30 cm apart into two randomized arrays of five plants, with 2 m between arrays. Arrays were located 50 m away from unmanipulated *M. ciliata* populations. Despite our original intention to manipulate flowering time outside of the normal flowering period, the 40 plants that flowered and could be included in the experiment all did so (June 23–July 20) within the blooming period of the surrounding wild population (Fig. 1A). Naturally occurring, unmanipulated *M. ciliata* populations adjacent to the study area flowered for six weeks (June 16–July 27; Appendix S2). To explore concurrent changes in the surrounding flowering community, we tracked the flowering phenology and floral abundance of all co-flowering species along two 50×1 m^2 transects bisecting the experimental arrays (Appendix S3).

Plants in each phenology week were left open to pollination for one week, and pollinator observations were conducted. We 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, open flowers per potted plant were counted. Visitors were counted as pollinators if they entered the corolla. For each plant, we calculated mean pollinator visitation rate as (total number of flowers visited) / (number of flowers available per hour of observations) averaged across the phenology week and mean percentage 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. The order of observations was randomized between the two arrays for a given week. We completed 15 hours of observations per phenology week, except in week four because of significantly lower flower abundance (Fig. 1, ANOVA with quasi-Poisson distribution, $\chi^2_{3,36} = 30.4$, $P < 0.0001$). To compensate for that difference in floral display, and because no pollinators were observed during the first 15 hours of observations in week four, we increased the number of available flowers by adding cut stems from the source population in florists' water picks to each pot and added 15 new hours of observations. We combined the data from both rounds of observations for week four in our analyses, because it was not possible to ascertain whether a given seed came from a visit in the first or second round. In total, we completed 75 hours of pollinator observation.

At the end of each week, individual flowers in each phenology week were labeled and bagged with fine mesh jewelry bags (Uline, Pleasant Prairie, Wisconsin, USA) to prevent further pollination and loss of seeds. To standardize conditions after pollination exposure, all plants remained in the field until seeds were collected. Seeds were collected once they matured. 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).

To the extent possible, we sought to eliminate differences in floral attractants due to resource variation, thus testing the effect of flowering time alone on 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). Although air temperature and rainfall varied across treatment weeks (Appendix S4), average volumetric water content in the pots was 25.4 ± 0.7% (Mean ± SE, measured weekly with 12 cm "HydroSense" probe, Campbell Scientific, Logan, Utah, USA) and did not differ significantly among phenology weeks (ANOVA, $F_{3,36} = 0.79$, $P = 0.5$). For an average of 4.3 flowers per plant, we measured corolla width, corolla length, nectar volume, and 48 h sugar concentration as described in Gallagher and Campbell, 2017;. Corolla length, nectar volume, and sugar concentration did not differ among phenology weeks (Appendix S5), but corollas in the last week were 1.2 ± 0.1 mm narrower than flowers in previous weeks (ANOVA, $F_{3,36} = 5.72$, $P = 0.003$).

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 ($P \geq 0.05$), 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 et al., 2008).

We tested whether experimental variation in flowering time affects pollination and plant fitness with the following response variables: pollinator visitation rate, pollinator type, seed set, and seed mass. For each response variable, we first looked at the effect of phenology week alone. 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; Galen, 2000; Thompson, 2001; Peat et al., 2005; 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. Initially we used generalized linear models (GLM) with Poisson distributions for the analyses of pollinator visitation rate and pollinator type, however the residual deviances greatly exceeded the residual degrees of freedom indicating over-dispersion; therefore, we repeated the analyses assuming a quasi-Poisson error distribution. 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, $P > 0.05$).

