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Consequences of variation in flowering time within and among individuals of *Mertensia fusiformis* (Boraginaceae), an early spring wildflower[1]

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Climate change is causing many plants to flower earlier in spring, exposing them to novel selection pressures, including—potentially—pollinator shortages. Over 2 years that contrasted in timing of flowering onset, we studied reproductive strategies, pollen limitation, and selection on flowering time in *Mertensia fusiformis*, a self-incompatible, spring-flowering perennial. Plants opened most of their flowers early in the flowering period, especially in 2007, the early year; but selection favored early-flowering individuals only in 2008. However, resource allocation to early vs. late seed production was flexible: In 2008, but not 2007, early flowers on a plant produced more and heavier seeds. Late flowers were capable of equal seed production if fertilization of early ovules was prevented, suggesting that late flowers serve a bet-hedging function. Evidence for pollen limitation was weak, although there was a tendency for early flowers to be pollen-limited in 2007 and for late flowers to be pollen-limited in 2008. Poor reproductive success in 2007 was likely attributable less to pollen limitation than to frost damage to flowers. We suggest that plasticity in floral longevity and resource allocation among flowers will make this species resilient to short-term pollinator deficits; whether this will help or hinder future adaptation is unclear.

Key words: Boraginaceae; climate change; floral longevity; flowering phenology; *Mertensia fusiformis*; phenotypic selection analysis; pollen limitation; pollination; resource allocation; Rocky Mountain Biological Laboratory; skewness.

Climate change has given a new urgency to the need to understand how flowering phenology affects plant reproductive success and the ability of populations to adaptively adjust flowering time in response to changing conditions (Elzinga et al., 2007). Timing of flowering has long been a popular research topic because it is such an important determinant of fitness in plants (e.g., Rathcke and Lacey, 1985; Pilson, 2000; Sandring et al., 2007): Within a growing season, an individual's flowering period must coincide with favorable climatic conditions, flowering of conspecifics, and availability of abiotic resources and mutualist partners. As global temperatures rise, the windows of climatic suitability, resource availability, and presence of interaction partners will likely all shift—though not necessarily in concert. Climate change has already altered flowering phenology of many species (Parmesan, 2006; Rosenzweig et al., 2007), presumably often because of plastic responses to changing environmental cues (although evolutionary change in flowering schedules has also been documented [Franks et al., 2007; Franks and Weis, 2008]). However, we still know little about the consequences of these changes in flowering phenology for plant populations. For instance, will plastic shifts to earlier flowering lead to reduced outcrossing or seed set, and, if so, will selection begin to favor later-flowering individuals?

In temperate habitats, plants that flower early in spring have always faced particular challenges associated with a harsh and unpredictable environment. Possible benefits of spring flowering include high levels of light and moisture and low levels of interspecific competition for pollinators. On the other hand, weather can be cold or erratic, and this might limit seed production directly by damaging floral tissue, or indirectly by reducing pollinator activity (Schemske et al., 1978; Kudo et al., 2008). Climate change may exacerbate these problems. For example, early snowmelt in recent years has been associated with increased frost damage to developing flowers of some subalpine plants—and a consequent reduction in recruitment (Inouye, 2008). Frost damage occurs because timing of plant development advances in response to earlier melt, but frost frequency does not necessarily decline along with the increase in mean temperatures (Hänninen, 1991; Inouye, 2008). Similarly, early melt and correspondingly early flowering might not be accompanied by early pollinator emergence if insect activity is regulated by different environmental cues (e.g., air temperature; Willmer and Stone, 2004). Plants growing in regions with naturally variable spring-time climate may be relatively resilient to these consequences of climate change, but this has been largely untested.

Plants may employ a variety of strategies to cope with an erratic early season environment in which pollinator visits are infrequent, including self-compatibility or a generalized pollination system (Lloyd, 1992; Waser et al., 1996). A prolonged flowering period, resulting from the production of many flowers or, alternatively, a few long-lived flowers, would increase chances of pollinator visitation (Primack, 1985; Rathcke, 2003; Elzinga et al., 2007). Cold temperatures can increase floral longevity (Yasaka et al., 1998; Vesprini and Pacini, 2005), and although a reduced rate of senescence might simply be a metabolic consequence of lower temperatures, it may also serve

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an adaptive function by maintaining attractiveness of flowers that are unlikely to have been visited. In many plants, corolla wilting and abscission is also affected by pollen receipt, such that flowers remain attractive for longer if unpollinated (van Doorn, 1997; Ishii and Sakai, 2000; Clark and Husband, 2007). This strategy has the benefit of tailoring both the longevity of individual flowers and, potentially, overall floral display size to variable pollinator availability (Harder and Johnson, 2005).

In plants that produce many flowers in a growing season, the temporal pattern of flower opening (i.e., the flowering schedule) and patterns of resource allocation among flowers can be viewed as strategies for maximizing offspring quantity and quality—though some elements of inflorescence architecture and development are no doubt phylogenetically constrained (Prusinkiewicz et al., 2007). A skewed flowering schedule, in which most flowers on a plant open early and a few open late, might serve to attract and maintain loyal pollinators throughout the flowering period (Thomson, 1980; Makino and Sakai, 2007)—or it might function simply to ensure the earliest possible development for the bulk of a plant's ovules. Typically, reproductive success of a strategy is measured at the scale of whole plants, but studying within-plant variation can be illuminating (Wesselingh, 2007). For example, declining seed set is often observed in later or distal flowers within inflorescences (Stephenson, 1981; Thomson, 1989). So far, most evidence supports two alternative (but not mutually exclusive) hypotheses to explain this observation: The earliest-fertilized ovules may take precedence in within-plant competition for resources (Stephenson, 1981; Medrano et al., 2000; Humphries and Addicott, 2004), or the earliest flowers may have structural advantages such as proximity to nutrient sources or possession of a greater numbers of ovules (Thomson, 1989; Diggle, 1997; Buide, 2008). However, temporal variation in pollinator availability could also generate within-plant variation in seed set. An ability to adjust resource allocation in favor of later flowers if early flowers are unsuccessful would presumably be beneficial under a changing environmental regime, while a fixed strategy of investment in early flowers would be risky. An ability to adjust flower number and duration of the flowering period would also be advantageous when the length of the growing season is unpredictable (Prusinkiewicz et al., 2007).

In this study, we investigated reproductive strategies of a many-flowered perennial, *Mertensia fusiformis* (Boraginaceae), that flowers in early spring in subalpine meadows. We compared the success of these strategies in a year with a relatively early start to the growing season (2007) and a relatively late year (2008). In particular, we tested whether plants are capable of autogamous self-pollination, whether they can adjust floral longevity in response to pollination, and whether they can adaptively reallocate resources to late flowers if early flowers are not pollinated. In light of this information, we asked how pollen limitation varied through the growing season and whether there was selection on timing of flowering. Our objective was to determine whether these early-flowering plants are vulnerable to early-season mismatch with the timing of pollinator activity, particularly in a year of early snowmelt, and whether this affects the pattern of selection on flowering time.

