PHENOLOGICAL LITERACY

UNDERSTANDING THROUGH SCIENCE & STEWARDSHIP

Seminar Module 3:

Plant-Climate Interactions

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Goals For Student Learning

This seminar module was created to help students:

- Understand how researchers use experiments to evaluate the potential phenological responses of plants to climate change
- Understand how phenological schedules in plant populations and communities are influenced by climate and may be affected by ongoing or predicted climate change

Plant-climate interactions

Phenological responses to climate change have been studied extensively in plants. Plants may be especially sensitive to climate change because they are sessile and therefore, must either withstand all climatic conditions that occur in their habitats or perish. Two of the studies presented in this module report plant phenological responses to climatic conditions. Haggerty and Galloway (2011) conducted a **reciprocal transplant** experiment in southwestern Virginia, transplanting plants from a high elevation site to a low elevation site (*and vice versa*). They took advantage of environmental differences between transplant sites in order to explore plant responses to changes both in the length of the growing season and in temperature, and to determine whether the observed **phenotypic plasticity** in phenological traits at the transplant sites was adaptive. In other words, did environmentally induced changes in phenology increase the fitness of the plants that exhibited them? The researchers found that at the low elevation site, where the growing season was expanded relative to the high elevation site, natural selection favored individuals with advanced flowering. The results of this study imply that this species' phenological schedule may evolve in response to climate change.

In sub-alpine habitats, the timing of snowmelt is an important environmental cue that influences plant phenology. Inouye (2008) studied three Rocky Mountains wildflower species whose flower buds are very susceptible to frost damage. His study, which spans 14 years, demonstrates that an earlier start of the growing season has exposed his study species to greater frost exposure in recent years than in years past.

Many plant species require animal **mutualists** to carry their gametes to and from potential mates (pollination) and/or to disperse their seeds across the landscape. If the phenological schedules of plants and their mutualistic partners shift in different ways as the climate changes, then any subsequent reduction in **phenological synchrony** may strongly (and negatively) affect both plant and animal populations. In a long-term study of the montane **geophyte** *Erythronium grandiflorum,* Thompson (2010) demonstrated that **pollen limitation** (the reduction in seed production due to inadequate pollination) of reproduction has increased over a 17-year period, suggesting that plant-pollinator asynchrony may be increasing over time.

Articles To Read

Haggerty, B. P., and L. F. Galloway. 2011. Response of individual components of reproductive phenology to growing season length in a monocarpic herb. Journal of Ecology 99:242-253.

Inouye, D. W. 2008. Effects of climate change on phenology, frost damage, and floral abundance of montane wildflowers. Ecology 89:353-362.

Thomson, J. D. 2010. Flowering phenology, fruiting success and progressive deterioration of pollination in an early-flowering geophyte. Philosophical Transactions of the Royal Society B-Biological Sciences 365:3187-3199.

Discussion Questions

1. What components of plant reproductive phenology did Haggerty and Galloway (2011) measure?

2. What is the benefit of **reciprocally transplanting** plants from high elevations and low elevations to evaluate phenological responses to climate change? How did growing conditions and the length of the growing season differ among the transplant sites?

3. What do the estimates of **phenotypic selection** reported by Haggerty and Galloway (2011) suggest about phenological responses to climate change in the American bellflower (*Campanulastrum americanum*)?

4. Which components of *C. americanum's* reproductive phenology would you expect to evolve in response to global warming? What evidence from the paper supports your assertion?

5. What empirical measurements did Inouye (2008) record on his focal plants? How were climate data for the study region obtained?

6. Which wildflower species was most vulnerable to "frost-kills"? What evidence from the paper supports your opinion? (Inouye 2008)

7. Describe the relationship between "day of year with bare ground" and flowering phenology for each of the three wildflower species. What do you think contributes to the breakpoints in the plots presented in Figures 2, 5, and 8? (Inouye 2008)

8. Describe the experimental design of Thompson's (2010) pollen limitation experiments. How did Thompson (2010) quantitatively estimate pollen limitation of **fruit set** and **seed set** in *Erythronium grandiflorum*?

9. Thompson (2010) detected pollen limitation of reproduction in *Erythronium grandiflorum.* In which “cohort” was pollen limitation most severe? Justify your answer using evidence from the paper.

10. In which of the three studies presented here were the researchers able unambiguously to attribute phenological shifts to climatic conditions? Do you agree with their conclusions?

11. In which of the three studies presented here were the researchers unable to clearly attribute phenological shifts to climatic conditions? Do you agree with their conclusions?

12. What are some challenges associated with simultaneously studying climate change and phenology? How might those challenges be overcome/addressed?

Glossary

- **Fruit set:** the proportion of flowers on a single plant that develop into fruits

- **Geophyte:** A perennial plant with an underground food storage organ, such as a bulb, tuber, corm, or rhizome. The above-ground parts of the plant die away during adverse conditions, such as winter or summer drought, and then re-grow from buds produced by the underground portion when conditions improve. A tulip is a geophyte.

- **Mutualist:** an organism that engages in an ecological interaction with another organism from which both participants derive some benefit. A bumblebee that pollinates a flower while consuming floral nectar is an example of a mutualist.

- **Pollen limitation of reproduction:** a reduction in plant reproductive output caused by insufficient pollination

- **Phenological synchrony:** the degree to which phenological traits coincide among individuals in a population or community

- **Phenotypic plasticity:** the expression of an altered phenotype in response to environmental conditions. For example, many plant species flower in the spring shortly after the average weekly temperature exceeds a certain threshold. In environments or years that warm up relatively early in the spring, plants may respond by flowering earlier than they would in cooler environments or years. In this case, flowering time would be a plastic response to local or temporary environmental conditions.

- **Phenotypic selection analysis:** a statistical approach that estimates the strength and direction of natural selection on individual traits by measuring relationships between trait phenotypes and organismal fitness.

- **Reciprocal transplant:** an experimental method where plants from different environments are planted in one another's environment. For example, Haggerty and Galloway (2011) planted seeds collected from populations of the American bellflower that occupied low elevation sites in locations occupied by the same species but at high elevations (and *vice versa*).

- **Seed Set:** the proportion of ovules in a single ovary that develop into seeds

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Response of individual components of reproductive phenology to growing season length in a monocarpic herb

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Summary

1. Climate change is causing the growing season to expand and many plants are flowering earlier. However we know less about whether other components of reproductive phenology are altered or whether these changes in phenology are adaptive.

2. We evaluated reproductive phenology and fitness components for populations of *Campanulastrum americanum* sampled across an elevation gradient and reciprocally transplanted into common gardens at high and low elevations.

3. The low-elevation planting site had an expanded growing season that induced the advance of bolting, flowering, average flower date, and time to fruit maturity relative to the high-elevation site for transplants. With the exception of flowering initiation, each successive stage of reproduction was advanced more than the previous one, resulting in a compressed phenology in the warmer environment.

4. In contrast, populations from low elevation had a longer reproductive cycle when grown at both sites, with each phenological component extended relative to populations from high elevation. Fruit production indicated populations were locally adapted to elevation, suggesting these differences in phenology are adaptive.

5. Selection on phenological characters was stronger on transplants in the expanded low-elevation growing season, favouring delayed bolting and advanced flowering. Plastic response to the longer growing season was adaptive for flowering time but maladaptive for bolt initiation.

6. *Synthesis*. The compressed reproductive phenology favoured in the expanded growing season expected under climate change will largely be achieved with adaptive plasticity of individual phenological traits. Traits under selection in the longer growing season were genetically differentiated between populations that currently differ in growing season length, suggesting evolutionary malleability and likely modification of reproductive phenology in response to climate change.

Key-words: elevation, flowering time, growing season length, local adaptation, phenology, phenotypic plasticity, phenotypic selection, plant–climate interactions, reciprocal transplant, reproductive phenology

Introduction

Warming climates have resulted in an earlier onset of spring temperatures which has led to earlier flowering in many plant taxa (e.g. Bradley *et al.* 1999; Fitter & Fitter 2002; Peñuelas, Filella & Comas 2002; Parmesan & Yohe 2003; Parmesan 2007). Warm summer temperatures are also persisting later (Linderholm 2006) and, in response, trees are holding their leaves longer into the autumn (Menzel & Fabian 1999; Peñuelas, Filella & Comas 2002; Richardson *et al.* 2006). In total, the warming climate has expanded the growing season. However, despite numerous observations of changes in the timing of bud break, flowering and leaf drop in the longer growing seasons, we have little knowledge of whether these changes are adaptive (but see Inouye 2008).

In addition, although there is a wealth of data on earlier flowering in warming climates, we know less about how

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accelerating the initiation of reproduction may change subsequent reproductive events (Post *et al.* 2008). Earlier flowering plants have been shown to mature fruit earlier (Peñuelas, Filella & Comas 2002; Post *et al.* 2008), especially in species that bloom early in the growing season (Sherry *et al.* 2007). However, it is not known whether this change reflects patterns of maturation of individual fruits or simply an earlier initiation of reproduction. For example, if reproduction is tightly integrated across the life cycle (cf. Pigliucci 2003), the earlier reproductive development induced by warmer spring temperatures would also advance flowering time, flower deployment and fruit maturation, shifting the entire reproductive schedule. However, if later reproductive stages, e.g. flower production and fruit maturation, can respond independently to favourable conditions in the longer growing season, reproductive phenology might expand in warmer climates. Alternatively, later phenological stages may be accelerated in novel, warmer conditions, resulting in a compressed reproductive phenology. Finally, other aspects of the environment, such as water availability, may change with warmer temperatures and also alter reproductive phenology (Giménez-Benavides, Escudero & Iriondo 2007; Jentsch *et al.* 2009). A focus on individual elements of reproductive phenology is needed to understand the duration of reproduction as well as potential fitness consequences of an altered reproductive schedule.

Studies across elevations provide particularly useful insight into the phenological changes expected in response to an expanded growing season. At high elevations the growing season starts late and ends early, whereas at low elevations it begins earlier and ends later. Initiation of reproduction tracks elevation-associated changes in spring warming in a number of species (reviewed in Stinson 2004). With warming climates, populations at higher elevations will be faced with longer growing seasons similar to those of current lower elevations. Studies across elevations permit a comparison of response to growing season length over short geographic distances allowing regional-scale weather patterns and photoperiod to be held constant.

We took advantage of the differences in growing season length associated with elevation to determine (i) the effect of growing season length on individual components of reproductive phenology and (ii) whether plastic responses to a longer growing season were adaptive. Plants from high elevation were transplanted to low elevation to create an expanded growing season. By also transplanting in the complementary direction, it was possible to determine the extent of phenological differentiation between populations from different seasonal environments. Such differentiation provides insight into any potential long-term evolutionary response to changes in growing season length. We used *Campanulastrum americanum*, a monocarpic herb for which previous work found elevation-associated differences in flowering time in a uniform environment (Galloway, Etterson & Hamrick 2003), reproductive traits that were tightly integrated when flowering time was manipulated in a natural population (Galloway & Burgess 2009), and for which artificial selection rapidly altered flowering time (Burgess, Etterson & Galloway 2007). Using a reciprocal transplant design, we addressed the following specific questions. (i) What are the effects of an expanded growing season on the suite of phenological traits that span the initiation and progression of reproduction? (ii) Do plastic responses to growing season length involve a shift in all components of reproductive phenology, or do individual phenological components respond separately? (iii) Do populations from different growing season lengths differ in phenology and fitness components, and if so, is there evidence of local adaptation? (iv) Does phenotypic selection on phenological characters differ between low- and high-elevation environments, and are phenological responses to an expanded growing season adaptive?