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 wild populations at Rustler Gulch (June 27–July 12) and Schofield Pass (July 12–29). Unopened flowering cymes on individual ramets were bagged with fine mesh jewelry bags 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. The first control group remained bagged throughout the experiment to serve as a control for self-pollination. Nonproduction of seeds by plants in the 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 were not observed to be visited, thus serving as a control for missed visits by observers. Each group of three ramets, including the single-visit ramet and two control ramets, belonged to the same *M. ciliata* clone, which was ensured by examining the base of the stems for connections. No clone was used for more than one group of three, which was ensured by selecting plants that were >2 m apart, the maximum diameter of a clone (Geber, 1985).

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. Plants were only used if they had newly opened flowers, which was determined by the presence of pollen on the anthers. 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 rebagged 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 single-visit ramets, we measured corolla width and length of marked flowers. If marked flowers 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 some cases, multiple flowers were visited on the single-visit ramet. When this happened, we marked the calyces 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 to an open-control ramet was observed, we redesignated that ramet as a single-visit ramet and marked the visited flower(s).

We monitored 235 marked flowers from 95 single-visit ramets, as well as 65 open-control and 74 bagged-control ramets. The most common pollinators, bumblebee queens and workers (*Bombus* spp.) and flies (Muscoidea, hereafter flies), accounted for 97.5% of visits, with solitary bees (all of which were *Osmia* spp. 2.1%) and a syrphid fly (Syrphidae 0.4%) making up the rest. We excluded the syrphid fly from our analyses because the low percentage of them as pollinators was statistically insignificant. 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 15.5 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 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 (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 maturation takes approximately 14–20 days, but developing fruits are clearly visible a few days after pollination (Pelton, 1961; Gallagher, unpublished data). We monitored developing fruits beginning 7–10 days after pollination and collected seeds as they matured. 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 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, after adding 1 if there were any zeros in the data set, 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. The GLM model with quasi-Poisson error distribution

provided a better fit than a zero-inflation model, as judged by a lower value for the Akaike information criterion (AIC). We included visitor identity and site as fixed 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 et al., 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), therefore, we expected single bumblebee visits would produce more seeds than visits by solitary bees. We hypothesized that bee-pollinated flowers (bumblebees, solitary bees, and combination) would produce more seeds than flowers visited by flies, because bees foraging for pollen and nectar are likely to move more outcross 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; Bischoff et al., 2013; Gallagher, personal observation). Finally, we tested for differences in seed set between open-control and bagged-control flowers.

To measure 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 per 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) (Appendix S2).

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 factors. Residuals of seed mass were approximately normally distributed.

Expected versus observed seed set

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 through 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 seed set with observed values from both the phenology manipulation experiment and natural phenology transects (Appendix S2), 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

Experiment controlling onset of flowering

Over four weeks, we observed 78 pollinator visits to plants and these pollinators made a total of 212 visits to flowers. Eighty-eight percent of pollinator visits to plants were bumblebees (*Bombus* spp.). Pollinator visitation rates varied significantly among phenology weeks ($\chi^2_{3,34} = 13.48$, $P = 0.004$), showing a five-fold decrease between week one and week four (Fig. 2A). We also detected a significant interaction between phenology week and corolla width ($\chi^2_{3,32} = 7.98$, $P = 0.046$). 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 (Fig. 2B), although this trend was only significant when we included