MATERIALS AND METHODS

Study species—*Mertensia fusiformis* Greene (alpine, spindle-root, or dwarf bluebells) is a long-lived, herbaceous perennial that grows abundantly in subalpine meadows around the Rocky Mountain Biological Laboratory (RMBL), near Crested Butte, Colorado, United States. It is one of the first species to flower in spring, typically blooming 7–14 d after snowmelt (Inouye et al., 2000). Early in its flowering period, the only other concurrently flowering species in these habitats is *Claytonia lanceolata* Pursh (Portulacaceae). Later in the season, flowering overlaps with *Viola praemorsa* Douglas ex Lindl. (Violaceae; sometimes identified as *V. nuttallii* in previous work at the RMBL), *Delphinium nuttallianum* Pritz. (= *D. nelsonii*; Ranunculaceae), *Lathyrus lanszwertii* Kellogg (= *L. leucanthus*; Fabaceae), *Taraxacum officinale* Weber ex F. H. Wigg (Asteraceae) and other species. Timing of peak flowering in *M. fusiformis* has advanced by approximately 5 d per decade since 1973, corresponding to a similar advance in snowmelt date in the same time-period (Miller-Rushing and Inouye, 2009).

Like other Boraginaceae, *M. fusiformis* has a determinate cymose inflorescence, in this case, composed of numerous densely clustered campanulate flowers. Flowers are ~1 cm long, changing from pink at the bud stage to blue as they open. We did not measure temporal variation in stigma receptivity or pollen viability, but anthers typically open within 24 h of corolla opening, and there are no obvious physical changes in the stigma over the course of flowering. In *Mertensia ciliata*, stigmas remain receptive even after corollas and stamens have been shed (Pelton, 1961; Geber, 1985); this may also be the case in *M. fusiformis*. The plant reaches a maximum height of approximately 15 cm, and later-opening flowers are borne lower on the plant and in smaller clusters than earlier flowers. The most conspicuous flower visitors are nectar-collecting bumble bee queens, primarily *Bombus bifarius* at our study sites; however, the species is also visited by solitary bees, including *Osmia* spp. Fruits are one-seeded nutlets that have no specialized dispersal mechanisms other than an elaiosome that is attractive to ants (Turnbull et al., 1983). Herbivore damage at our study sites was rare, but stems were occasionally grazed, presumably by deer. Many plants showed signs of water stress during the fruiting period, and vegetative growth ceases well before the start of autumn frosts, likely in response to summer drought.

Study sites—We established two study sites 2.4 km apart, near the RMBL. The first (Avery; 38°58.27′N, 106°59.73′W) was a 40 × 60 m area of meadow with numerous *M. fusiformis* that included dry, rocky sections and more level, wetter areas. At this site, on 9 May 2007, we selected and marked with red pin flags 195 plants that were not yet in flower, along with the only six plants in the study area that were already flowering (five of these apparently having begun to flower that day). Because we were particularly interested in early-flowering plants, these were over-represented in our sample; estimates of population flowering distributions were therefore not calculated for this site. At the end of the season, all plants were permanently marked with metal tags and their locations mapped so they could be recovered in 2008. However, only a subset of these plants were relocated in 2008, so an additional 100 plants were newly flagged in the second year.

The second site (South Gothic; 38°57.13′N, 106°58.88′W), studied in 2008 only, was limited to a 20 × 50 m area of more uniformly dry, rocky ground. Snow melted early here in comparison both with the surrounding lower-lying areas and the Avery site. Two hundred plants were selected on 23 May 2008, before they flowered, by placing three 30 cm × ~50 m transects and flagging all plants with buds that lay within the transect. We also flagged an additional 20 plants that appeared likely to flower soon to ensure a larger sample of early-flowering plants; these nonrandomly selected plants were not included in measurements of population-level flowering patterns.

Field methods, 2007—Plants were monitored daily, except on 3 d of especially snowy weather. All newly flowering plants were noted and the number of open flowers per plant on each day was counted for a subset of plants that included at least five newly flowering plants per day, when possible.

Pollen limitation—For the first 25 plants to flower (excluding the six already in bloom), all flowers to open over the first 5 d of flowering were marked on sepals with a black permanent marker to indicate flower rank within that plant (i.e., flowers opening on the first day were rank 1, second-day flowers were rank 2, etc.). After the fifth day of flowering, all flowers on a plant were grouped as rank 6. Of these 25 plants, 12 were randomly assigned to a pollen-supplementation treatment. Open flowers on these plants received outcross pollen on each day the plants were monitored. For six of these plants, all flowers on the plant were pollinated; but the other six plants were prohibitively large (plants can produce as many as 900 flowers), so that only a subset of flowers (those on one or two stems) received supplemental pollen (see *Data*

analysis). For pollinations, we collected dehiscing anthers from plants growing outside the study plot and brushed these across the stigmas of the selected flowers—though within-population variation in style length meant that stigmas were often concealed beneath the ring of anthers, making precise placement of outcross pollen difficult. Multiple pollen donors were used each day, and all selected flowers were pollinated on each day the flowers were open, so that each outcrossed flower was likely to receive pollen from multiple pollen donors over the course of its lifespan.

Mating system and floral longevity—To test for self-compatibility and the effect of pollen receipt on floral longevity, we selected 37 additional plants and covered these with 1-mm mesh bags prior to flowering to exclude pollinators. Bagged plants were assigned to three treatments: control (unmanipulated), self-pollination, and cross-pollination. On pollinated plants, in the first 5–6 d of flowering, newly opened flowers were marked as already described to indicate flower rank. Flowers were considered open when the corolla had opened sufficiently to make all anthers fully visible; typically this coincided with the flower turning from pink to blue (anther dehiscence was not used as a criterion, but anthers usually dehisced later the same day). Approximately half of the monitored flowers received a black mark on the pedicel and were hand-pollinated; the remainder were handled for marking but otherwise unmanipulated. Outcross-pollination was conducted as described earlier for unbagged plants. Self-pollination was accomplished with forceps to transfer pollen from the anthers of the same flower or other flowers of the same plant to stigmas of the selected flowers. We noted the day on which each flower shed its corolla. Bags were removed after plants had finished flowering.

Seed counting—We monitored fruit set in 76 unmanipulated plants that spanned the range of first flowering dates (~5 per first flowering day between 9 and 26 May, plus the single plant that was already in flower, assigned an estimated first flowering date of 8 May) as well as in the 12 hand-pollinated and 37 bagged plants. Nutlets were considered to have successfully matured once they dropped from the plant, allowing us to determine seed set by examining abscission scars in cases where nutlets detached while we were absent. Abscission scars were easily distinguished from undeveloped ovules and were therefore a reliable indicator of the number of matured fruits. Because *Mertensia* spp. have four ovules per flower, the percentage seed set could be calculated as (100% × number of mature nutlets)/(4 × number of flowers). This value is likely an overestimate of the number (or proportion) of viable seeds, however, because some of the nutlets that did detach were soft. We did not expect differences in seed mass or viability between fruits that fell naturally from plants, and those we collected manually, because whether a nutlet detached on its own seemed to depend only on the orientation of the flower pedicel and whether the infructescence was physically disturbed. However, in 2009, we tested this assumption explicitly by attaching a vial around the inflorescences of 12 plants just before fruit set to collect any abscised nutlets. Vials were monitored for seeds approximately every 2 d. At 5-d intervals (the average frequency at which seeds were collected in 2007 and 2008), we also hand-collected mature nutlets that had not dropped into the seed traps. In total, we collected 216 nutlets from 12 plants by hand and 146 nutlets from 11 plants in the seed traps. We found no difference between seeds collected manually or passively, either in terms of mean seed mass (0.87 ± 0.06 mg [mean ± 1 SE] for manually collected seeds vs. 0.86 ± 0.04 mg for seeds in traps; paired t test, $t_{10} = 0.40$, $P = 0.70$) or proportion of seeds that could be crushed with forceps (0.070 ± 0.027 for manually collected seeds vs. 0.104 ± 0.048 for seeds in traps; Wilcoxon signed-rank test, $P = 0.95$, $V = 21.5$, $N = 11$).