Materials and methods

STUDY SYSTEM

Campanulastrum americanum Small (= *Campanula americana* L; Campanulaceae) is an insect-pollinated outcrossing herb common to open deciduous woods, moist borders and steep slopes throughout eastern and central North America (Shetler 1962; Galloway, Cirigliano & Gremski 2002; Galloway, Etterson & Hamrick 2003). The present study was conducted in southwest Virginia, where natural populations of *C. americanum* are found across a range of elevations from *c.*500 m to 1400 m a.s.l. *Campanulastrum americanum* is monocarpic; rosettes must be vernalized prior to initiating bolting in the spring. Therefore seeds that germinate in the autumn, such as in the present study, have a winter annual life-history. Plants flower in mid- to late-summer with compact inflorescences at reproductive nodes on the main stem and lateral branches. Flowers typically last for 2 days (Evanhoe & Galloway 2002). Fruits contain 10–40 seeds, ripen after *c.*6 weeks (Galloway 2002), and are persistent, allowing fruit production to be assessed at the end of the season.

PLANTING SITE ENVIRONMENTS

Reproductive phenology and fitness components were assessed for plants grown in low- and high-elevation planting sites. The high-elevation planting site was located in the understorey of a mixed deciduous forest (1194 m) and the low-elevation site in a riparian mixed deciduous forest (514 m, see Table S1 in Supporting Information). Seasonal patterns of temperature and precipitation during the experiment were described with data from the Mountain Lake Biological Station (MLBS) and Kentland Farm meteorological data bases (3 and 0.5 km from and similar in elevation to the high- and low-elevation planting sites respectively). Temperature during the experiment was compared to a long-term mean (1972–1997) at each site. For the high-elevation planting site, data were obtained from the National Climatic Data Center for MLBS (NCDC Co-Op ID 445828); the nearest NCDC station to the low-elevation planting site was 10 km away and similar in elevation (NCDC Co-Op ID 440766). Photosynthetically Active Radiation (PAR) was measured at each planting site in cloud-free conditions using a portable PAR-meter on August 12 and 13. Measurements were taken at 1.5 m heights (slightly taller than most plants) at the four corners and middle of the eight planting blocks per site.

EXPERIMENTAL DESIGN

Fruits were collected by maternal plant from five natural populations located on an elevation gradient (see Table S1). Two populations, L1 and L2, were sampled from low-elevation sites on the flood plain of the New River; an intermediate elevation population was sampled at the base of Salt Pond Mountain; and two populations were sampled from high elevation, H1 and H2 (near the high-elevation planting site), near the tops of Beanfield and Salt Pond Mountains, respectively. Tests of the effect of planting site and population origin on phenology and fitness only included populations from the high and low elevations as only those populations were transplanted reciprocally. The phenotypic selection analysis also included the intermediate population to create a broader phenotypic distribution. Fruits were collected from approximately 60 haphazardly selected maternal plants per population.

Seeds were germinated in autumn 2004 under controlled conditions. Three seeds were sown per plug in plug trays filled with MetroMix 200® (Sun Gro Horticulture, Vancouver, British Columbia, Canada) for 50 maternal families in each population (30 for L2). This was replicated six times (eight for L2). The seeds were germinated under near-optimal conditions in a growth chamber (23 °C day/14 °C night; 12:12 L:D). The first seedling to germinate in each plug was retained and subsequent seedlings were removed. Seeds from the low-elevation populations germinated on average 1.5 days earlier than those from the high-elevation populations. A month after planting, seedlings were placed outdoors for 1 week to acclimate to ambient temperatures.

Seedlings were reciprocally transplanted into the two sites in the late autumn in a randomized block design. Eight blocks were created per site, and fenced to exclude mammalian herbivores. All blocks were cleared of existing vegetation before transplanting and weeded regularly throughout the experiment. In total, 1440 plants were transplanted with approximately 150 from each source population in each planting site distributed across families. Individuals were spaced 0.33 m from each other. Season extenders (plastic cups with bottoms cut-off, staked upside down) were affixed around each seedling to help reduce transplant shock in cool fall weather. Transplant mortality was assessed when season extenders were removed 1 week later; individuals that did not survive transplant were dropped from the data set (2.1%). Fall rosette size, measured as the number of leaves multiplied by the length of the longest leaf, was also assessed at this time. Survival (presence/absence) was determined prior to bolting in the spring and again when plants began flowering.

Reproductive phenology was followed from bolting through seed maturation. Bolting phenology was assessed by measuring plant height every 2 weeks until the opening of the first flower. Bolt initiation was recorded as the date of stem height > 1.5 cm. Plants were censused every 3 days for flowering initiation, defined as the first open flower. Bolting duration was the number of days from bolt initiation to flowering initiation. Floral display, scored as the number of open flowers, was censused every 6 days throughout the flowering season. Flowering duration was the number of days between an individual's first and last flower. Seasonal patterns of flower production for each plant were summarized with 'average flower date,' the mean date that an individual's flowers were produced. Average flower date was calculated by weighting each census date by the proportion of an individual's total flower production that was open on that date and summing over all census dates (cf. Nuismer & Cunningham 2005). A small average flower date indicates most of an individual's flowers are produced early in the season, whereas large values indicate greater late-season floral production. Finally, one open flower was tagged on each plant at weekly intervals. Because flowers are typically open for 2 days, the date flowers were tagged approximates the date of fruit initiation. Tagged fruits were checked approximately every 4 days for the opening of lateral pores which indicated maturity. Fruit maturation time was the number of days from when the flower was tagged until the fruit matured.

An index of reproductive duration was estimated from these sequential phenological traits as the sum of bolting duration, the time from flowering initiation to the average flower date and the maturation time of the first fruit. Fruit maturation time was only available for 41% of the plants due to an error in data collection. Therefore mean fruit maturation time, estimated for each population in each planting site, was used when data for an individual was missing. Calculating reproductive duration using average flower day, rather than flowering duration, results in estimates that are more conservative for demonstrating differences between planting sites and elevation of population origin.

Plants were harvested when almost all fruits had dehisced. They were dried, weighed for above-ground biomass and fruits counted. Cumulative fitness was estimated by number of fruits; individuals that did not survive were assigned a fitness of zero. Therefore cumulative fitness combines both survival and reproduction.

STATISTICAL ANALYSIS

Planting site environments

Meteorological variables and light availability were compared between planting sites. A difference between sites for PAR was determined in an ANOVA with planting site as a fixed effect and block nested within planting site as a random effect. Temperature and daily-accumulated precipitation were compared between planting sites by performing a repeated measures ANOVA with planting site as a fixed effect.

Comparison of sites and populations

ANCOVA was used to evaluate differences between planting sites and populations from low and high elevation for phenological characters and fitness components. Planting site, elevation of population origin (only populations from low and high elevation), population nested within elevation, the planting site × elevation interaction, and the planting site × population interaction were treated as fixed effects, and block nested within planting site was included as a random effect. Differences between sites correspond to environmental effects and those between elevations or populations represent genetic effects. A planting site × elevation interaction indicates that populations from different elevations differ in their plastic response to the environments of the planting sites, i.e. genetic differences in plasticity. Fall rosette size, a measure of variation among individuals at planting, was included as a covariate for all traits. Any differences in size due to the slightly earlier germination of the low-elevation populations will be accounted for by the covariate, rather than contributing to the results of adult traits. Survival between the fall rosette, spring rosette and flowering initiation stages was analyzed with a loglinear analysis using a comparable model to the above ANCOVA, assuming a binomial distribution and a logit link function. Fruit maturation time was analyzed with repeated measures ANOVA using the above analysis with the inclusion of fruit initiation date. Unfortunately the maturation date of a number of the later fruits is missing; therefore an additional analysis was conducted using the first tagged fruit to mature on each plant (fruits initiated in the first 2 weeks of flowering).

To evaluate whether phenological traits respond to local environments independently, they were reanalyzed using the previous phenological stage as an additional covariate. Path analysis is an alternate approach to addressing the interrelated phenological traits (see Table S2). However, path analysis is only effective when a branched diagram can be constructed; the linear nature of the sequential phenological traits measured here precluded this analytical approach.

To meet assumptions of normality, biomass and cumulative fitness + 1 were ln-transformed while fruit production was square-root transformed. The significance testing is approximate for cumulative fitness due to the large number of zeros in the data set. However, analyses of fitness means across groups of individuals yielded qualitatively similar results.

Phenotypic selection analysis

Phenotypic selection acting on phenological traits was estimated for each planting site to evaluate whether plastic changes in trait expression between environments were adaptive (Lande & Arnold 1983; Mitchell-Olds & Shaw 1987). Individuals from all five populations were included to expand the phenotypic distribution and enhance the ability to detect selection (Wade & Kalisz 1990; Conner & Hartl 2004). The phenological traits, date of bolt initiation, date of flowering initiation, average flower date and flowering duration were included in the analysis. Bolting duration was not included because it is not independent of bolt initiation and flowering initiation. Analyses initially included fruit maturation time, but that variable was dropped due to lack of significant selection combined with missing data for a number of individuals. Biomass was included in the model to control for any covariance between phenology and plant size, and population was included to account for the lack of independence among plants from the same population. Traits were first standardized to a mean of zero and variance of unity. Pearson correlation coefficients were then calculated to determine correlations between characters (see Table S2). To ensure that multicollinearity among these traits did not influence results, we estimated variance inflation factors and found all were less than five, implying limited multicollinearity (Kutner, Nachtsheim & Neter 2004).

Relative fitness was calculated by dividing individual reproductive success (fruit production) for plants that survived to flowering by the site-specific mean (Lande & Arnold 1983). Selection differentials (S_i) were calculated as the covariance between relative fitness and each standardized character (i), and are measures of direct linear selection on character i as well as any indirect effects from selection on correlated characters. Standardized linear selection gradients (β_i) were calculated as partial regression coefficients from the multiple regression of relative fitness on all traits. Thus, β_i is a measure of the effect of each trait i on relative fitness, holding all other traits fixed. Standardized nonlinear selection gradients (γ_i) were obtained as double the parameter estimates from a multiple regression analysis of relative fitness on all traits and their squares (Stinchcombe *et al.* 2008). Negative values of γ_i indicate a decelerating relationship between trait values and fitness, with stabilizing selection present when intermediate trait values have the greatest fitness. Positive values of γ_i indicate accelerating selection where a unit change in the trait is associated with a greater fitness increase for more extreme trait values.

We determined the significance of the selection gradients using 95% confidence intervals estimated by creating 10 000 replicate data sets in a bootstrap of the original data. The patterns of significance for both linear and quadratic selection gradients in each of the two planting sites were consistent with those of parametric tests, so the results of the original regressions are reported. ANCOVAS were performed to determine whether the magnitude of linear or quadratic selection differed between sites with phenological traits as covariates and planting site a fixed effect. Significant interactions between planting site and the phenological traits indicate that the pattern of selection differs between environments.

Results

PLANTING SITE ENVIRONMENTS

The planting sites at high and low elevation differed in temperature but not in accumulated precipitation (Site $F_{1,686} = 2.97$, $P = 0.09$; Site × Month $F_{11,686} = 0.12$, $P = 0.99$) or light (PAR Site: $F_{1,64} = 2.37$; $P = 0.15$). Repeated measures ANOVA revealed that mean temperatures during the experiment were warmer at the low-elevation site ($F_{1,686} = 78.4$; $P < 0.001$) and fluctuated synchronously at both sites throughout the experiment (Site × Month: $F_{11,686} = 0.21$; $P = 0.99$). As a consequence, warm spring temperatures occurred earlier and fall frosts later at the low-elevation site. Temperatures at the planting sites during summer growth and reproduction were representative of long-term patterns at those locations (April–August 2005, elevation mean: high 14.99 °C, low 18.02 °C; long-term elevation mean ± SE: high 14.73 ± 1.83 °C, low 17.58 ± 2.00 °C).

REPRODUCTIVE PHENOLOGY

In both planting sites, bolting phenology differed between populations from high and low elevations. Populations from high elevation initiated bolting late and rapidly produced a fully developed flowering stem in both sites, whereas those from low elevation initiated bolting early and required more time to produce a flowering stem (Table 1, Fig. 1a and d). On average, the bolt initiation date in the low-elevation site was advanced by about 7 days relative to the high-elevation site (Fig. 1a). However, planting site had no average effect on bolting duration because populations had opposite plastic responses to changes in elevation. Populations from low elevation had shorter bolting duration in the low-elevation site whereas populations from high elevation had shorter bolting duration high-elevation site (Table 1, Fig. 1d). As a result of these combined variables, there is less difference between populations from high and low elevations for flowering initiation date than for bolting traits. Similar to bolt initiation, flowering initiation was advanced by about 7 days at the low-elevation site (Fig. 1b). There were no differences between populations from low and high elevations for flowering initiation in the low-elevation site. However at the high-elevation site, populations from low elevation flowered significantly later than those from high elevation (Table 1, Fig. 1b).