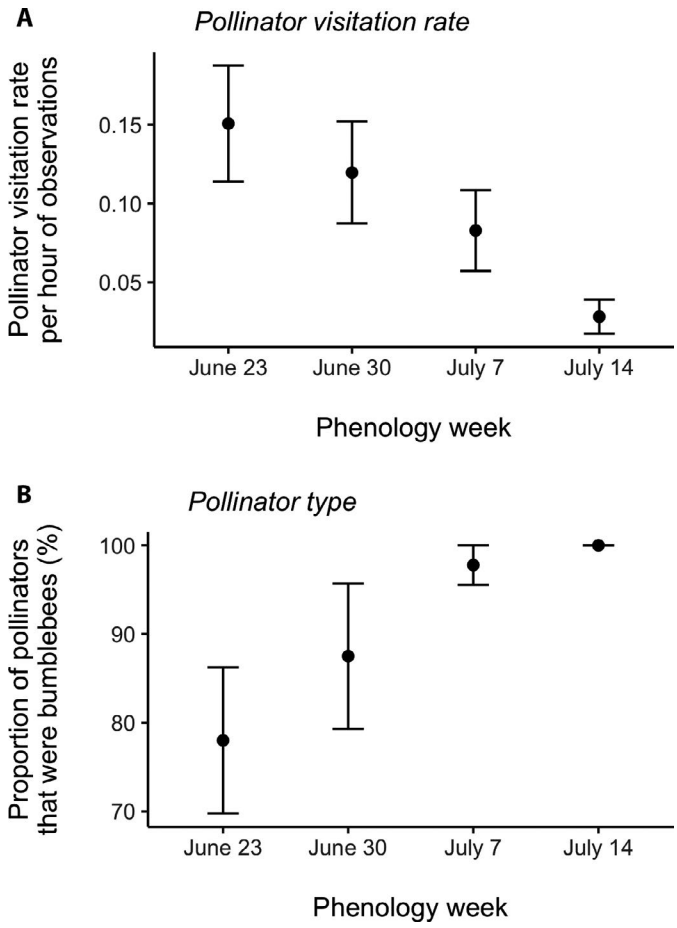

FIGURE 2. Effects of variation in *Mertensia ciliata* flowering time on (A) mean pollinator visitation rate per plant and (B) pollinator type of manipulated plants. Mean ± SEM for each phenology week were calculated from averages of 10 plants per week (N = 40).

corolla width in the model ($\chi^2_{3,28}$ = 7.91, P = 0.048). The percent of pollinators that were bumblebees was not significantly influenced by corolla width ($\chi^2_{1,28}$ = 1.23, P = 0.3). Despite these shifts in pollination, seed set of experimental plants did not vary detectably among phenology weeks (Fig. 3A, $F_{3,36}$ = 0.9, P = 0.5), with plants producing an average of 2.1 ± 0.2 seeds per flower (Mean ± SEM) across the four weeks. Seed mass, however, did vary with phenology ($F_{3,34}$ = 2.9, P = 0.049). Seeds of plants open during week one weighed 0.86 ± 0.3 mg more than those in the last week (Fig. 4).

Species richness of coflowering species remained between 10–14 species each week (Appendix S6). Among species that share pollinators with *M. ciliata,* total flower abundance peaked around July 14 (Appendix S6; Fig. 1B), although the variation among weeks was not significant (ANOVA with quasi-Poisson distribution, $\chi^2_{4,24}$ = 2.9, P = 0.6).

Single-visit pollinator effectiveness

Pollinator identity was a significant predictor of conspecific (Fig. 5A, $F_{3,58}$ = 6.9, P = 0.0005) and heterospecific pollen receipt (Fig. 5B, $F_{3,58}$ = 20.9, P < 0.0001), as well as seed set per flower (Fig. 5C, $\chi^2_{4,230}$ = 169.2, P < 0.0001). Although flowers of both bagged and open-control ramets produced seed, a priori contrasts indicate that seed set per flower was higher for an insect-pollinated flower than for a control flower (Z = –8.44, 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), a bee visit produced 63% more seeds per flower than a fly visit (all bees, Z = 4.33, P < 0.0001). Seed set did not differ significantly between bagged and open-controls (Z = 1.56, P = 0.1). Combined, bagged and open-control ramets produced an average of 0.06 ± 0.01 seeds per flower, confirming that autogamy contributes relatively little to seed set in *M. ciliata* (Geber, 1985).