Field methods, 2008—We visited the Avery site four times during snowmelt in 2008 to map the boundaries of the retreating snow. This allowed us to assign approximate snowmelt dates to individual plants, based on their mapped locations (see *Data analysis*). When flowering began, plants at both sites were visited every second day (except for one day missed because of snow), instead of every day as in 2007. All open flowers were counted on each census day. We monitored fruit set on a subset of approximately 10 unmanipulated plants per first flowering date per site, for a total of 84 or 85 plants per site; however, at the South Gothic site, one plant was grazed, and data from another two were omitted following an error in data entry, leaving us with a sample size of 81. As many seeds as possible were collected as they matured for later weighing. Thus, in 2008, we had mean seed mass (per seed) and seed set as response variables.

Pollen limitation—At each site, 40 of the first plants to flower were alternately assigned to the pollinated or the control treatments. Newly opening flowers were marked as described, except that rank 1 now included flowers opening on the first 2 d of the flowering period, rank 2 flowers opened on days 3 and 4, and so on. Each day, a subset (up to half) of the new flowers on pollinated plants received supplemental pollen; the other flowers on these plants, and the control plants, were unmanipulated. This experimental design allowed us to compare seed set between control and hand-pollinated flowers on the same plant and between unmanipulated flowers on control and (incompletely) hand-pollinated plants. Hand-pollinated flowers received an additional black mark on the pedicel but were otherwise indistinguishable from unmanipulated flowers. Because flowers are tightly clustered within the inflorescence, pedicels are typically concealed, and these marks are unlikely to have influenced pollinator behavior. Pollination techniques were the same as described for 2007. Seeds were collected to obtain mean seed mass for flowers of each rank and treatment on each plant.

Resource allocation between early and late flowers—To test for inherent differences in fecundity and trade-offs in allocation between early and late flowers, we conducted a second bagging experiment at the Avery site in 2008. Before plants began to flower, we bagged 12 sets of three similar-sized plants that each had at least 30 buds. On all plants, we marked the first 10 and the last 10 flowers to open. Within each set of three, plants were arbitrarily assigned to one of three treatments: early pollination (first 10 flowers received outcross pollen), late pollination (last 10 flowers pollinated), or both early and late pollination. Seeds were weighed to obtain an average seed mass for each cohort (early or late) of each plant.

Data analysis—Analyses were conducted with the program R (R Development Core Team, 2007).

Flowering patterns—In comparing flowering patterns between years, we calculated flowering duration for individual plants as last flowering day − first flowering day + 1 in 2007, and last flowering day − first flowering day + 2 in 2008 (we assume that sampling only every second day in the latter year would cause us to underestimate flowering duration by one day, on average). Skewness in flowering schedules of individual plants (g) was calculated as the sample skewness using the npde package in R, while population skewness (G) was calculated and tested using the moments package.

We used multiple linear regression to find the best predictor of flowering date among permanently tagged plants at the Avery site, including 2008 snowmelt date (at the location of the individual plant), plant size, and the date on which the plant flowered in 2007 as explanatory variables. We had mapped snowmelt boundaries at 2- to 4-d intervals (on 26 and 29 May, and 2 and 4 June 2008), giving us lower temporal resolution for snowmelt dates than for flowering dates. Therefore, to avoid underestimating the explanatory power of snowmelt date, we inferred an additional snow contour for 31 May, positioned midway between the observed contours for 29 May and 2 June. Individual plants were assigned one of the five possible snowmelt dates according to their locations relative to these contours. The maximum number of open flowers on a single day (log-transformed) was used as an estimate of plant size, as it was strongly correlated with the total number of flowers produced by a plant (square-root transformed; Pearson's $r > 0.85$, $P < 0.0001$ in both years), but was more easily obtained. Flowering date in 2007 was rank transformed to reduce the influence of one late-flowering outlier. We selected the best model as the one having the lowest AIC (Akaike information criterion) value, and we used likelihood-ratio comparisons of nested models to evaluate the significance of additional terms. Despite moderate correlations among predictor variables, all variance inflation factors (VIFs) were less than 1.4, indicating that our parameter estimates are robust (Quinn and Keough, 2002).

Floral longevity—Differences in floral longevity between pollinated and unpollinated flowers on the same plant were evaluated with paired t tests (conducted separately for self- and cross-pollinated plants).

Pollen limitation—Because of the large number of zero values and unequal sample sizes, these data did not lend themselves to parametric statistical analyses. We therefore tested for treatment effects on flowers of each rank separately, using nonparametric tests, and applied sequential Bonferroni correction to account for the multiple testing. In 2007, we used Kruskal–Wallis tests to compare seed set of all flowers of a given rank on pollen-supplemented and unmanipulated plants, even though some of the former plants included flowers that did not receive supplemental pollination. In 2008, we were able to test for an effect of pollen supplementation within manipulated plants; for flowers of a

given rank, we tested whether the differences in seed set or seed mass between hand-pollinated and open-pollinated flowers differed significantly from zero, using a Wilcoxon signed-rank test. We also compared seed set and seed mass of open-pollinated flowers on manipulated plants to those of unmanipulated plants, to ensure that enhanced seed set of supplemented flowers had not caused a corresponding reduction in seed set of control flowers on those plants (Zimmerman and Pyke, 1988). To better allow comparison between years, we reanalyzed 2007 data after lumping flowers into ranks that corresponded with three of those used in 2008 (rank 1: flowers from days 1 and 2, rank 2: days 3 and 4, rank 3: day 5 and beyond). We tested for trends in seed set and seed mass with increasing flower rank by calculating rank correlations (Spearman's ρ) for each plant and then testing whether the set of correlation coefficients for each treatment deviated from a mean of zero using a Wilcoxon signed-rank test.

Resource allocation—Mean seed masses were normally distributed, so differences between treatments were tested by one-way ANOVA. Percentage seed set values were not normally distributed, even after arcsine-transformation, so a Kruskal–Wallis test was used instead.

Selection on flowering schedules—We used number of seeds per plant and total seed mass (the product of number of seeds per plant and mean seed mass, measured in 2008 only), proxies for fitness via female function, to estimate the strength of phenotypic selection on flowering time and skewness of the flowering schedule. Lifetime fitness measures were beyond the scope of our 2-year study; however, these shorter-term measures of selection allowed us to evaluate whether selection differed between years and between sites. Linear selection differentials were calculated as the covariance between relative fitness (an individual's seed production divided by the population mean in that year) and the trait; significance was tested by Pearson correlations. Linear selection gradients were obtained from multiple regressions of relative fitness on flowering date, flower number, and skewness of a plant's flowering schedule. The partial regression coefficient for a trait is a measure of the strength of selection acting directly on that trait, instead of indirectly via an effect on the other measured traits (Lande and Arnold, 1983). We checked that all VIFs were below 2, suggesting that our parameter estimates were not affected by problems of multicollinearity. Selection gradients (β) are reported in SD units. Given our relatively small sample sizes and the absence of obvious nonlinearity in the relationships between traits and fitness, we did not attempt to measure nonlinear selection. For significance testing, number of seeds per plant and total seed mass per plant were 4th-root transformed, and flower number was square-root transformed.