Seasonal flowering patterns differed between the planting sites (Fig. 2). The low-elevation environment induced an advance in average flower date by *c.*14 days. In both sites, populations from high elevation had an earlier average flower date than those from low elevation (Table 1, Fig. 1c), indicating that populations from high elevation produce the majority of their flowers early in the season, whereas those from low

Table 1. ANCOVA of reproductive phenology for populations of *Campanulastrum americanum* sampled from high and low elevations and reciprocally transplanted into common gardens at those elevations. *F*-values are reported for fixed effects and *Z*-values for random effects. (*)$P < 0.10$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

	Planting site	Elevation of origin	Pop(Elev)	Site × Elev	Site × Pop(Elev)	Rosette size	Block(Site)
Bolt initiation	57.00***	143.24***	2.05	7.81**	5.77**	14.53***	1.38
Bolting duration	0.21	222.28***	2.68(*)	25.86***	5.86**	4.61*	1.48
Flowering initiation	155.95***	18.76***	4.57**	13.27***	0.72	6.82**	2.11*
Average flower date	240.53***	29.77***	13.02***	0.00	1.12	0.76	2.11*
Flowering duration	55.12***	12.79***	4.63**	19.14***	1.66	1.00	2.08*
Fruit maturation	127.44***	5.06*	1.00	1.13	0.78	0.01	1.35(*)
Reproductive duration	347.91***	202.85***	10.62***	9.88**	9.87***	3.89*	1.62(*)
Numerator d.f.†	1	1	2	1	2	1	–

†*F* Statistics: Denominator d.f. = 230–646, except site denominator d.f. = 14.

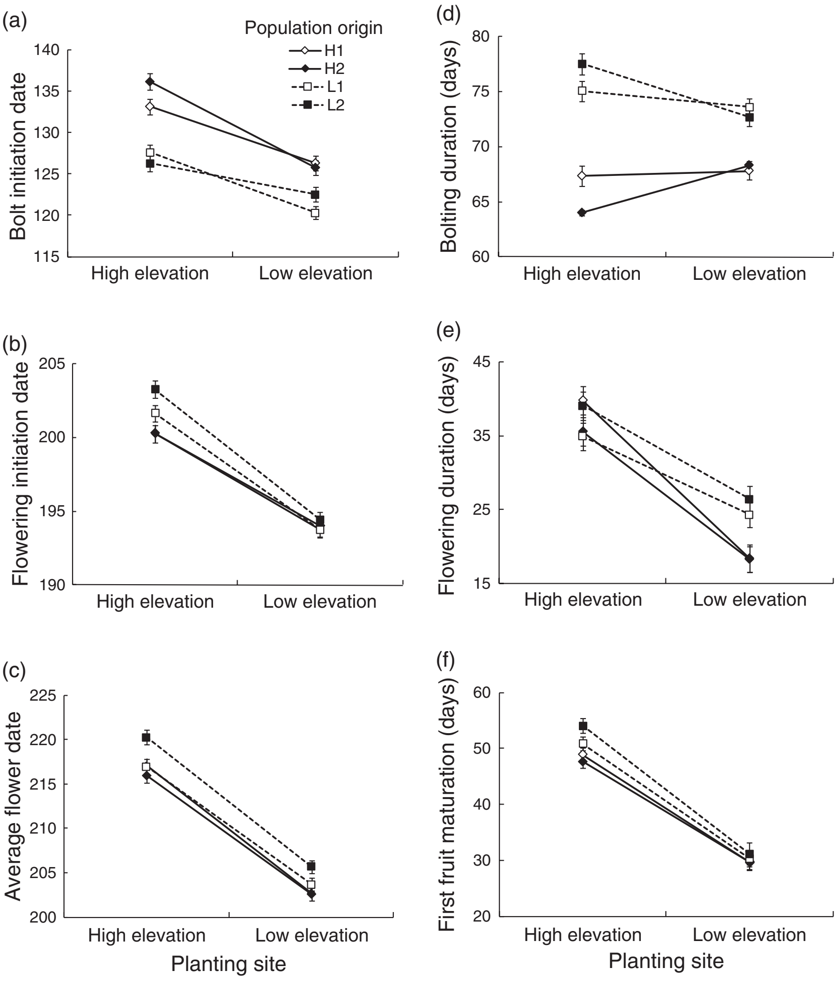

Fig. 1. The effect of elevation on reproductive phenology (lsmean ± SE) for populations of *Campanulastrum americanum* collected from high and low elevations and reciprocally transplanted to common gardens at high- and low-elevation sites. Populations H1 and H2 originated from high-elevation sites while L1 and L2 came from low-elevation sites. Traits include the date of (a) bolt initiation, (b) flowering initiation, and (c) the average flower, as well as duration of (d) bolting, (e) flowering, and (f) maturation of the first tagged fruit. Dates are given in days of the year.

elevation produced more flowers late in the season (Fig. 2). Flowering duration was almost twice as long at the high-elevation site than the low-elevation site (Table 1, Figs. 1e and 2). The flowering duration of populations from low and high elevations was similar when plants were grown at high elevation. However in the low-elevation site, flowering duration of populations from high elevation was less than those from low elevation. On average, populations from high elevation had shorter fruit maturation time than those from low elevation (Tables 1 and 2; Figs. 1f and 3). Fruit maturation time was also more rapid at the low-elevation site with the first fruit taking an average of 20 days less time to ripen than at the high-elevation site (Table 1; Fig. 1f). Over the season, time to fruit maturation of high- and low-elevation populations was similar when grown at low elevation ($F_{1,178} = 0.59$, $P = 0.44$) but in the high elevation site was shorter for

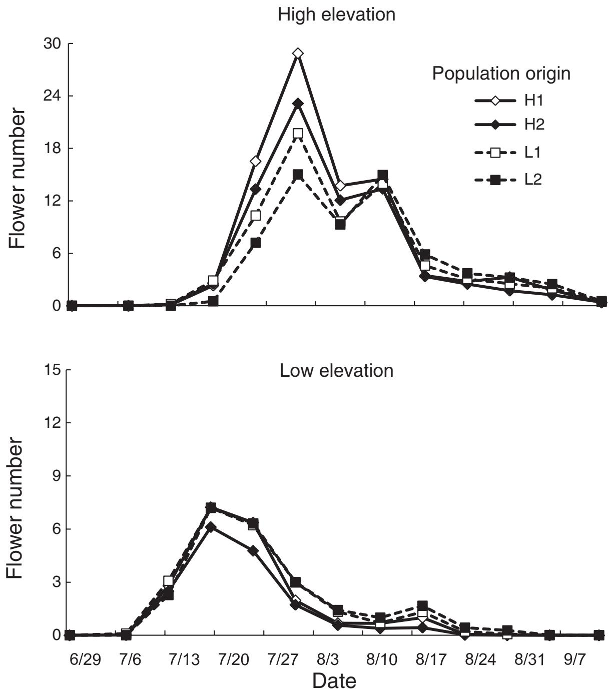

Fig. 2. Seasonal floral display size of populations of *Campanulastrum americanum* collected from high (H1, H2) and low (L1, L2) elevations and reciprocally transplanted to common gardens at high- and low-elevation sites. Note that the scale of the *y*-axis for the high-elevation site is twice the size of the low-elevation site.

Table 2. Repeated measures ANOVA to evaluate effects of planting site and elevation of origin on the maturation time of *Campanulastrum americanum* fruit initiated at weekly intervals over the reproductive season. *F*-values are reported for fixed effects and *Z*-values for random effects. Denominator degrees of freedom = 273 (Planting site denominator d.f. = 14)

	Num d.f.	*F*/*Z*	*P* <
Planting site	1	227.07	0.001
Elevation of origin	1	12.88	0.001
Population(Elevation)	2	0.86	0.424
Site × Elevation	1	4.89	0.028
Site × Population(Elev)	2	1.99	0.139
Date fruit initiated	4	35.74	0.001
Date*Planting site	2	2.57	0.078
Date*Elevation	4	2.86	0.024
Date*Population(Elev)	8	1.83	0.074
Date*Site*Elevation	2	2.68	0.070
Block(Site)		1.26	0.104

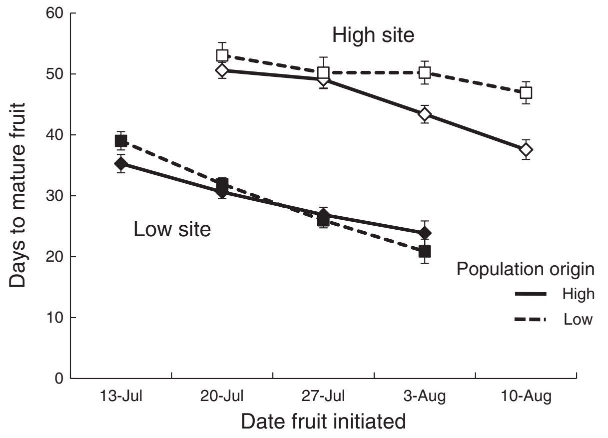

Fig. 3. The number of days for fruit maturation over the reproductive season for *Campanulastrum americanum* collected from high and low elevations and reciprocally transplanted to common gardens at high- and low-elevation sites. Populations that originated from the same elevation were combined for presentation.

high-elevation than low-elevation populations ($F_{1,91}$ = 5.59, P = 0.02; site × elevation Table 2). Fruit maturation time shortened over the season at both planting sites, with final fruit maturation time over a week shorter than initial maturation time (Table 2, Fig. 3).

The differences in phenology between plants grown in high- and low-elevation sites and between populations from high and low elevations were found for each successive reproductive stage. Patterns of differentiation between sites and between populations from different elevations changed little when the previous phenological stage was included as a covariate (Table 3). The exception was average flower date. After accounting for differences in initiation of flowering, greater divergence in average flower date was found between populations from low and high elevation in the low-elevation site than the high-elevation site (Table 3). However, for most traits populations from low- and high-elevation were more similar when grown in the low elevation site. Least-square means from the other analyses were very similar to Fig. 1, indicating that successive phenological components each responded to the local environment, but the pattern of response was similar across the reproductive phenology. As a consequence of the different responses among traits, reproductive duration was 21% shorter in the low-elevation site than the high-elevation site and 11 days shorter in plants that originated from high elevation than those from low elevation (Table 1, Fig. 4).

FITNESS COMPONENTS

The probability of an individual surviving to flower was a function of the planting site and the elevation from which it originated, but not their interaction (Table 4). Survival to flowering can be divided into two life cycle stages: the overwinter interval and the spring growth interval. Although statistically similar during the winter interval, survival was significantly greater in the low-elevation site during the spring interval and over the entire fall to flowering period (Table 4, Fig. 5a). Across both planting sites, populations from low elevation had a greater proportion of individuals survive to flowering than those from high elevation.

Although overall fruit production was greater in the high-elevation site, fruit production within each planting site was greater for plants grown at their native elevation (Table 4,

Table 3. ANCOVA to evaluate stage-specific effects of planting site and elevation of origin on reproductive phenology of *Campanulastrum americanum* sampled from high- and low-elevation populations and reciprocally transplanted into common gardens at those elevations. For each trait, rosette size at transplant is the upper covariate and the previous phenological stage is the lower covariate. *F*-values are reported for fixed effects and *Z*-values for random effects. (*)$P < 0.10$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

	Planting site	Elevation of origin	Pop(Elev)	Site × Elev	Site × Pop(Elev)	Covariate	Block(Site)
Flowering initiation	125.86***	39.96***	4.40*	19.31***	1.53	3.54(*)	2.09*
Bolt initiation						25.95***	
Average flower date	103.89***	15.29***	9.07***	4.34*	1.18	0.25	2.09*
Flower initiation						177.52***	
Flowering duration	79.54***	19.70***	6.42**	13.46***	2.02	0.25	2.07*
Flower initiation						24.69***	
Fruit maturation	158.17***	8.85**	0.91	3.04(*)	0.34	0.41	1.34(*)
Fruit initiation						33.58***	
Numerator d.f.†	1	1	2	1	2	1	–

†*F* Statistics: Denominator d.f. = 288–597, except site denominator d.f. = 14.