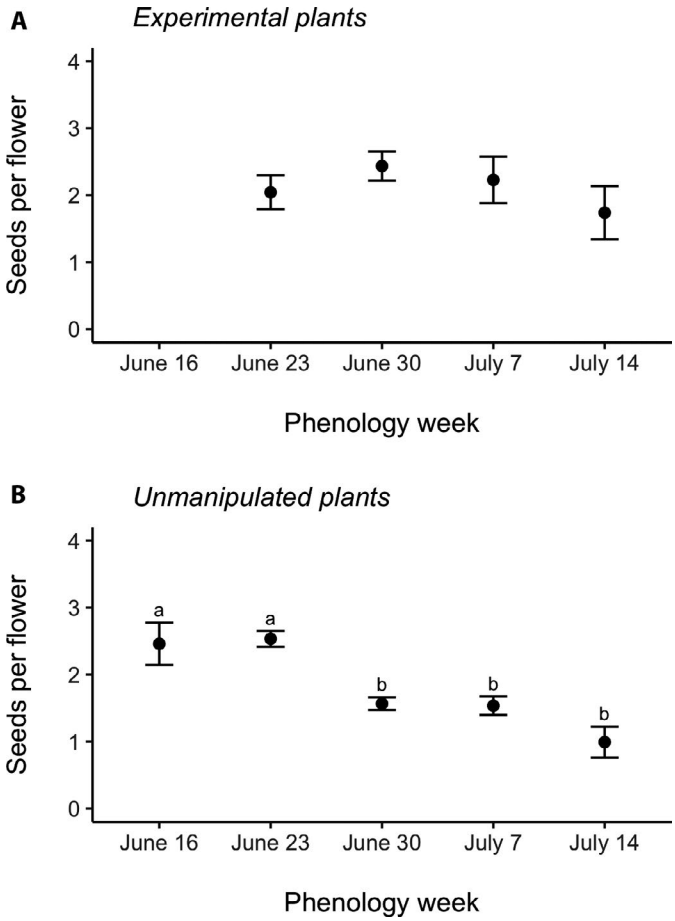

FIGURE 3. Effects of variation in flowering time on *Mertensia ciliata* seed set of (A) manipulated plants and (B) unmanipulated ramets (see Appendix S2). Mean ± SEM of manipulated plants were calculated from seed set of 10 plants per week (N = 40). Mean ± SEM of unmanipulated ramets was calculated from the average number of seeds produced per week by 10 ramets in each of five transects (N = 25; see Appendix S2). For unmanipulated ramets, means with the same letter were not significantly different based on Tukey's HSD pairwise comparisons (P < 0.05).

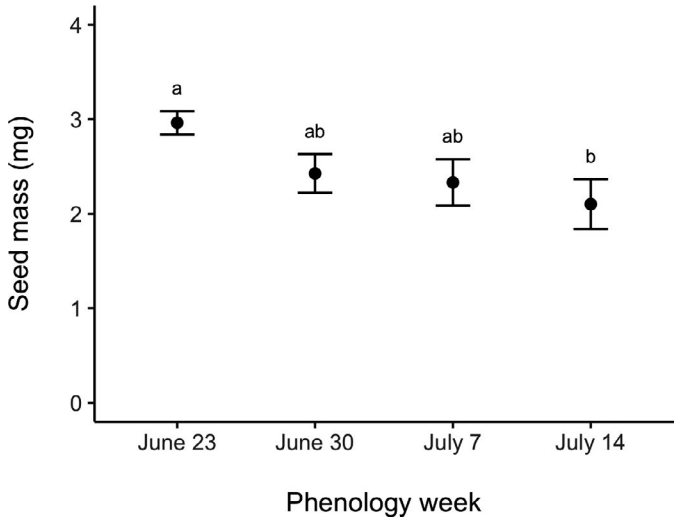

FIGURE 4. Effects of variation in *Mertensia ciliata* flowering time on seed mass of manipulated plants. Mean ± SEM were calculated from seed set of 10 plants per week (N = 40). Means with the same letter were not significantly different based on Tukey's honest significance test (HSD) pairwise comparisons (P < 0.05).