To determine the extent to which estimates of selection via female function (seed set) also reflect selection via the male component of fitness (seed siring), we estimated the level of assortative mating by flowering date based on unmanipulated plants at the South Gothic site (2008), using the method of Weis and Kossler (2004). For all flowers open on a given maternal parent on a given day, we estimated the mean first flowering date of potential pollen donor plants by weighting first flowering dates of all other censused plants in the population by the number of flowers they had open on that day. The values for each day were weighted by the proportion of open flowers on the maternal parent on that day to obtain the mean first flowering day of pollen donors for that plant. The correlation in first flowering dates between potential pollen donors and recipients (ρ) is a measure of assortative mating. Spatial proximity of prospective mates was not taken into account. Similarly, because we had no evidence of dichogamy, it was not considered in calculations of assortative mating. However, not all flowers on a plant are equally likely to set seed; temporal variation in fecundity would change the distribution of potential sires from that predicted based on the flowering schedule alone (Weis and Kossler, 2004). We therefore calculated a second estimate of ρ, weighting flowers by their expected contribution to a plant's seed production (estimated from the mean seed set observed for flowers of different ranks on the control plants at this site).

RESULTS

Population-level flowering patterns—Weather patterns in the 2 years of our study differed dramatically: Of the last 34 years (1975–2008), 2007 was the year with the fifth-earliest date of spring snowmelt at the RMBL, while 2008 ranked 27th (RMBL, 2009). This difference in timing of snowmelt caused permanently tagged and relocated plants ($N = 67$) at the Avery site to flower on average 27 d later in 2008 than they did in 2007. Larger plants (those with a larger peak floral display) tended to flower earlier, but the strength of this relationship varied between sites and years, being strongest at the Avery site in 2007 (the site/year with the earliest start to the flowering season; Pearson's $r = -0.57$, $N = 76$, $P < 0.0001$), weaker at the South Gothic site ($r = -0.29$, $N = 160$, $P = 0.0002$), and nonsignificant at Avery in 2008 (the latest site/year; $r = -0.12$, $N = 133$, $P = 0.17$). This pattern was unchanged if total number of flowers was used as the response variable instead of maximum floral display. Nevertheless, date of first flowering was highly correlated between years for individual plants (Spearman's $\rho = 0.62$, $N = 67$, $P < 0.0001$). Peak flowering displays of tagged plants were larger in 2008 (paired t test, $t_{34} = 2.89$, $P = 0.0067$; Fig. 1), but there was no detectable difference in total flower number per plant between years (paired t test, $t_{24} = 0.84$, $P = 0.41$), suggesting that the increased display size was driven at least in part by the compressed flowering season in the later year. Indeed, duration of flowering for individual plants was, on average, 9.5 d shorter in 2008 (paired t test, $t_{34} = 10.2$, $P < 0.0001$; Fig. 1).

Snowmelt date at the location of individual plants was a good predictor of flowering date in 2008, but better models also included the date on which a plant flowered in 2007, regardless of whether plant size was also included in the model (Table 1). That the inclusion of 2007 flowering date significantly improved model fit suggests that plant attributes other than size and position relative to snowmelt gradients contributed to determining flowering time.

The randomly selected plants at the South Gothic site in 2008 showed a positively skewed flowering distribution, with most flowers in the population opening early in the flowering period ($G = 0.229$; D'Agostino skewness test, $P < 0.0001$). This skew

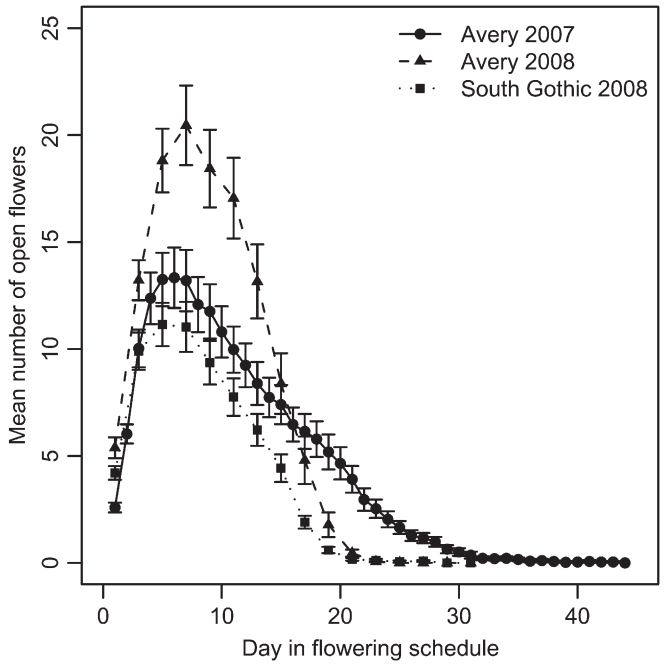

Fig. 1. Average flowering schedules, showing mean number of open flowers per plant on each day of the flowering period (± 1 SE); note that the first flowering day for each plant is treated as day 1 of the flowering schedule regardless of the calendar date of first flowering. For Avery 2007, $N = 75$; for Avery 2008, $N = 133$; for South Gothic, $N = 161$.

TABLE 1. Model predicting flowering time for plants at Avery site in 2008 ($N = 65$), based on snowmelt date in 2008, plant size (as ln[maximum floral display size]) in 2008, and 2007 flowering date (fl.date) (rank-transformed). Likelihood-ratio χ^2 statistics (on 1 df) and P-values test the significance of the last model term relative to the next-simplest nested model. Interaction terms did not significantly improve model fits.

Predictors (Sign of estimated effect)	R^2	AIC	χ^2	P
Size (–)	0.00	385	0.2	0.665
2007 fl.date (+)	0.38	353	32.1	<0.0001
Snowmelt (+)	0.66	314	70.9	<0.0001
Snowmelt (+), size (–)	0.68	311	5.7	0.017
Snowmelt (+), size (–), 2007 fl.date (+)	0.74	297	15.2	0.0001
Snowmelt (+), 2007 fl.date (+)	0.73	299	16.9	<0.0001
Snowmelt (+), 2007 fl.date (+), size (–)	0.74	297	4.1	0.043

Notes: AIC = Akaike information criterion

resulted from a weakly positively skewed distribution of first flowering dates ($G = 0.116$, $P = 0.66$) in combination with skewed flowering schedules of individual plants (pollinated plants excluded; mean within-plant $g = 0.123$, one-sample t test against a hypothesized mean of 0, $t_{159} = 4.63$, $P < 0.0001$; Fig. 1). Individual plants also showed positively skewed flowering schedules at the Avery site in both years (mean within-plant $g = 0.305$ in 2007, $g = 0.168$ in 2008, both $P < 0.0001$; Fig. 1). Skewness was moderately consistent between years for individual plants ($r = 0.476$, $N = 35$, $P = 0.0038$), but most plants had more positively skewed flowering schedules in 2007 than in 2008 (paired t test, $t_{34} = 3.84$, $P = 0.0005$; Fig. 1).