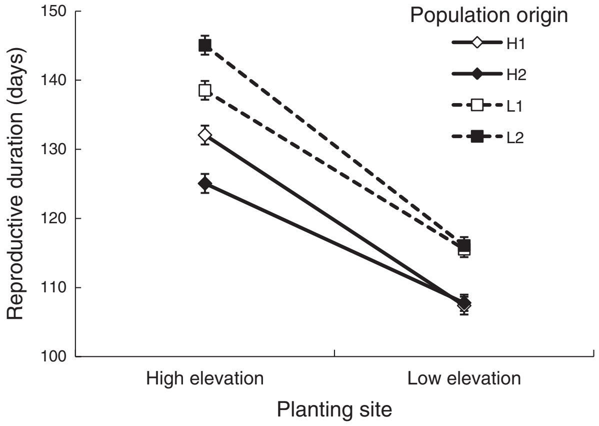

Fig. 4. Index of reproductive duration (± SE) of populations of *Campanulastrum americanum* collected from high (H1, H2) and low (L1, L2) elevations and reciprocally transplanted to common gardens at high- and low-elevation sites.

Fig. 5b). Cumulative fitness was more similar between sites than its components due to opposite responses to the sites for survival and fruit production (Fig. 5c). At the high-elevation site cumulative fitness did not depend on a population's origin, while populations from low elevation had greater cumulative fitness at the low-elevation site (Table 4, Fig. 5c). As a result, overall cumulative fitness was greater for populations from low elevation.

PHENOTYPIC SELECTION ANALYSIS

Phenotypic selection analyses revealed stronger total and direct selection on phenological traits in the expanded growing season of the low-elevation site than in the high-elevation site (Table 5). Total selection (S) favoured earlier bolt and flowering initiation, and a longer flowering duration at both sites, along with a later average flower date at the low-elevation site (Table 5). Direct linear selection (β) in the low-elevation site favoured later bolt initiation and earlier flowering (Table 5). The magnitude of selection on flowering initiation was *c.*2.5 times greater than on bolt initiation. Direct linear selection was not detected for any phenological traits in the high-elevation site.

Nonlinear selection (γ) was only detected in the low-elevation site (Table 5). A positive quadratic selection gradient for flowering initiation indicated that relative fitness was an

Table 4. Log-linear analysis of survival and analysis of covariance of fruit production and estimated fitness for populations of *Campanulastrum americanum* sampled from high and low elevations and reciprocally transplanted into common gardens at those elevations. Chi-square statistics reported for survival; for other traits *F*-values are reported for fixed effects and *Z*-values for random effects. **$P < 0.01$, ***$P < 0.001$

	Planting site	Elevation of origin	Pop(Elev)	Site × Elev	Site × Pop(Elev)	Rosette size	Block(Site)
Survival							
Fall to Spring	0.15	0.62	4.00	0.08	0.10	30.07***	251.56***
Spring to Flower	8.26**	9.64**	3.19	0.55	1.93	29.41***	16.65
Fall to Flower	30.14***	11.29***	0.90	0.73	1.07	35.56***	207.99***
Fruit production	17.23***	0.36	0.83	16.66***	0.36	26.06***	2.34**
Cumulative fitness	0.81	20.70***	0.22	9.26**	0.35	38.16***	2.55**
Numerator d.f.†	1	1	2	1	2	1	–

†*F* Statistics: Denominator d.f. = 415–934, except site denominator d.f. = 14.

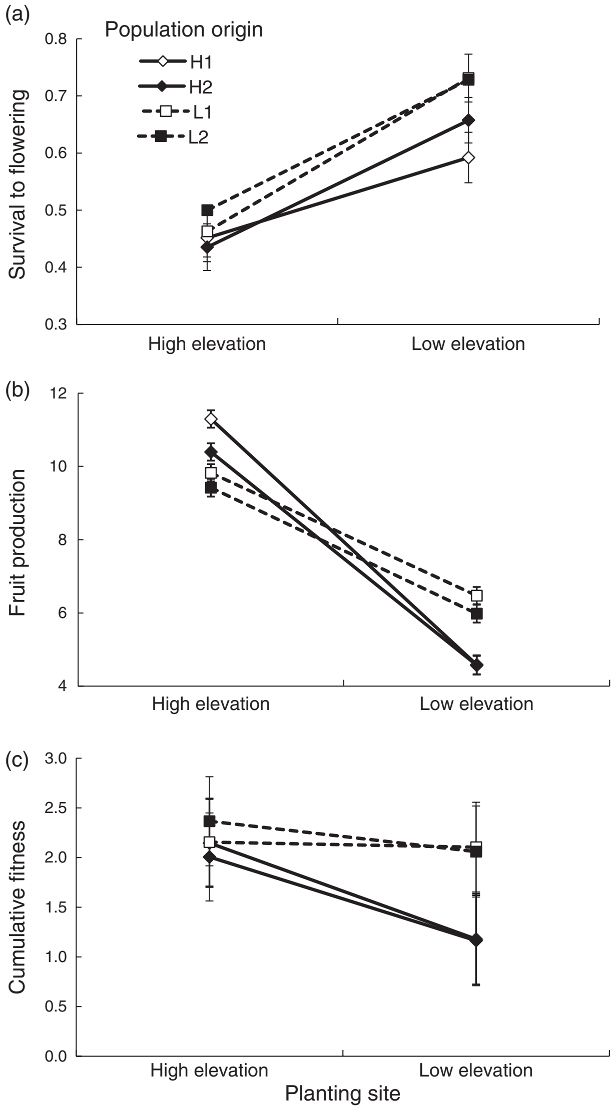

Fig. 5. The effect of elevation on mean (± SE) (a) survival to flowering, (b) fruit production (lsmeans), and (c) cumulative fitness (lsmeans) for populations of *Campanulastrum americanum* collected from high (H1, H2) and low (L1, L2) elevations and reciprocally transplanted to common gardens at high- and low-elevation sites.

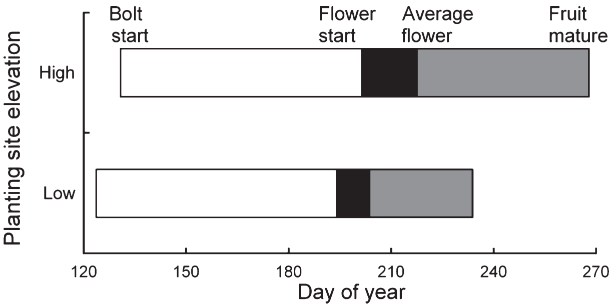

Fig. 6. Summary of reproductive phenology at the low- and high-elevation planting sites. Each segment indicates the length of time between successive phenological stages. Total bar length represents reproductive duration, from initiation of bolting to the maturation of a fruit open on the average flower date at each site.

accelerating function of earlier flowering such that earlier flowering resulted in a larger increase in fitness for early-flowering plants than for later-flowering ones. A negative quadratic selection gradient was found for average flower date. Graphical inspection revealed a peak within the phenotypic distribution, indicating stabilizing selection on patterns of flower deployment.

Discussion

RESPONSE TO EXPANDED GROWING SEASON

The low-elevation site had an expanded growing season, resulting from an earlier spring and a later fall, that induced the advance of a suite of phenological characters in *C. americanum*. Bolting, flowering and average flower dates of experimental plants were each at least 1 week earlier in the low-elevation site (Fig. 6). The duration of flowering and the time required to mature a fruit were also substantially shorter when plants were grown at low elevation. *C. americanum*'s earlier reproductive phenology in a longer growing season is similar to results of other studies that have experimentally manipulated growing season length or the onset of warm, spring-like temperatures (e.g. Clausen, Keck & Hiesey 1940, 1948; Galen & Stanton 1993; Price & Waser 1998; Dunne, Harte & Taylor 2003; Aerts

Table 5. Standardized selection differentials (S), linear gradients (β), and quadratic gradients (γ) for populations of *Campanulastrum americanum* sampled along an elevation gradient and planted in common gardens at high and low elevations. Source population was also included in the models as a blocking term. Selection is compared between the planting sites with *F*-values in the Site × S, Site × β and Site × γ columns. High-elevation site $N = 294$, low-elevation site $N = 245$. (*)$P < 0.10$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

	S			β			γ		
Trait	High site	Low site	Site × S	High site	Low site	Site × β	High site	Low site	Site × γ
Bolt initiation	−0.207***	−0.274***	7.87**	0.0002	0.070*	1.98	−0.017	−0.010	0.01
Flower initiation	−0.180***	−0.269***	2.48	−0.029	−0.120**	3.96*	−0.016	0.096**	8.68**
Ave flower date	−0.032	0.339***	19.65***	−0.0006	0.073	0.80	0.016	−0.097*	5.59*
Flowering duration	0.232***	0.676***	44.50***	0.040(*)	0.023	0.01	0.033	0.032	0.10
Biomass	0.761***	0.942***	42.10***	0.752***	0.900***	15.63***	−0.041(*)	0.037	3.21(*)

et al. 2004; Stinson 2004; Sherry *et al.* 2007; Hovenden *et al.* 2008). The advance in reproductive phenology also matches the phenological shifts observed in warming climates (e.g. Peñuelas, Filella & Comas 2002; Walther *et al.* 2002; Parmesan & Yohe 2003; Miller-Rushing & Primack 2008; Gordo & Sanz 2009). Finally, the advance in reproductive phenology in the warmer low-elevation site supports expectations from our knowledge that temperature is the main driver of phenology (Ratchke & Lacey 1985; Badeck *et al.* 2004; Gordo & Sanz 2010; Hülber, Winkler & Grabherr 2010), in part because it accelerates plant development (reviewed in Saxe *et al.* 2001). Photoperiod, the second most important trigger of spring phenology (Badeck *et al.* 2004), which accurately predicts onset of reproduction in winter annual *Arabidopsis thaliana* when combined with temperature (Wilczek *et al.* 2009), is constant across the two planting sites in the present study. The fact that our results are consistent with experimental and observational studies as well as expectations from physiology suggests that the low-elevation conditions in the present study simulated a warming climate relative to high-elevation conditions, despite the potential for additional abiotic factors to differ between the sites.

The expanded growing season resulted in a nonlinear shift in reproductive phenology. Bolting and flowering were each about 7 days earlier under the warmer low-elevation conditions indicating the advance of reproductive initiation by a week. Average flower date was 14 days earlier in the longer growing season, a week beyond that due to initiation of flowering, in part because flowering duration was reduced by about 12 days. Finally, fruits matured on average 20 days faster under the warmer conditions. In total, combining advances in initiation of flowering and average flower date with the reduced time to fruit maturity yielded a reproductive phenology in which fruit initiated on the average flower date matured on average 34 days earlier at the low-elevation site than the high-elevation site (Fig. 6).

Therefore in *C. americanum*, earlier initiation of bolting does not simply shift the reproductive phenology earlier. Nor is there an expansion of the phenological schedule to match the longer growing season as seen in some woody plants (Aerts *et al.* 2004; Chmielewski, Muller & Kuchler 2005). Instead, the reproductive phenology is compressed such that progression through the later phenological stages is accelerated relative to the earlier stages. Because advances in early phenological traits may move subsequent phenological events into cooler temperatures, reducing environmental differences between the sites, we estimated the growing degree days (GDD) accumulated in each site for each phenological event. We found greater GDD accumulations in the low-elevation site for each phenological stage ($\text{GDD}_{\text{low site}}$–$\text{GDD}_{\text{high site}}$: bolting initiation 6.2, flowering initiation 153.9, average flower date 129.8, estimated maturation date of a fruit initiated on the average flower date 159.6). This indicates that individual phenological events occurred at warmer temperatures in the low-elevation sites which likely resulted in the non-linear shift in phenology, with reproduction occurring earlier, more rapidly and occupying less of the growing season. When flowering initiation date was manipulated in a natural *C. americanum* population, reproductive phenology was highly integrated and shifted to follow flowering time (Galloway & Burgess 2009). The exception was plants that flowered a month earlier than the natural population which had a compressed reproductive phenology similar to that seen in this study. By comparison, an investigation of *in situ* warming in 12 prairie species found the total reproductive period was shortened in four species, expanded in three, and unchanged in five (Sherry *et al.* 2007). Also, duration of the initial stages of reproductive phenology, examined under control and warmed conditions in a low-arctic site, revealed a compression of individual reproductive stages in two of three species (Post *et al.* 2008). These studies support the possibility that the nonlinear shift reported here may be found in other systems.