Relative to open-pollinated plants, a single bumblebee visit contributes 81.5% of the average reproductive success of a 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% of the average reproductive success of a flower in a single visit (Spears measure, Solitary bees: N = 6). We found no effect of corolla size on conspecific pollen receipt (Corolla width: $F_{1,39}$ = 0.26, P = 0.6; Corolla length: $F_{1,39}$ = 1.22, P = 0.3) or seed set from a single visit (Corolla width: $\chi^2_{1,74}$ = 0.06, P = 0.8; Corolla length: $\chi^2_{1,74}$ = 0.23, P = 0.6). There was no significant difference in seed mass among visitor or control groups (Fig. 5D, $F_{4,57}$ = 0.93, P = 0.5).

Expected versus observed seed set

Combining visit rate with single-visit pollinator effectiveness was useful in predicting relative seed set (Table 1). Both expected and observed mean seed set of experimental plants peaked in the week of June 30 and then declined, although not significantly for the observed data (Fig. 3A, Table 1). Seed set of unmanipulated plants peaked in the week of June 23 and declined significantly thereafter (Fig. 3B, Table 1). Although expected and observed seed set followed similar trends, expected seeds per flower fell off much more sharply the last two weeks.

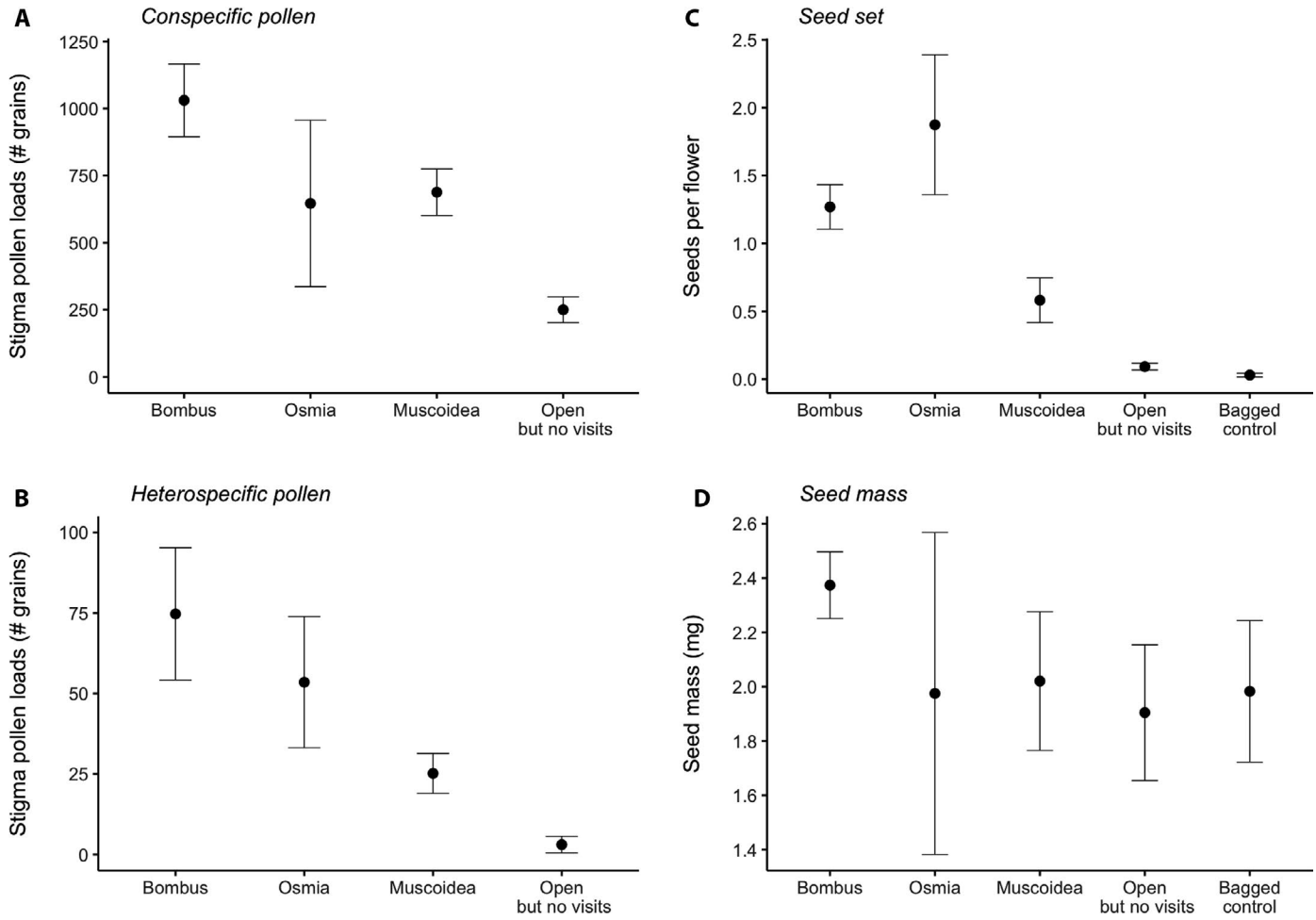

FIGURE 5. Single-visit pollinator effectiveness of different *Mertensia ciliata* visitors in terms of (A) conspecific and (B) heterospecific pollen receipt (N = 62), (C) seed set (N = 232), and (D) seed mass (N = 69). Results of two kinds of control ramets are included when available. "Open but no visit" control ramets were made available to pollinators during observation periods but not observed to be visited, serving as a control for missed visits by observers. Bagged-controls remained bagged throughout the experiment to serve as control for self-pollination. Error bars indicate ± 1 standard error.

TABLE 1. Relative expected and observed effects of variation in flowering time on seed set per flower of *Mertensia ciliata*.

		Observed	
Week	**Expected**	**Experimental**	**Unmanipulated**
June 23	0.72	0.84	1
June 30	1	1	0.62
July 7	0.32	0.91	0.61
July 14	0.05	0.71	0.39

Note: Observed seed set calculated for both experimental and unmanipulated *M. ciliata* plants (Appendix S2). Expected seed set is 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 expected seed set with observed values, relative values were calculated by dividing the value for a particular week by the highest value observed for any week.