Mating system and floral longevity—Unmanipulated flowers on bagged plants (Avery site, 2007) set a negligible number of seeds, and hand-self-pollinated flowers set none (Table 2), indicating self-incompatibility. Flowers that were outcross-pollinated by hand successfully set seed; and these plants shed corollas, on average, 2 d earlier than control flowers on the same plant (Table 2; paired t test, $t_{10} = 6.2$, $P < 0.0001$). Interestingly, despite its inability to fertilize ovules, self-pollen also reduced floral longevity (by 1.5 d; paired t test, $t_9 = 4.6$, $P = 0.0012$). This reduction does not differ significantly from that produced by outcross pollen (t test on differences, $t_{19} = 1.0$, $P = 0.32$).

Pollen limitation—Among the early cohort of plants that were used to test pollen limitation at the Avery site in 2007, seed set was low overall and did not differ significantly between treatments at the level of whole plants (6.0 ± 1.0% [mean ± 1 SE] for control plants, 10.4 ± 2.4% for pollinated plants; Kruskal–Wallis $\chi^2 = 0.855$, df = 1, $N_1 = 13$, $N_2 = 12$, $P = 0.36$). The earliest flowers showed the greatest difference in seed set between control and pollinated treatments (Fig. 2). This difference was not significant, particularly after Bonferroni correction (Kruskal–Wallis $\chi^2 = 3.19$, df = 1, $N_1 = 13$, $N_2 = 12$, $P = 0.074$, $\alpha = 0.05/6 = 0.0083$), but the trend for pollen limitation in the earliest flowers remained when flowers from the first 2 days were combined for analysis, for comparison with 2008 results (6.2 ± 2.0% seed set in unmanipulated vs. 13.4 ± 3.0% in pollinated plants, Kruskal–Wallis $\chi^2 = 3.00$, df = 1, $N_1 = 13$, $N_2 = 12$, $P = 0.083$). In control plants, seed set increased with flower rank (mean $\rho = 0.21$, Wilcoxon signed-rank test on correlation coefficients, $V = 64.5$, $N = 12$, $P = 0.050$); pollinated plants, in contrast, showed no temporal trend in seed set (mean $\rho = -0.11$, $V = 24$, $N = 11$, $P = 0.45$).

In 2008, seed set among experimental plants was higher at both sites than it was at Avery in 2007 (22.7 ± 2.9% for pollinated and 20.3 ± 2.3% for control plants at Avery; 35.3 ± 3.1% for pollinated and 27.7 ± 3.6% for control plants at South Gothic). Overall, the differences between pollen supplemented and unmanipulated plants were not significant (Kruskal–Wallis $\chi^2 \leq 3$, df = 1, $0.20 > P > 0.05$ at both sites); however, the strength of the treatment effect appeared to vary with flower rank (Fig. 2). Among the latest-opening (rank 6) flowers at both sites, hand-pollinated flowers set more seed than their unmanipulated counterparts, although this was not significant after Bonferroni correction (Wilcoxon signed-rank tests, $V = 78$, $P = 0.025$ at Avery, and $V = 72$, $P = 0.011$ at South Gothic; $\alpha = 0.05/6 = 0.0083$). Increased seed set by pollinated flowers was not associated with a decline in seed set of unmanipulated flowers, compared to flowers of the same rank on control plants (Kruskal–Wallis $\chi^2 < 2.5$, df = 1, $P > 0.1$ for rank 6 flowers at both sites). At both sites, seed set of unmanipulated flowers declined strongly with increasing flower rank (Fig. 2; mean rank correlations = –0.41 and –0.67 at Avery and South Gothic respectively; Wilcoxon $P < 0.01$ in both cases). This decline was alleviated somewhat in pollen-supplemented flowers at Avery (mean $\rho = -0.22$, Wilcoxon $V = 36$, $N = 17$, $P = 0.058$), but not at South Gothic (mean $\rho = -0.62$, Wilcoxon $V = 12$, $N = 19$, $P = 0.0009$).

Mean seed mass (per seed) followed a similar decline with increasing flower rank as was observed for seed set in 2008 (the only year in which seeds were weighed). This decline was consistent across the two sites and both flower treatments (mean within-plant $\rho < -0.4$, all Wilcoxon $P < 0.02$, Fig. 3). There was no effect of pollination treatment on mean seed mass, regardless of flower rank (Wilcoxon signed-rank tests, all $P > 0.1$).

Resource allocation—When pollinators were excluded and only the 10 earliest and/or 10 latest flowers were pollinated (2008 bagging experiment), there was no difference in seed set or mean seed mass between early and late flowers (Table 3), indicating that the declines in seed set and seed mass observed in open-pollinated plants were not due to structural differences between early and late flowers. Furthermore, pollination of the 10 earliest flowers did not reduce seed size or seed production in the latest flowers (Table 3), suggesting there was not a strong trade-off in resource allocation between early and late flowers.

TABLE 2. Floral longevity and fruit and seed set of bagged plants (2007). Plants were assigned to three treatments (control, self-pollinated, or outcross-pollinated). Within pollinated plants, flowers opening on the first 5–6 d of flowering were assigned either to hand-pollination or control treatments. Floral longevity was not monitored in control plants. Values are means ± 1 SE, with sample sizes (numbers of plants) in parentheses.

	Control	Self-pollinated		Outcross-pollinated	
Variable	Control	Pollinated	Control	Pollinated	Control
Longevity (d)	—	3.3 ± 0.3 (10)	4.8 ± 0.4 (10)	3.2 ± 0.2 (11)	5.2 ± 0.5 (11)
Fruit set (%)	1.0 ± 0.6 (13)	0.0 ± 0 (11)	0.10 ± 0.07 (11)	44.2 ± 5.3 (12)	0.5 ± 0.3 (12)
Seed set (%)	0.3 ± 0.2 (13)	0.0 ± 0 (11)	0.03 ± 0.02 (11)	27.3 ± 3.6 (12)	0.3 ± 0.2 (12)