Such nonlinear dynamics among phenological traits can affect reproductive success and ecological interactions. Plants grown in the low-elevation site had reduced fecundity relative to those with longer reproductive schedules at the high-elevation site. This may be because plants initiated reproduction earlier at the low-elevation site. Timing of reproduction often determines final size, therefore earlier flowering may reduce fecundity (Roff 2002; in *C. americanum*, Burgess, Etterson & Galloway 2007). Alternatively, the compressed reproductive phenology, with reduced flowering duration, may have resulted in the production of fewer flowers if the rate of flower production was unchanged. The compressed reproductive schedule may also alter biotic interactions such as with pollinators or herbivores (Elzinga *et al.* 2007; Hegland *et al.* 2009) or other abiotic factors (Giménez-Benavides, Escudero & Iriondo 2007; Inouye 2008; Jentsch *et al.* 2009), thereby reducing fecundity. Finally, the reduced fecundity at the low-elevation site could be partially an experimental artefact because plantings at the two sites were initiated at the same time whereas in nature the timing of germination may differ between the sites. However, it is not known whether germination would be earlier or later at the low-elevation site making it difficult to predict its effect on fecundity. Further study to identify the specific cause of reduced fecundity would aid in predicting demographic responses to warmer conditions.

EVOLUTIONARY RESPONSE TO GROWING SEASON LENGTH

Reproductive phenology was differentiated between *C. americanum* populations from low and high elevations. Regardless of planting site, populations from low elevation initiated bolting earlier and reached reproductive maturity more slowly, whereas populations from high elevation initiated bolting later and developed more quickly. As a result of rapid development, the late-bolting high-elevation populations initiated flowering at the same time as earlier-bolting low-elevation populations in the low-elevation site, and even earlier in the high-elevation site. Populations from high elevation produced more of their flowers toward the beginning of the reproductive season and flowered for a shorter duration on average than those from low elevation. Finally, fruits matured more rapidly in populations from high elevation than those from low elevation. In total, reproductive phenology was shorter for populations from high elevation with more rapid development, more condensed and earlier deployment of flowers, and faster fruit development.

Differences between low- and high-elevation populations likely reflect genetic divergence and suggest that local growing season length has selected for different patterns of reproductive phenology. Maternal environmental effects may also contribute to the differentiation of populations from low and high elevation (Roach & Wulff 1987). However, in other taxa we also find that the short growing seasons of alpine or high-latitude environments are mitigated by rapid development and reproduction (Arroyo, Armesto & Villagran 1981; Ratchke & Lacey 1985; Arft *et al.* 1999; Blionis, Halley & Vokou 2001; Olsson & Ågren 2002; Stinson 2004). The generality of these patterns reveals a common evolutionary response of reproductive phenology to growing season length and suggests that we may expect widespread evolution of a slower reproductive phenology in response to warmer climates.

Because high-elevation populations initiated bolting up to a week later than low-elevation populations, it is possible that the differences in bolting duration are due to environmental factors rather than genetic effects. Specifically, the later-bolting high-elevation populations experienced warmer temperatures when bolting which may have accelerated development and reduced bolting duration. This possibility is supported by a negative association between bolting duration and accumulated growing degree days, $R = -0.78$; $P < 0.001$ (see also Inouye, Morales & Dodge 2002). However, populations from high elevation still have a shorter bolting duration than those from low elevation after statistically accounting for these environmental effects, indicating a genetic basis to differences in bolting phenology.

Differentiation of a suite of traits across a physical gradient, such as elevational differences in reproductive phenology, is termed ‘ecotypic differentiation’ (Turesson 1922; Clausen, Keck & Hiesey 1948) and is typically the product of local adaptation. Fruit production in *C. americanum* was locally adapted to the environmental differences associated with elevation. Populations from high elevation had greater fruit production than those from low elevation when grown in the high-elevation site, and populations from low elevation produced more fruit than those from high elevation in the low-elevation site. However, cumulative fitness did not show local adaptation because survival of low-elevation populations was greater at both planting sites. Divergence in reproductive phenology between low- and high-elevation populations is expected to influence reproductive fitness components more than survival. Therefore ecotypic differentiation in reproductive phenology between high- and low-elevation populations is likely adaptive. Because common gardens were not replicated within an elevation, and unmeasured environmental factors may contribute to differences between high- and low-elevation sites, temperature cannot be isolated as the sole factor underlying local adaptation or phenological differences. However, previous work on a forest herb identified temperature and light availability as the major environmental factors influencing reproductive phenology (Dahlgren, von Zeipel & Ehrlen 2007). Therefore shifts in mean temperature and growing season length across elevations, but similar light and water availability, suggest that differences in temperature are likely to have led to adaptive differentiation of reproductive phenology between high and low elevation *C. americanum*.

Reproductive phenology of populations from high and low elevation was more similar when plants were grown under the expanded growing season of the low-elevation site. For all phenological traits except average flower date, there were differences in the response to growing season length between populations from low and high elevation. In most traits including overall reproductive duration, this difference in plasticity resulted in less phenotypic divergence in the low-elevation site than the high-elevation site. As a result, there was a phenotypic resemblance between the populations from high elevation and the local low-elevation populations in the low-elevation site. Whether this phenotypic similarity reveals historic selection of high-elevation populations in a longer growing season (reviewed in Ghalambor *et al.* 2007) or simply a common physiological response of accelerated growth and development under warmer conditions is not known. Temperatures during the year of study were representative of the twenty-five year average at each site. Regardless, convergence on a similar phenotype suggests adaptive plasticity of populations from high elevation to the warmer low-elevation climate.

SELECTION ON PHENOLOGY IN AN EXPANDED GROWING SEASON

Selection on phenological characters was stronger in the expanded growing season. We might imagine, all else being equal, that a longer growing season would favour later bolting and flowering, because that would allow plants to grow larger prior to flowering and therefore reproduce more per unit time (Roff 2002; Metcalf, Rose & Rees 2003). Following this expectation, in uniform growth conditions plants from lower latitudes, where growing seasons are longer, often flower later than those from higher latitudes (e.g. Weber & Schmid 1998; Olsson & Ågren 2002; Etterson 2004; Griffith & Watson 2005). A longer growing season might also be expected to favour individuals with a longer reproductive period and later average flower dates because these individuals would use the longer season to produce more reproductive structures. Following these expectations, in the longer growing season direct linear selection favoured later bolting (larger size). However, in contrast to expectations, earlier flowering individuals had greater fitness. Flowering duration did not affect fruit production, but plants that deployed their flowers with a similar timing to those currently growing naturally in the low-elevation site, i.e. with the same average flower date, had the greatest fitness. Thus, in an expanded growing season there was direct fitness advantages associated with attributes of populations from high elevation: delaying bolting and advancing flowering initiation.

Phenotypic plasticity modified flowering initiation and average flower date in the direction favoured by selection in the low-elevation site. Therefore adaptive plasticity for these traits provided populations beneficial short-term responses to the selective pressures imposed by the longer growing season (see also Etterson 2004). However, the plastic response of earlier-bolting in the low-elevation site was maladaptive. Maladaptive plasticity to a longer growing season suggests that evolutionary change will be required for this trait to enhance fitness under the projected warmer conditions. The relatively later initiation and shorter duration of bolting in the high-elevation populations suggest that such evolution is possible, despite the positive phenotypic correlation between bolting and flowering initiation (cf. Etterson & Shaw 2001; Hellmann & Pineda-Krch 2007).

In total, the results of this reciprocal transplant study suggest that individual components of reproductive phenology will play a central role in plant reproductive success as the global climate continues to warm. The combination of adaptive plasticity, expressed as nonlinear responses of reproductive phenology to warmer temperatures, and selection for changes in the timing and relative duration of phenological traits, suggests that we must consider the ecological and potential evolutionary changes of individual phenological components to forecast response to climate change.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Locations and elevations of the populations of *Campanulastrum americanum* from which seeds were collected and the sites at which the experiment took place.

Table S2. Phenotypic correlations among phenological traits for high and low elevation *Campanulastrum americanum* grown in common gardens at both elevations.

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EFFECTS OF CLIMATE CHANGE ON PHENOLOGY, FROST DAMAGE, AND FLORAL ABUNDANCE OF MONTANE WILDFLOWERS

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Abstract. The timing of life history traits is central to lifetime fitness and nowhere is this more evident or well studied as in the phenology of flowering in governing plant reproductive success. Recent changes in the timing of environmental events attributable to climate change, such as the date of snowmelt at high altitudes, which initiates the growing season, have had important repercussions for some common perennial herbaceous wildflower species. The phenology of flowering at the Rocky Mountain Biological Laboratory (Colorado, USA) is strongly influenced by date of snowmelt, which makes this site ideal for examining phenological responses to climate change. Flower buds of *Delphinium barbeyi*, *Erigeron speciosus*, and *Helianthella quinquenervis* are sensitive to frost, and the earlier beginning of the growing season in recent years has exposed them to more frequent mid-June frost kills. From 1992 to 1998, on average 36.1% of *Helianthella* buds were frosted, but for 1999–2006 the mean is 73.9%; in only one year since 1998 have plants escaped all frost damage. For all three of these perennial species, there is a significant relationship between the date of snowmelt and the abundance of flowering that summer. Greater snowpack results in later snowmelt, later beginning of the growing season, and less frost mortality of buds. Microhabitat differences in snow accumulation, snowmelt patterns, and cold air drainage during frost events can be significant; an elevation difference of only 12 m between two plots resulted in a temperature difference of almost 2°C in 2006 and a difference of 37% in frost damage to buds. The loss of flowers and therefore seeds can reduce recruitment in these plant populations, and affect pollinators, herbivores, and seed predators that previously relied on them. Other plant species in this environment are similarly susceptible to frost damage so the negative effects for recruitment and for consumers dependent on flowers and seeds could be widespread. These findings point out the paradox of increased frost damage in the face of global warming, provide important insights into the adaptive significance of phenology, and have general implications for flowering plants throughout the region and anywhere climate change is having similar impacts.

Key words: climate change; Delphinium barbeyi; Erigeron speciosus; *flowering; frost; growing season;* Helianthella quinquenervis; *phenology; Rocky Mountain Biological Laboratory; snowmelt, subalpine.*

Introduction

The phenology of reproduction is an important life history trait that influences fitness in a variety of ways. Reproducing at the wrong time, in advance of or after the appropriate season, may lead to failure in finding mates, failure to match demands of growing offspring with temporal peaks in food resources (e.g., Visser et al. 1998), or failure by a pollinator to find pollen and nectar, or failure of a flower to be pollinated. Given these potentially severe consequences, it is not surprising that in many cases the phenology of reproduction has evolved to rely on environmental cues that have proven to be reliable indicators of appropriate timing of reproductive effort. An ecological and evolutionary dilemma is posed to a variety of organisms now because of the environmental changes accompanying global climate change. Can they respond in appropriate ways to these ongoing changes so that their phenology remains synchronous with other species with which they interact? And can they adjust their responses to previously reliable environmental cues for timing of reproduction? These questions are difficult to answer without long-term observations and experiments.