DISCUSSION

We manipulated the onset of flowering, and measured the rate of pollinator visitation to those plants and relative frequencies of visits by different species. 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, and bumblebee queens to 100% worker bumblebees by the final week. Seed set of unmanipulated natural plants declined over the season, which reflects a commonly observed pattern that late-blooming flowers on the same plant produce fewer seeds (Austen et al., 2015). Seed set of experimental plants, however, did not vary significantly among treatment weeks. The differences in seasonal patterns for visitation and seed set 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 time had no significant effect on *M. ciliata's* reproductive fitness. These results highlight the need for studies that go beyond examination of pollinator visitation rate to determine the effects of phenology on reproductive success.

Causes of seasonal shifts in pollinator visitation and assemblage

Changes in the frequency and diversity of pollinator visitors over the summer (Fig. 2) were associated with concurrent changes in floral neighborhood (Appendix S6). Floral neighborhood composition and abundance can affect both quantity and 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 14 (Appendix S6; Fig. 1B). Over that same period, both total visitation rate and percent of visits by non-bumblebees declined, either because these early-season visitors switched to other floral resources (or other life-stages) as the season progressed or because bumblebees became more abundant. While it is possible that decline in pollinator visits may have stemmed from an overall seasonal decline in pollinator activity, we think this change is more likely because of concurrent changes in the floral neighborhood. *Mertensia ciliata* is a relatively low-rewarding flower, producing less nectar on average then midseason co-flowering plants like *Delphinium barbeyi* (Wright, 1988). Bumblebees are known to switch their preferred floral resources opportunistically to gain the highest reward (Ogilvie and Forrest, 2017). In the relatively generalized plant-pollinator communities around RMBL, species interactions form and dissolve rapidly across the season (CaraDonna et al., 2017).