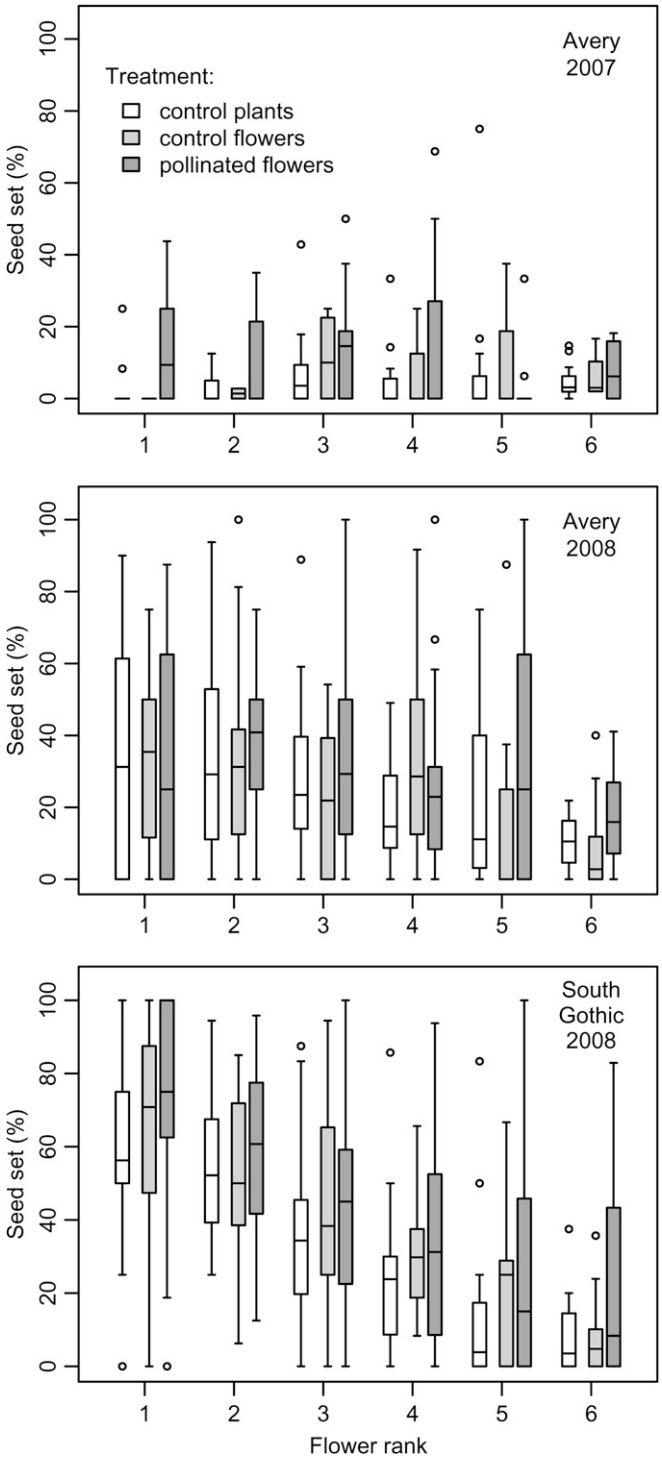

Fig. 2. Tukey box-plots showing percentage seed set of pollinated and control plants vs. flower rank at each site/year. Flowers on pollinated plants either received supplemental pollen (dark gray bars) or did not (light gray bars); no flowers on control plants received supplemental pollen (open bars). Flower rank in 2007 refers to the day in the plant's flowering schedule on which the flower opened; in 2008, ranks are 2-d periods instead of single days (see *Field methods 2008*, *Pollen limitation*). Boxes show the median and interquartile range.

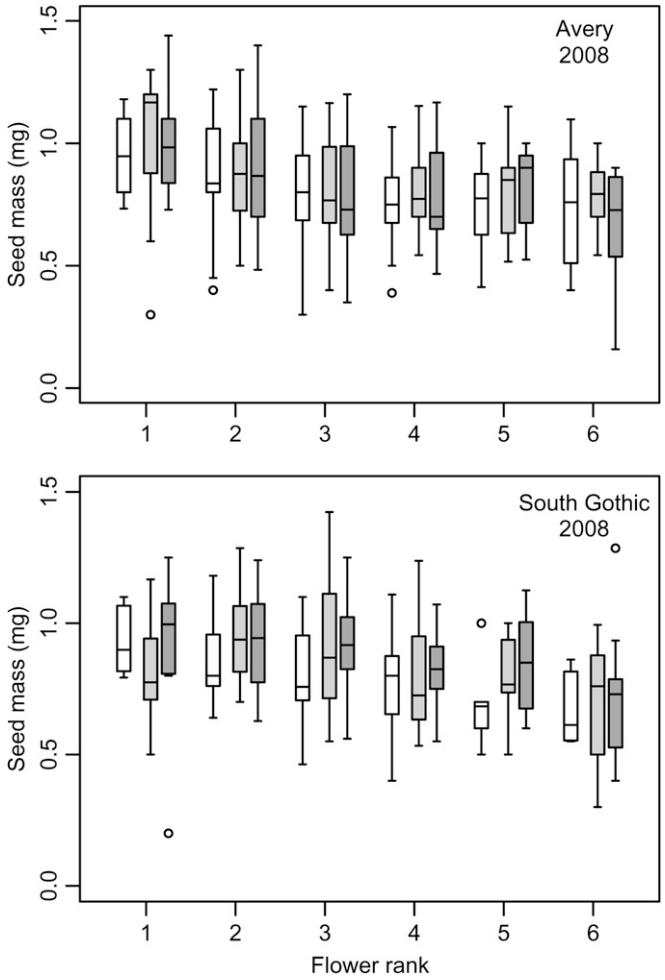

Fig. 3. Seed mass of pollinated and control plants vs. flower rank at each site/year. Shading as in Fig. 2.

Mean seed mass of late (rank 6), pollinated flowers on unbagged plants at the same site was lower than that of late flowers on the late-treatment bagged plants (planned comparison; Welch's $t = 2.33$, df = 15.6, $P = 0.034$), while there was no such difference between early (or rank 2) flowers of bagged and unbagged plants (Welch's $t = 1.14$, df = 22.1, $P = 0.27$). This may indicate that the higher pollination levels achieved by unbagged, pollen-supplemented plants reduced seed mass of later flowers. However, in terms of percentage seed set, there were no significant differences between the four treatment-cohort combinations (Kruskal–Wallis $\chi^2 = 0.043$, df = 3, $P = 0.998$) or between flowers of bagged and unbagged plants (Kruskal–Wallis $\chi^2 < 2$, df = 1, $P > 0.1$ for both early and late flowers).

Phenotypic selection on flowering schedules—Plants that began flowering later in the season generally produced fewer seeds; this resulted in a significant selection differential for flowering time at Avery in 2007 ($s = -1.1$ d) and at South Gothic in 2008 ($s = -1.6$ d), but not at Avery in 2008 (Table 4, Fig. 4). However, much of this effect was due to indirect selection, acting through flower production; after accounting for number of flowers, direct selection for earlier flowering was significant only at South Gothic (Table 4). Considering seed mass (mea-

TABLE 3. Seed mass and seed set for plants at the Avery site in 2008. Only a subset of flowers on bagged plants (the 10 earliest, 10 latest, or both) were pollinated by hand. Unbagged plants were open to insect pollination; data presented here are for flowers that also received supplemental outcross pollen. Values are means ± 1 SE, $N = 12$ bagged plants per pollination treatment and 18 unbagged plants. Letters denote values that were significantly different by Tukey's HSD ($P < 0.05$). See text for details.

	Bagged plants				Unbagged plants	
	Early pollination	Early and late pollination		Late pollination	Hand-pollination	
Variable	Early cohort	Early cohort	Late cohort	Late cohort	Early cohort	Late cohort
Mean seed mass (mg)	1.01 ± 0.06 a	1.02 ± 0.08 a	1.00 ± 0.09 a	0.93 ± 0.10 ab	0.899 ± 0.08 ab	0.667 ± 0.06 bc
Seed set (%)	30.2 ± 7.2 a	27.7 ± 5.7 a	31.3 ± 8.4 a	28.7 ± 6.9 a	35.7 ± 5.5 a	16.8 ± 3.2 a

sured in 2008 only) did not qualitatively change this result; direct selection via total seed mass also favored early plants only at South Gothic ($\beta' = -0.15$, $P = 0.030$). There was no detectable selection on skewness of a plant's flowering schedule (Table 4).

At the South Gothic site, most plants showed at least partial flowering overlap with all other plants in the sample because of the extended flowering duration of individual plants and the relative topographical homogeneity of the sampled area. Nevertheless, the correlation in first flowering dates between potential mates, inferred from the flowering schedules of all individuals in the sample, was high ($\rho = 0.714$, $N = 166$), owing to the positive skew in most flowering schedules (i.e., an individual plant produced most of its flowers shortly after its first flowering day). This estimate was increased slightly by incorporating the observed decline in seed set in later flowers ($\rho = 0.741$). Thus, selection on first flowering date, when it occurs, would be manifested through both male and female components of fitness.