The phenology of flowering by herbaceous wildflowers at high altitudes where there is significant snowfall is primarily a consequence of one environmental event, the disappearance of the snowpack (Inouye and Wielgolaski 2003). This event is in turn influenced by a variety of factors, including global, regional, and local climate. Global influences include ongoing changes in temperature and precipitation regimes, with high-altitude environments warming and receiving more precipitation as rain instead of snow (Beniston and Fox 1996, Johnson 1998). Regional influences on snowpack in the western United States include the El Niño/Southern Oscillation

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TABLE 1. Study species.

Species (family)	Common name	Average flowering dates	Units counted
Delphinium barbeyi (Ranunculaceae)	subalpine larkspur	mid-July	flowers and inflorescences
Helianthella quinquenervis (Asteraceae)	aspen sunflower	mid to late July	capitulae and inflorescences
Erigeron speciosus (Asteraceae)	aspen fleabane	late July	capitulae

(ENSO; Diaz et al. 2003) and the North Pacific Oscillation (Pacific Decadal Oscillation; Grissino-Mayer et al. 2004). Local influences include topographic variables such as slope and aspect, which affect the accumulation and melting of snowpack (Miller 1982, Kudo and Hirao 2006), and the occurrence of cold air drainage that creates thermal and phenological inversions (Lynov 1984). At present, the net result of these environmental changes seems to be a trend toward earlier snowmelt, and hence earlier arrival of spring in the western United States (Cayan et al. 2001) and other mountain areas (Dankers and Christensen 2005). The phenology of high latitudes may show many of the same characteristics that high altitudes do (Wielgolaski and Inouye 2003).

Earlier beginning of the growing season due to earlier snowmelt can have multiple consequences. It could increase the length of the photosynthetic period, if the end of the season remains fixed or changes to a later date. If drought is a problem at the end of the growing season, however, earlier snowmelt and longer snow-free periods may increase exposure of plants to this stress (Giménez-Benavides et al. 2007). Earlier snowmelt can significantly alter the dates on which species may come into bloom throughout the summer (Inouye and McGuire 1991, Inouye et al. 2002, 2003, Saavedra et al. 2003) because the ground and air will warm up when the snow disappears. For some species there may also be a correlation between timing of snowmelt and the abundance of flowering (e.g., *Delphinium* species [Inouye et al. 2002, Saavedra et al. 2003]).

One of the factors linking dates of snowmelt to flowering abundance is frost (Inouye 2000, Inouye et al. 2002). If the probability of spring frost on a particular calendar date remains fixed, but leaf or flower buds are being initiated at earlier dates and thus are more vulnerable when frosts occur, the frequency of frost damage to frost-sensitive species is expected to increase. Frost damage might also increase even if the date of last spring frost is becoming later, if the rate of change in frost dates is slower than that of change in snowmelt dates.

In this study, I report data for three species of high-altitude herbaceous wildflowers that have flower buds susceptible to frost damage (Table 1). All three of these long-lived perennials can experience total mortality of flower buds due to late spring frost events. The availability of a long-term data set on flowering phenology is used to look for evidence in the past few decades of changes suggested above in the timing of snowmelt relative to flowering, and possible influences on timing and abundance of flowering.

METHODS

Study site.—An ongoing long-term study of flowering phenology is being conducted at the Rocky Mountain Biological Laboratory (RMBL), in the Colorado Rocky Mountains (38°57′ N, 106°59′ W). RMBL is located at 2886 m elevation in the East River valley of the West Elk Mountains, approximately 9.5 km north of the town of Crested Butte, Colorado, USA. In 1973, several sets of 2 × 2 m plots were established by a group of researchers at RMBL for monitoring flowering phenology. For a separate study, two larger plots were established (1974 and 1975) to monitor abundance of flowering by *Helianthella quinquenervis*.

Focal species.—This study reports on data for *Delphinium barbeyi* and *Erigeron speciosus* (see Plate 1) from two subsets of the total of 30 phenology plots, one set in a mesic meadow on level ground (altitudes 2864–2870 m) adjacent to the junction of the East River and Copper Creek (originally established and monitored by Graham Pyke) and the other on dry rocky meadows at slightly higher elevations (2927–2970 m), along the Copper Creek trail and the portion of Forest Service trail #401 that crosses RMBL property. Data on flower abundance for *Helianthella quinquenervis* have been collected each year since 1974 from one plot (lower plot, 10 × 45 m; mean altitude about 2893 m) or 1975 for a second plot (upper plot, 10 × 36.5 m; mean altitude about 2905 m). GPS coordinates for the two plots, located above and below the Copper Creek trail in the Gothic town site, are available at the RMBL web site, and a map is presented in Fig. 1.

Empirical design.—Every other day for most or all of the growing season, all flowers in the 2 × 2 m phenology plots are counted, typically as number per inflorescence or ramet. A map, GPS coordinates for plot corners, and altitudes for the individual plots are available at the RMBL web site (*available online*).[4] For *Helianthella*, the number of flowers per stalk is counted on all inflorescences in each plot annually in July, and the number of inflorescences cut or broken off, and those with missing flowers (typically due to herbivory by deer or pocket gophers), is also counted. Since 1989, the annual mean number of flowers per stalk has been used to estimate the number of missing flowers (typically fewer than 1% of stalks were cut and/or missing flowers), to calculate a

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[4] ⟨www.rmbl.org⟩

total number of flowers produced in each plot. Each year since 1994, counts have also been made of the number of frost-killed inflorescences. The inflorescences are typically developed enough to identify frost-killed ones easily (a stalk starts developing instead of just petioles on a vegetative rosette).

Environmental measurements.—Snowmelt data are from daily observations by billy barr of snowpack at a measurement station at the north edge of the RMBL, within 1 km of the plots. Temperature data are from the Crested Butte NOAA weather station.

Analytical methods.—Data for each phenology plot in each year are stored in individual spreadsheets. Statistical analyses were accomplished using SigmaPlot (Systat Software, San Jose, California, USA).

RESULTS

The initiation of the flowering season at this study site is highly variable. Data from three additional species from the phenology plots that represent two of the earliest species and the latest to flower illustrate this, and help to set the context for variation and patterns shown by the three focal species. The first flowers each spring are typically *Claytonia lanceolata* (Portulacaceae), which bloom within a few days after snow melts; its first flowering dates have been as early as 14 April (2002) and as late as 9 June (1995) in the same 2 × 2 m plot (Rocky Meadow #7). The correlation between date of snowmelt and first flowering date is highly significant for species that flower early (e.g., for *Delphinium nuttallianum*, $r^2 = 0.734$, $P < 0.0001$; data for seven plots, 1975–2006, 1990 missing, mean flowering date 11 June, range 27 May–2 July) and late (e.g., for *Artemisia tridentata* [sagebrush], $r^2 = 0.600$, $P = 0.0001$; data only available from one plot, 1975–2006, 1989–1990 missing, mean flowering date 14 August, range 29 July–30 August).

Delphinium barbeyi

This species flowered in 3–12 plots/yr (mean 8.8; including frost-killed buds as years with flowering) between 1973 and 2006 (data were only collected on first flowering and not peak flowering for 1976, and no data were collected in 1990); non-flowering plants were present in most of the 12 plots in most years, but in some years most or all flower buds on plants that developed inflorescences were killed by frost, reducing the sample size for flowering dates. The average number of years (out of 32) that each plot had flowers was 25.2 (range 14–32). The earliest annual average for flowering (the first flower in all plots with flowers) was 1 July (day of year 182.7, in 2006; $n = 6$ plots) and the latest was 5 August (day of year 217.7, in 1995; $n = 10$ plots). The mean date of first flower (mean of annual means) was 14 July (day of year 195.7; median 15 July). For years with early snowmelt (before 19 May, day 139), there is no significant correlation between flowering date and snowmelt date (mean flowering date = day 189, 8 July), but for years with later snowmelt there is a significant correlation between these variables ($r^2 = 0.745$, $P < 0.0001$; Fig. 2). This split in the data set (made by visual inspection of the data) makes sense biologically as it indicates that there is a threshold effect between snowpack melt date and timing of flowering. This effect could be mediated by a requirement to accumulate a certain number of degree days before flowering occurs, with it taking longer to accumulate that heat sum in years with early snowmelt.

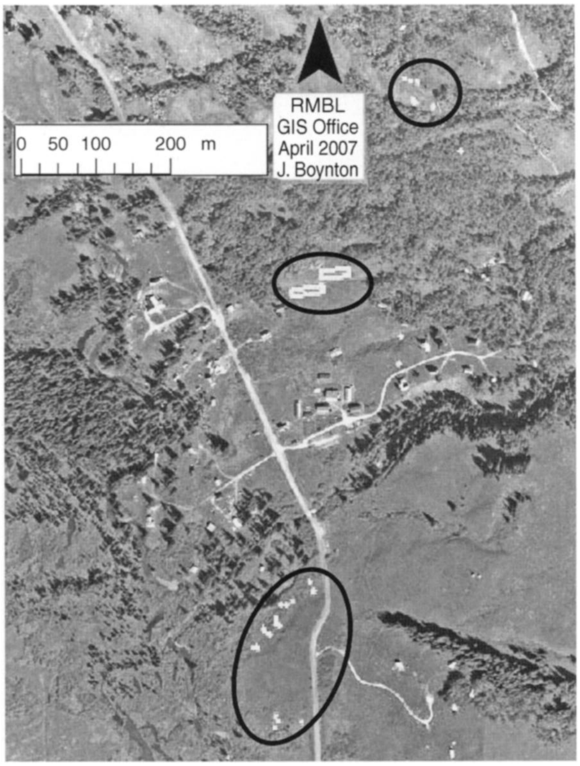

FIG. 1. Aerial view of the Rocky Mountain Biological Laboratory. The vertical road is Gunnison County Road 317, and north is indicated by the arrowhead. The plots used for *Delphinium barbeyi* and *Erigeron speciosus* are included in the upper and lower ellipses, and the *Helianthella quinquenervis* plots are the two larger plots in the middle ellipse.

As was reported in Inouye et al. (2002), there is a significant correlation between winter snowpack and the abundance of flowering for *Delphinium barbeyi*. Fig. 3 shows this relationship, using snowpack remaining on 30 April and including the seven additional years of data collected since that paper appeared; data for peak flowering were incomplete for 1973–1976. One plot (Veratrum Removal #1) had an unusually large number of flowers in 2004, causing that year to appear as an outlier.

Erigeron speciosus

This species is found in both dry, rocky meadow plots ($n = 7$ plots) and wet meadow plots ($n = 9$ plots), and because these tend to melt out at different times (rocky meadow plots are earlier) some correlations are shown

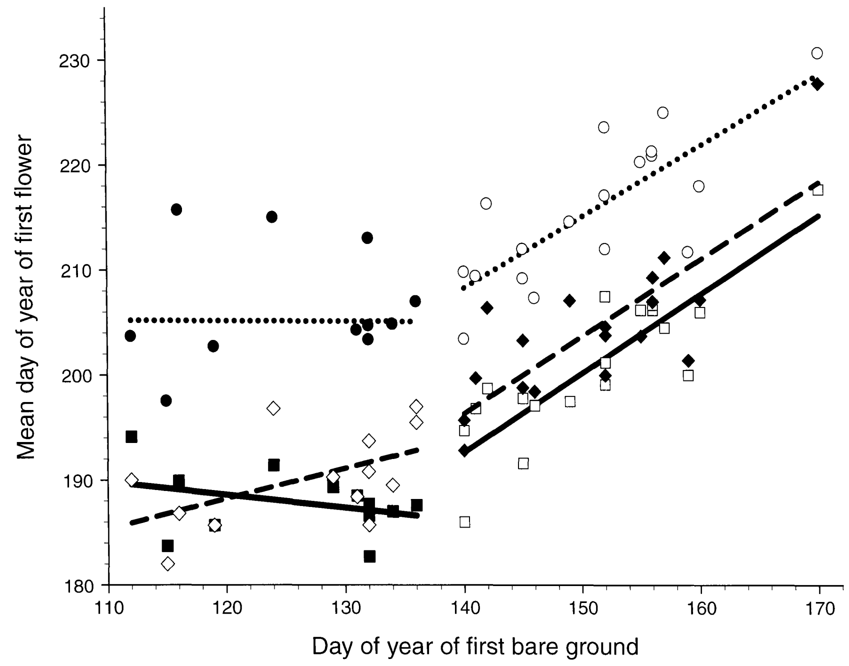

FIG. 2. The relationship between mean day of year of the first flowers of *Delphinium barbeyi* and *Erigeron speciosus* in the phenology plots and the day of year of first bare ground. The data were broken into two subsets by visual inspection; the early set (through day 139) has no significant slope or correlation for either species, and both are significant for the later set ($r^2 = 0.745$, $P < 0.0001$, $N = 18$ years). *Delphinium* data are shown with squares (solid for 12 early years, open for late years), and solid lines indicate the best fits. Data for *Erigeron* are shown separately for the seven dry, rocky, meadow plots (diamonds, open for 13 early years, solid for 18 late years) and nine wet meadow plots (circles; solid for early years and open for late years). For *Erigeron speciosus*, the equation for the later snowmelt dates for rocky meadow plots is $y = 0.734x + 93.506$ (dashed line, $r^2 = 0.629$, $P < 0.0001$); the equation for later snowmelt dates for wet meadow plots is $y = 0.679x + 113.223$ (dotted line, $r^2 = 0.620$, $P < 0.0001$).