Our finding of little change in seed set over the season contrasts with the published results from other systems in which phenology has been manipulated. Late-flowering plants experienced lower seed set in the spring blooming, *Claytonia lanceolata,* (Gezon et al., 2016) and higher seed set in the prairie wildflowers, *Tradescantia ohiensis* and *Asclepias incarnata* (Rafferty and Ives, 2011, 2012). In our system, seed mass dropped over the season, suggesting that there could be fitness effects of phenology mediated through other mechanisms. The change in seed mass could potentially reflect changes in weather conditions, a drop in genetic quality of pollen moved by pollinators, or an artifact of forcing plants to delay flowering. It remains unclear what determines whether phenological shifts will result in positive, negative, or neutral fitness consequences in plant-pollinator systems, and how flexible those interactions may be under climate change. Negative fitness consequences may be more likely in highly specialized systems in which a plant species depends on one or a few pollinators that are not substitutable (Rafferty et al., 2015). Predicting the consequences will presumably be advanced by understanding more about the mechanisms, including a consideration of pollinator effectiveness along with visitation.

Predicting seed set by incorporating pollinator effectiveness

Like many studies in pollination, studies of the impact of phenology on plant reproductive success tend to focus on pollinator visitation (Parsche et al., 2011; Gezon et al., 2016), ignoring the potential for changes in effectiveness of those pollinators. Our study illustrates that it is necessary to incorporate differences in per-visit effectiveness of pollinators to understand seasonal patterns in seed set, specifically why seed set did not differ much with phenology week (Fig. 3A), despite the five-fold monotonic decrease in pollinator visitation rate (Fig. 2A). In our system, a late-season replacement of less-effective pollinators by more-effective ones compensated for the drop in overall visitation.

Incorporating effectiveness, along with visitation rate into calculations, led to a predicted seasonal pattern that was qualitatively similar to the observed pattern in seed set for manipulated plants, with maximum values in the middle of the season. There were, however, quantitative differences because seed set did not drop off late in the season as rapidly as predicted (Table 1). There are at least four potential reasons for this discrepancy: unmeasured differences between some pollinator taxa in effectiveness, seasonal changes in effectiveness for particular insects, uncertainty in our estimates of effectiveness and visitation, and a change in effectiveness with number of visits.

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 et al., 2003; Sahli and Conner, 2007). Although we were unable to test for differences in effectiveness between bumblebee workers and queens, it is possible that they differed in their effectiveness, either because of differences in the morphological match between flowers and bumblebees of different castes (Suzuki, 1994) 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 increases in per-visit pollinator effectiveness of bumblebees that would have dampened the drop-off in seed set.

Second, pollinator effectiveness may have increased over the season because of an increase in outcrossing in this species that exhibits inbreeding depression, or an increase in floral specialization. Worker bumblebees tend to specialize more on specific floral resources compared to bumblebee queens (Heinrich, 1976). As the abundance of *M. ciliata* in the floral neighborhood increased (Fig. 1), worker bumblebees may have specialized more on *M. ciliata* flowers, potentially decreasing handling time and increasing compatible conspecific pollen deposition. Increased specialization might also reduce their usually high heterospecific deposition that otherwise might interfere with seed production (Morales and Traveset, 2008). While we did not examine whether pollinator effectiveness changes over time, there is 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. In generalist-pollinator systems, such changes in pollinator effectiveness across the flowering season may be an important mechanism affecting reproductive success.