DISCUSSION

***Determinants of flowering phenology in* M. fusiformis**—Timing of snowmelt and (at least at Avery in 2007 and South Gothic in 2008) plant size had a strong influence on date of first flowering. After accounting for snowmelt date and size, which is likely a function of age as well as genetic and environmental factors, individual plants differed predictably in flowering time. Many studies have demonstrated a genetic basis to flowering phenology (Mazer and LeBuhn, 1999; Van Dijk and Hautekèete, 2007; Franks et al., 2007; Wilczek et al., 2009), and some of the observed individual differences in flowering time in our plants may reflect heritable variation. Unmeasured environmental gradients may also be acting; for instance, our estimates of snowmelt date were coarse, and we lack data on small-scale heterogeneity in air temperatures. However, predictability can be viewed as setting an upper limit for heritability.

At our study sites, the shorter 2008 growing season led to shorter, but more intense, flowering periods for individual plants. The compressed season may also explain the lack of a detectable relationship between plant size and date of first flowering at the (later) Avery site. The longer flowering season of 2007 produced more positively skewed flowering schedules; that is, individual plants had longer "tails" of late-opening flowers. It is unlikely that these late flowers represent a plastic response to low seed set in early flowers; total flower number did not differ significantly between years for individual plants. Because the flowering peaks of individual plants—not only the first flowering dates—shift earlier in early snowmelt years, most of a plant's flowers are exposed to early season conditions, although the tail of late flowers provides some insurance against reproductive failure of early flowers. If this were a general pattern, it could also explain why first flowering dates can be an adequate predictor of peak flowering dates in these communities, in spite of fluctuations in population size (Miller-Rushing et al., 2008).

Because timing and duration of flowering in *M. fusiformis* are highly responsive to variation in snowmelt date (this study; Inouye et al., 2000; Dunne et al., 2003), plants can experience dramatically different conditions during the flowering period, depending on whether snow melts early or late. To some extent, this variation was captured in the 2 years of our study, which differed markedly in the timing of onset of the growing season. However, *M. fusiformis* possesses traits that, in combination with a perennial habit, confer some resilience to year-to-year fluctuations in climate and synchrony with pollinators, thereby limiting the strength of selection on flowering time. These include extended longevity for unpollinated flowers, and production of many later flowers that probably serve an "insurance" or bet-hedging function. Below, we elaborate on the benefits and limitations of these strategies.

Determinants of seed set—Overall, seed set was lower in 2007 than in 2008. Numerous factors might be responsible, but more severe frost damage in 2007 was undoubtedly important. Two hard frosts in May 2007 damaged many of the flowers at the study site (one frost event, occurring shortly after the peak of flowering, reduced the number of open flowers by ~40%). More frosts occurring after the start of the growing season are a projected consequence of climate warming (Hänninen, 1991), and long-term data from the RMBL suggest that they are an increasingly important problem for frost-sensitive species of

TABLE 4. Estimates of standardized linear selection differentials (s') and gradients (β'), ± SE. Flower number was square-root transformed for analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

	Avery 2007 ($N = 76$)		Avery 2008 ($N = 85$)		South Gothic 2008 ($N = 81$)	
Character	s'	β'	s'	β'	s'	β'
Flowering date	–0.25*	0.13 ± 0.12	–0.042	0.078 ± 0.073	–0.51***	–0.19 ± 0.071**
Total no. of flowers	0.71***	0.78 ± 0.12***	0.99***	1.02 ± 0.072***	0.848***	0.775 ± 0.070***
Skewness	–0.087	–0.024 ± 0.10	–0.11	0.070 ± 0.073	–0.019	0.0023 ± 0.063

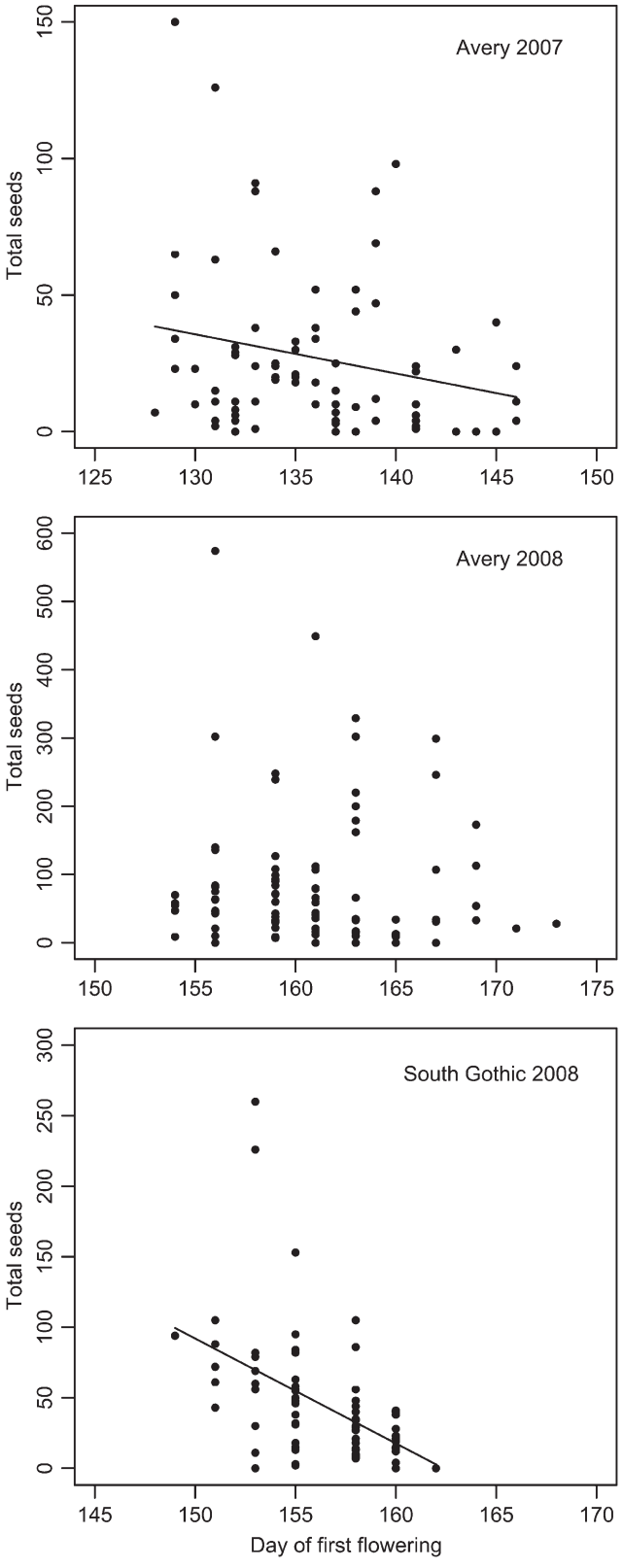

Fig. 4. Total seed production vs. day of year of first flowering for unmanipulated plants (150 = May 30 in 2007, or May 29 in 2008, a leap year). For Avery 2007, $N = 76$; Avery 2008, $N = 85$; South Gothic, $N = 81$. Regression lines are shown only if significant selection differentials were detected. Note variation in *y*-axis scale.

subalpine wildflowers (Inouye, 2008). Despite this potential cost to early flowering, in the plants we studied, seed production was typically greatest in early-flowering individuals. This pattern arose because the frosts were not confined to the beginning of the growing season and because early plants produced more flowers (cf. Ehrlén and Münzbergová, 2009). After accounting for flower number, we detected insignificant selection on flowering time in 2007, although later plants tended to make more seeds. We observed direct selection for earlier flowering in 2008, the late-snowmelt year—but only at one site (South Gothic). Thus, the differences in environmental conditions between years did not have a strong or consistent effect on the relative success of early- vs. late-flowering individuals, and we cannot conclude that climate warming will lead to strong selection on flowering time in this species. Although warmer springs may be accompanied by a greater risk of frost damage and a consequent overall reduction in seed set, we have no evidence that escape via later flowering will be favored.