separately for each habitat (Fig. 2). *Erigeron* flowered in 6–15 plots/yr (mean 11.0) between 1973 and 2006 (missing data for rocky meadow plots for 1976 and for both habitats in 1990); non-flowering plants were present in most of these plots in most years, but in some years most or all flower buds were killed by frost, reducing the sample size for flowering dates. The average number of years (out of 30) that these 15 plots

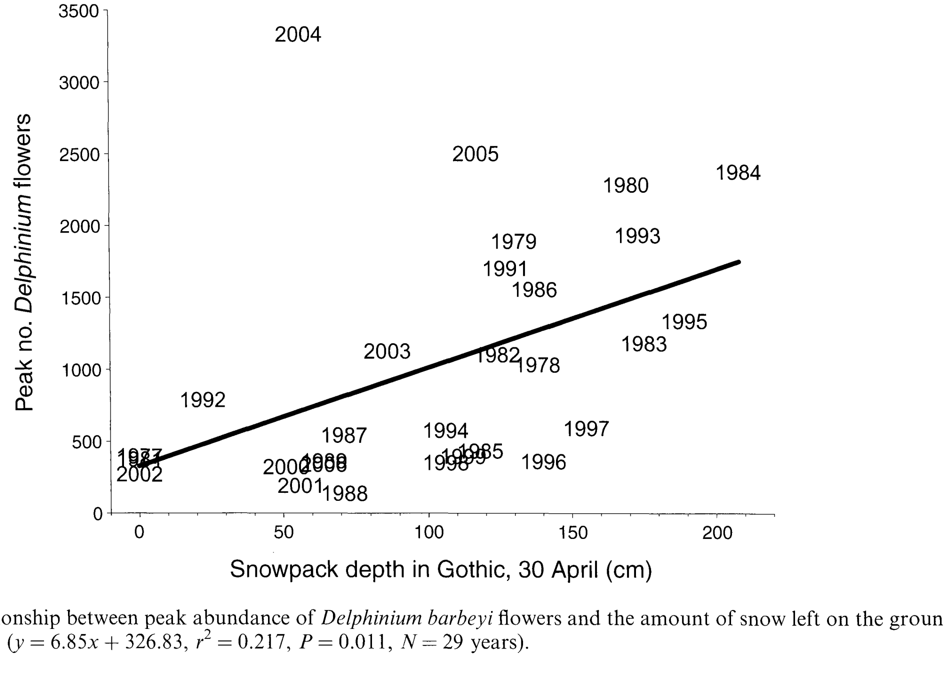

FIG. 3. The relationship between peak abundance of *Delphinium barbeyi* flowers and the amount of snow left on the ground on 30 April of that year ($y = 6.85x + 326.83$, $r^2 = 0.217$, $P = 0.011$, $N = 29$ years).

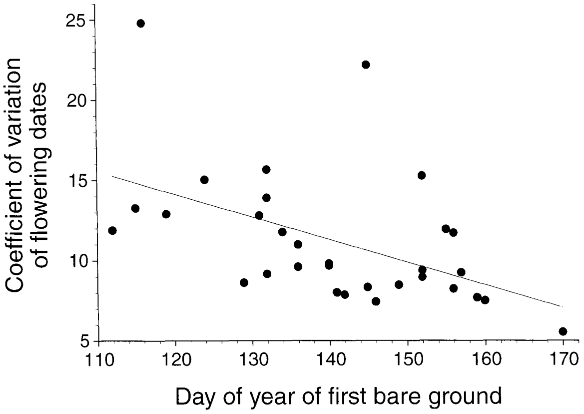

FIG. 4. The relationship between variability of flowering date of *Erigeron speciosus* and the date of snowmelt. Coefficient of variation is calculated using data from both habitats (dry and wet meadow.

had flowers was 22.3 (range 1–30). The earliest annual average for flowering (the first flower in all plots with flowers) was 9 July (day of year 190.2, in 2004; $n = 9$ plots) and the latest was 17 August (day of year 229.3, in 1995, $n = 12$ plots). The mean date of first flower (mean of annual means) was 30 July (day of year 210.5; median 25 July), and annual dates of first flower are dependent on snowmelt date. For wet meadow plots, in years with early snowmelt (before 19 May, day 139, $n = 12$ plots), there is no significant correlation between flowering date and snowmelt date (mean flowering date = 205, 24 July), but for years with later snowmelt there is a significant correlation between these variables ($r^2 = 0.620$, $P < 0.0001$, $n = 18$ plots; Fig. 2). For rocky meadow plots, in years with early snowmelt there is no significant correlation between flowering date and snowmelt date (mean flowering date = 190, 9 July, $n = 13$ plots), but for years with later snowmelt there is a significant correlation between these variables ($r^2 = 0.629$, $P < 0.0001$, $n = 18$ plots; Fig. 2).

There is a significant correlation between the date of snowmelt and the coefficient of variation of flowering date ($r^2 = 0.247$, $P = 0.005$; Fig. 4), with earlier snowmelt correlating with increased variability in flowering date among plots. There is also a clear pattern between the first date of bare ground and the abundance of flowers the following summer. For years with early snowmelt (before 19 May, day 139), there is no significant correlation between number of flowers and snowmelt date (mean = 204 flowers), but for years with later snowmelt there is a trend between these variables ($r^2 = 0.131$, $P = 0.14$; Fig. 5). This split in the data set makes sense biologically as it indicates that there may be a threshold effect between date of snowmelt and frost damage. It appears that if snow melts out before 19 May (or there is less than a meter of snow left on the ground on 30 April) there is a strong likelihood of frost damage the following summer.

Helianthella quinquenervis

The number of flower heads of the aspen sunflower in the two plots combined has varied over four orders of magnitude from 1975 to 2006, ranging from 1 (2004) to 4448 (1982) (Fig. 6). Since 1992, when I first began quantifying frost damage, the percentage of flower buds

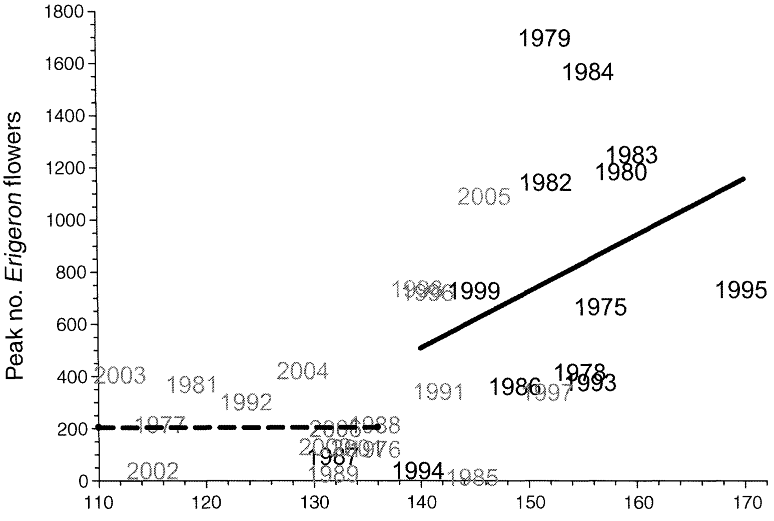

FIG. 5. The relationship between peak abundance of *Erigeron speciosus* flowers and the first date of bare ground of that year. The data were broken into two subsets by visual inspection; the early set (through day 139) has no significant slope or correlation. The dashed line indicates the mean number of flowers for years with snowmelt dates earlier than 19 May (day 139). The equation for the later snowmelt dates (solid line) is $y = 21.65x - 2523.30$, $r^2 = 0.131$, $P = 0.14$). The driest summer from 1925 to 2006 was 1994, and most flower buds dried up before opening. Years in gray are those in which I recorded evidence of frost damage in my field notes.

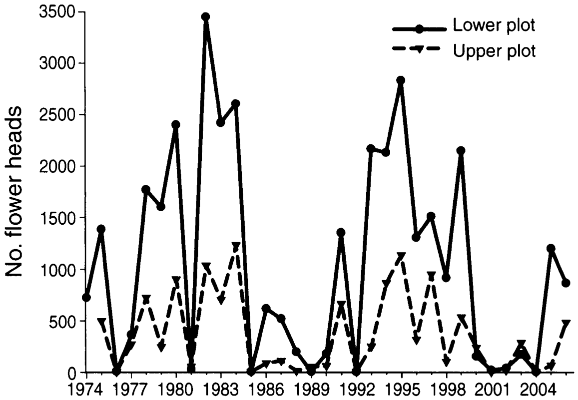

FIG. 6. The number of unfrosted *Helianthella quinquenervis* flower heads in two plots (lower plot, 450 m^2; upper plot, 365 m^2) at the Rocky Mountain Biological Laboratory, Colorado, USA. Years with very few flowers are typically years in which frost killed most flower buds.

killed by frost has ranged from 0% to 100% (Fig. 7). Over the past eight years, bud mortality has been zero in one year; in the other seven years it has ranged from 65% to 100%. The probability and degree of frost damage appears to be correlated with the previous winter's snowpack. For years with early snowmelt (before 19 May, day 139), there is no significant correlation between the number of unfrosted flower heads and snowmelt date, but for years with later snowmelt there is a significant correlation between these variables ($r^2 = 0.363$, $P = 0.008$; Fig. 8).

DISCUSSION

Collectively, these results provide evidence for significant and detrimental impacts of current climate trends on some subalpine flowers, mediated by their phenological responses to snowmelt. The impacts are variable among species, but are clearly related to life history, and have the potential to result in demographic changes in the populations due to lack of seed production. All three of the three focal wildflower species are long-lived perennials, with life spans that can probably reach multiple decades (estimates based on excavation of roots and tagging of individual *Helianthella* plants). This confers an element of stability to their presence in these plots, although there is evidence of turnover. For example, in one phenology plot (Willow-Meadow Interface #2) *Delphinium barbeyi* has only flowered in one year since 1988, and in another (Willow-Meadow Interface #5) it has not flowered since 1993 (although there were aborted flower stalks in 1994). It first appeared in *Veratrum* Removal Plot #1 in 1979 (possibly a consequence of the removal of *Veratrum tenuipetalum* (Melanthiaceae (Liliaceae)) beginning in 1974).

During this study, there has been an increase in the frequency of frost damage. For example, during the first 11 years of the *Helianthella* study (1974–1984) there were two years with significant frost damage (inferred as years with almost no flowers), while there have only been two years without significant frost damage in the past 11 years (Figs. 6 and 7). Biologically, it makes sense that there might be a threshold level of snow that will delay flower bud development beyond the time when frost is still likely to occur. The data reported in this paper are consistent with the interpretation that the likelihood and degree of frost damage to flower buds are strongly affected by snowmelt date.