A third potential reason for why expected and observed seed set differed is uncertainty in estimates of visitation and pollinator effectiveness. Apparent differences between expected and observed values may not be detectable when considering the error propagation in our estimates of visit rate and single-visit effectiveness. If we had a larger sample size and less variance around our estimates, we might have found a closer match between our predicted and observed values. Moreover, the extremely low visit rate in week four resulted in lower seed set predictions than were observed (Table 1). Week four also had unusually low flower abundance in the potted plants, leading us to supplement them with cut stems. The low flower abundance may have attracted fewer pollinators (Geber, 1985), potentially leading to an underestimate of visitation during week four.

Finally, effectiveness of a pollinator making a second or third visit to a plant may differ from that in a single visit (Galen and Stanton, 1989). Because *Mertensia ciliata* has only four ovules per flower, seed set could asymptote relatively quickly with number of visits, enabling relatively high seed set despite the low visitation rates observed, particularly at the end of the season. Indeed,

the single-visit pollinator effectiveness study indicates that flowers likely require few visits to receive enough pollen to develop all four ovules. In addition, because flowers are typically open for six days, even with the very low visitation rate of 0.03 visits per flower per hour in week four, we would expect an average flower to receive at least four visits in its lifetime. On average, however, both single-visit and open-pollinated plants produced less than half of the possible four seeds per flower even though flowers can receive more than 1000 pollen grains (Figs. 3, 5C). Because seed set in *M. ciliata* increases with water availability (Gallagher and Campbell, 2017), it is possible that discrepancies between ovule number and seed set may be due to resource limitation rather than pollen limitation even though 2015 was a wetter year than average (~17% higher total precipitation in 2015 compared to average total precipitation between 1999–2018, National Atmospheric Deposition Program, 2018).

CONCLUSIONS

This study demonstrates that changes in the time of flowering onset can alter pollination success for plants visited by a broad array of pollinator species, but that those changes do not necessarily correspond with changes in reproductive success. In our system, high visitation rates from a diverse array of pollinator taxa early in the season shifted to low visitation rates from more effective worker bumblebees late in the season. This compensatory effect underscores how important it is to assess pollinator effectiveness in addition to visitation rate, whether studying natural selection on flowering time or evaluating the effect of phenological mismatches associated with environmental change (Memmott et al., 2007; Forrest, 2015; Rafferty et al., 2015).

ACKNOWLEDGMENTS

We thank A. Barth, H. Clements, A. Faidiga, A. Hacker, S. Haque, B. Ottoson, E. Park, W. Recart, A. Siah, P. Sosenski, C.S. Whiting, and C.E. Whiting for assistance with field and laboratory work; and T. Huxman, D. Inouye, J. Martiny, K. Mooney, J. Powers, W. Recart, A. Sakai, S. Weller, and anonymous reviewers for comments on the manuscript. The Botanical Society of America, NSF DEB-1601191, Rocky Mountain Biological Laboratory, and Sigma Xi supported this work.

AUTHOR CONTRIBUTIONS

M.K.G. and D.R.C. designed the research, conducted fieldwork, analyzed the data and wrote the manuscript.

DATA AVAILABILITY

Data available from the Dryad Digital Repository: https://doi.org/10.7280/d19x0d (Gallagher and Campbell, 2019).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Methods for collecting and overwintering *Mertensia ciliata*.

APPENDIX S2. Phenology and abundance of naturally occurring, unmanipulated *Mertensia ciliata*.

APPENDIX S3. Floral neighborhood diversity and abundance.

APPENDIX S4. Weekly (A) mean high (closed circles) and low (open circles) air temperature (°C) and (B) total precipitation (in millimeters) between June 16–July 27, 2015.

APPENDIX S5. Mean ± SEM corolla length and width (N = 40) as well as 48-h nectar volume and percentage sugar concentration (N = 20) of *Mertensia ciliata* plants in each of four experimental phenology treatment groups, calculated from an average of 4.3 ± 0.3 flowers per plant.

APPENDIX S6. Flowering phenology of co-flowering species found along two 50 × 1 m^2 transects bisecting flowering onset manipulation experimental arrays.

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