Furthermore, despite the year-to-year variation in abiotic conditions, in neither year did pollinator availability appear to be the major factor limiting seed production, at least not among the early-flowering plants used in our pollen supplementation experiments. However, an absence of pollen limitation at the whole-plant level can mask variation at the level of individual flowers—variation that can shed light on patterns of pollen supply and resource allocation within plants (Wesselingh, 2007). In our study, early flowers tended to be more pollen limited in 2007, and late flowers (at both sites) more pollen limited in 2008. Why did these patterns not translate into pollen limitation at the whole-plant level? The large number of late flowers held in reserve, coupled with flexibility in allocating resources to a subset of successfully pollinated flowers (discussed later) buffer this species from short-term variation in pollinator availability. Results from this species, therefore, do not provide much support for the hypothesis that the earlier springs expected with future climate change will disrupt plant–pollinator interactions (Price and Waser, 1998; Dunne et al., 2003; Hegland et al., 2009). We did not quantify pollinator visitation at our study sites, but we did note that bumble bees were visiting flowers by the end of the first week of flowering, even in 2007. This may explain the observation that any pollen limitation in early plants was short-lived. Flowers on plants from which pollinators were excluded often lasted 5 d or more if unpollinated, something that could have played a role in preventing pollen limitation (assuming that stigmas remained receptive). However, we rarely observed such long-lived flowers on open-pollinated plants, even early in the season, suggesting that most flowers were receiving some insect visits.

Why were late-flowering individuals selected against at the South Gothic site in 2008? Declining fecundity in later plants mirrored the pattern of reduced seed set of late flowers within plants, and both may owe something to reduced pollinator visitation later in the season. However, declining resource availability (e.g., soil moisture) might also be responsible. At the Avery site, where there was more topographic variation and a greater number of plants growing in relatively moist microhabitats, we did not observe any decline in seed set in later plants (after accounting for flower number). Direct pollinator observations, or pollen-supplementation experiments with previously unvisited plants exposed to pollinators at different times during the flowering season, would help in understanding temporal patterns of pollinator visitation and pollen limitation in these populations.

Within plants, the decline in seed set and seed mass seen in later flowers in 2008 was probably due mainly to resource limitation. No reductions in seed quantity or quality were observed in 2007, when early flowers set few seed, or in bagged plants on which few flowers were pollinated. Declining fecundity in late flowers has been observed in many plants (Nicholls, 1987; Medrano et al., 2000; Humphries and Addicott, 2004; Kliber and Eckert, 2004), although in some cases it may be due to structural constraints (e.g., distal positioning of late flowers when flowering is acropetal [Diggle, 1997], or declining ovule number in later flowers [Thomson, 1989]). In *Cryptantha flava* (also Boraginaceae), for example, seed set is higher in early flowers, but does not increase in later flowers if early flowers are prevented from setting seed (Casper, 1984). The greater success of early flowers in that species may be due to their location closer to the main axis of the plant, suggesting that resource supply to late flowers, rather than the amount of resources available to the whole plant, is the important limiting factor. In *M. fusiformis*, limited resource availability, and not structural constraints, seems to prevent high seed set and mass in late flowers when pollen is unlimited. Thus, later flowers probably serve as a backup in the event of poor pollination of earlier flowers.

Reproductive strategies—Many temperate-region perennials appear to use stored resources to produce as many flowers and fruit as possible early in the growing season. Plant populations often show positively skewed flowering distributions, perhaps because most individuals begin flowering in response to a discrete environmental cue, while the end of flowering is not similarly truncated (Rathcke and Lacey, 1985). In many perennials, larger plants flower earlier (Widén, 1991; Pettersson, 1994; Bishop and Schemske, 1998; Ollerton and Lack, 1998; Lacey et al., 2003; Sandring et al., 2007; Sola and Ehrlén, 2007)—a pattern we also observed here—suggesting that early reproduction is favored by those plants with sufficient belowground reserves to allow rapid growth as soon as conditions permit. Flowering distributions at the level of individual plants have received less attention than population-level patterns, but these can be positively skewed, too (Thomson, 1985; Blionis et al., 2001). Finally, allocation of resources within plants also favors early flowers, these almost invariably having higher levels of fruit set if pollination is adequate (Stephenson, 1981; Thomson, 1989; Diggle, 1997; Ladio and Aizen, 1999; Medrano et al., 2000; Humphries and Addicott, 2004; Kliber and Eckert, 2004; Brown and McNeil, 2006; Buide, 2008).

This strategy of preferential investment in early reproductive structures appears to be the one employed by *M. fusiformis* in our study populations, with most plants opening most of their flowers early in the growing season. This pattern was stronger in 2007, the early snowmelt year. In principle, such a strategy carries with it the risk that poor conditions in early spring (e.g., early season frosts or pollinator inactivity) might have a devastating impact on the population. However, we did not detect selection on skewness of the flowering schedule in either year, indicating that, at least within the context of currently existing variation in flowering schedules, plants with a strong pattern of preferential allocation to early flowering are not at a disadvantage. Furthermore, although reproductive success was lower in 2007 than in 2008, we found only weak evidence for pollen limitation—and this only in the earliest flowers, suggesting that mismatches with the timing of pollinator activity are not a major threat for this species. Our data also suggest that the possession of a large number of flowers, and the ability to adjust resource allocation among flowers in response to pollination, make *M. fusiformis* somewhat insensitive to variation in environmental conditions. Given the great interannual variation in snowpack in these habitats, it makes sense that high-elevation plants would have evolved resilience.

Responses to future climate change—There may nevertheless be a limit to how well these strategies buffer the plant against effects of future climate warming. Further reductions in snow accumulation are predicted for western North America (Mote et al., 2005; Christensen et al., 2007), and these may bring an increased risk of frost damage in spring (Inouye et al., 2002; Inouye, 2008) along with decreased soil moisture later in the summer. Because summer drought already appears to be a factor limiting the duration of the growing season for *M. fusiformis*, it is uncertain to what extent plants will be able to maintain seed set by reallocating resources to later flowers if early flowers fail. Plasticity in the duration of flowering and the pattern of resource allocation among flowers may prevent large fluctuations in reproductive success despite climatic variation; this plasticity (along with a long generation time) may limit the capacity for evolutionary change in flowering phenology, even if the trait is strongly heritable. Indeed, we did not detect consistent selection on flowering schedules in either year of our study, despite the dramatic differences in climate between the two years. On the other hand, future, more extreme variation in climate has the potential to produce novel phenotypes through plastic changes in flowering patterns; these phenotypes would then be exposed to selection and might provide material for future adaptation (Price et al., 2003; Donohue, 2005). The interaction between plastic and evolved responses to climate change deserves further study.

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