Radiation frost (exposure to the cold night sky) alone does not seem to cause significant damage to flowers at

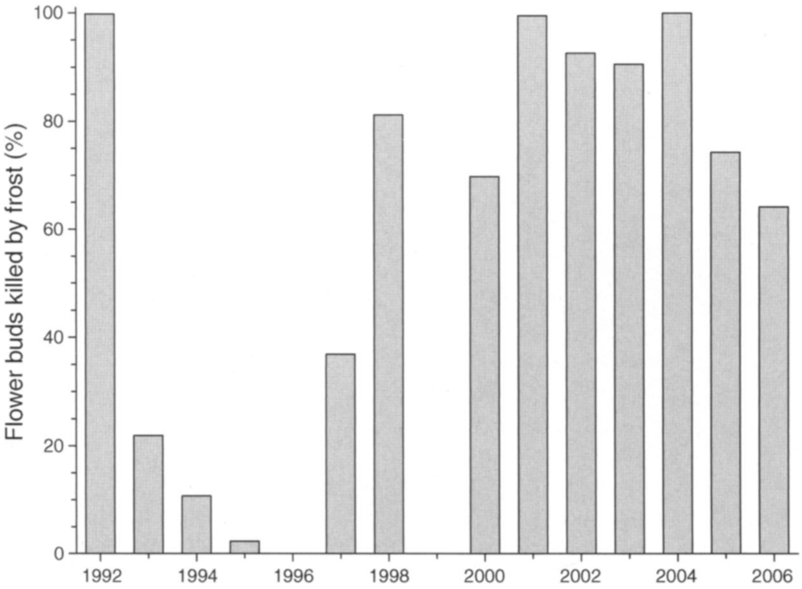

FIG. 7. The percentage of *Helianthella quinquenervis* flower buds that were killed by frost, 1992–2006. Data are from both plots (upper and lower) combined.

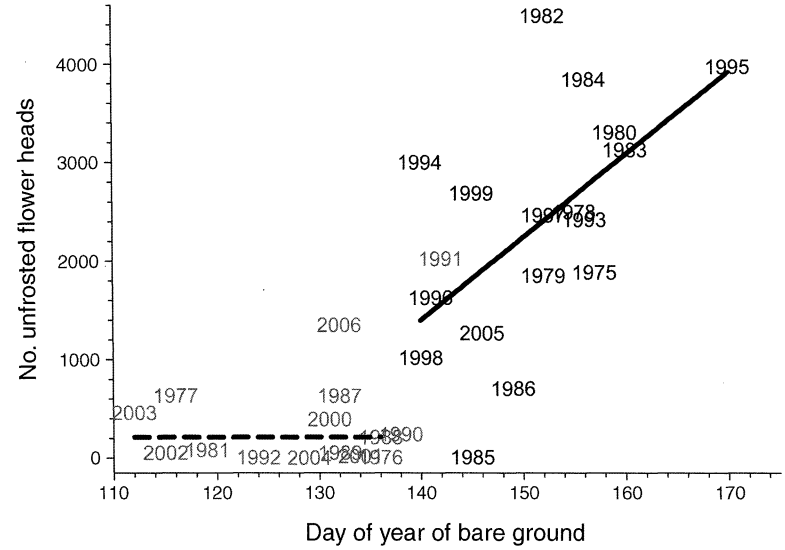

FIG. 8. The relationship between production of *Helianthella quinquenervis* flower heads that were not killed by frost in two plots and the first date of bare ground. The data were broken into two subsets by visual inspection; the early set (through day 139, in gray) has no significant slope or correlation, and both are significant for the later set ($r^2 = 0.313$, $P = 0.02$). The five partially overlapping early snowmelt data points are (counterclockwise from 2004) 1989, 2001, 1976, 1990, and 1988.

the study site; it is primarily convective frost (cold air masses) that affects them. The minimum temperature in June, when the frost damage occurs, has been trending ($r^2 = 0.077$, $P = 0.06$; data for the Crested Butte NOAA weather station, 1960–2005) toward lower temperatures; during the time of this study (1973–2005) the June minimum has averaged −4.3°C (range −1.7°C to −8.3°C). Unless this trend is reversed, potentially through global warming, frost damage is likely to continue to be a common event.

Several other species in my study site at RMBL are affected by spring frost that kills leaf buds, inflorescences, and developing fruits. For example, frost can damage new growth on Engelmann spruce (*Picea engelmannii*; Pinaceae) and subalpine fir (*Abies lasiocarpa*; Pinaceae), leaves of *Heracleum lanatum* (Apiaceae), fruits of *Erythronium grandiflorum* (Liliaceae), and inflorescences of *Ligusticum porteri* (Apiaceae), *Heuchera parviflora* (Saxifragaceae), *Veratrum tenuipetalum, Thalictrum fendleri* (Thalictraceae), and *Lupinus argenteus* (Fabaceae). There can be differences within a genus; for example *Delphinium nuttallianum*, which flowers much earlier than *D. barbeyi*, is not sensitive to frost, and *Erigeron flagellaris*, *E. elatior,* and *E. coulteri* do not seem to suffer frost damage.

In some cases, when most but not all flower buds are killed by frost, it appears that it may be the later-developing buds that survive, as flowering may be delayed beyond what would otherwise be predicted from the time of snowmelt. This could be responsible for the observed correlation between the coefficient of variation of flowering date by *Erigerón* and snowmelt date (Fig. 4). In this species some buds may survive frost, particularly in the rocky meadow plots, which are at a higher altitude and may escape effects of cold air drainage, and the combination of these flowers that may open at a "normal" date and those late-developing buds on plants on which most buds were killed by frost would generate a larger range of flowering dates. Kudo et al. (2008) found that flowering dates of early spring plants were more variable than those of later-flowering species, and attributed this to their dependence on timing of snowmelt.

The effects of frost on wildflowers at this study site are highly variable on a small geographic scale. Cold air drainage appears to play an important role in affecting low-lying areas, and the few degrees difference that this can make over a small scale of altitude was evident in the 2006 frost. In four of the five years in which there was more than a 10% difference between the upper and lower plots in frost kill of flower buds of *Helianthella* plants, the lower plot had the greater level of damage. In 2006, for example, the lower plot had 70% frost kill, and the upper plot 47%. There is 12.3-m difference in altitude between these plots (difference between the mean altitudes of upper and lower edges of each plot), but the minimum June temperatures was −3.37°C in the lower plot and −1.51°C in the upper plot (on 23 June 2006 for both plots; data recorded every 15 minutes with Hobo Pro Series data loggers [Onset, Pocasset, Massachusetts, USA]). The temperature in one of the phenology plots (Wet Meadow 1), which is at 2870 m, was −3.37°C on the same night (but −4.3°C on 16 June), and in this area all of the *Helianthella* flower buds were killed in 2006. In contrast to the high mortality in these plots, there was almost no frost damage in 2006 to

PLATE 1. (Above) *Erigeron speciosus* (Asteraceae) is an important nectar resource for the butterfly *Speyeria mormonia* (Mormon fritillary); (below) a frostkilled bud of *E. speciosus*. Photo credits: D. W. Inouye. A color photograph of *Helianthella quinquenervis* (Asteraceae) is available in the *Bulletin* of the Ecological Society of America 88(4).

Helianthella plants along trail 401, a few hundred meters away from the *Helianthella* plots and about 89 m higher, no frost damage to plants along County Road 317 in Mount Crested Butte (altitude about 2895 m, 5.8 km from RMBL), but 100% mortality at Horse Ranch Park (altitude 2706 m, 18.5 km from RMBL). This variation, even within very similar altitudes, indicates the importance of microclimate in determining both patterns of snowmelt and later air temperature.

Because these plant species are long-lived perennials, it is possible that the loss of reproductive potential due to frost damage to flower buds may not play a significant role in the long-term demography of their populations, if they are not limited by seed input. However, preliminary analysis of data for *Helianthella* from a demographic study at RMBL (D. Inouye, *unpublished data*) shows that the number of plants in a set of six 1.5 × 5 m plots has decreased significantly over the past nine years. During this period there has been significant recruitment of seedlings in only two years (1998, 2000); no seedlings have been found since 2000, following the last year without significant frost damage to flower heads (1999, see Fig. 6). If this trend of significant frost damage were to continue for many years, the population decline would probably continue. Even without recruitment, local extinction would take many years given the longevity of the plants.

Although it may seem paradoxical that a consequence of global warming is an increase in the frequency of frost damage, for the species described in this paper, and for those others mentioned that also suffer frost damage, there has been an increase in the past several years in the frequency of frost that damages vegetative or reproductive parts. The observed trend toward lower June minimum temperatures over the past few decades is not predicted by models of global warming, which in fact predict that night-time temperatures may be warming faster than daytime temperatures (Easterling et al. 2000). The phenomenon of earlier snowmelt and greater frost exposure may be a general phenomenon at high altitudes and high latitudes, as it has also been documented in a subarctic tundra community (Wipf et al. 2006). Bannister et al. (2005) suggested that the dependence on day length and temperature for development of frost tolerance of the alpine New Zealand species they examined was likely to confer protection even in the face of global warming, but assumed that incidence of frosts would be reduced. Scheifinger et al. (2003) found that frost events (last occurrence of daily minimum temperatures below a certain threshold) in Europe have been moving faster to earlier occurrence dates than have phenological phases during the preceding decade, and suggested that the risk of late spring frost damage should have been lower as a consequence.

Some animal species may be similarly reliant upon melting of the snowpack to set phenological clocks. For example, laying date and clutch size of American Pipets in alpine Wyoming are correlated with snowmelt date (Hendricks 2003). At my study site, the phenology of bumble bee queen emergence (from spending the winter underground) is probably tied to snowmelt in a fashion similar to that of plant development and flowering (D. Inouye, *personal observation*). Species of seed predators such as the tephritid flies that use *Helianthella* flowers as a host, and overwinter as adults, are probably also linked to snowmelt in their emergence. The abundance of these seed predators seems to have declined significantly in recent years (compared to levels reported in Inouye and Taylor [1979]; D. Inouye, *personal observation*), probably due to loss of opportunities for oviposition in flower heads. It is likely that other species of pollinators and herbivores are also tied phenologically to snowmelt dates.

One recent event that seems to have a significant effect on winter snowfall at my study site, and therefore plays a role in frost damage, is the change of phase of the North Pacific Oscillation (Pacific Decadal Oscillation), which has also been shown to influence precipitation and fire regimes in the Rocky Mountains (Schoennagel et al. 2005). The state of this 50–75 year sea surface temperature cycle has influenced winter precipitation at RMBL (data from 1935 to 2004, $P < 0.05$), and may be responsible in part for the trend toward more precipitation falling as rain instead of snow (Knowles et al. 2006). The phase change in 1998 falls about half-way through the data set for percentage of *Helianthella* flower buds killed by frost. The mean from 1992 to 1998 is 36.1% of buds killed by frost, and for 1999–2006 the mean is 73.9% (t test, $P = 0.06$). This appears to be an example of a regional climate change that is having an effect on phenology and, mediated by the effects of frost, on flowering and potentially plant demography and other species (pollinators, seed predators, parasitoids) involved in the trophic cascade starting with these wildflowers. Climate change at local and global scales may also be having an effect, but is more difficult to discern in this study, although the trend toward lower June minimum temperatures may be an effect at the local scale.

CONCLUSIONS

Both the timing and abundance of flowering by the species described in this paper are highly variable, and this variation is strongly influenced by differences among years in the amount of winter snowfall and subsequent snowmelt. Winter precipitation is likely to continue to be relatively light for the next couple of decades, until the next phase change of the North Pacific Oscillation. This supports the conclusion that frost is likely to be an important factor affecting the abundance of flowering in sensitive species, and that a continued reduction in seed production is likely to have demographic consequences.

This and other studies provide strong evidence for ecological constraints on phenological responses to rates of environmental change. Of course not all ecosystems experience frost, and in some cases frost may not be an important factor even if it does occur (e.g., Kudo et al. 2008), but a general message from this study and all the others in this Special Feature is that long-term records may be required to tease out the environmental variables that affect phenology. Non-scientists can contribute to these efforts (Miller-Rushing and Primack 2008), and participation by this audience is a goal of the National Phenology Network. Although I have focused on herbaceous species, it may be important to consider how phenology of woody species may differ (e.g., Rich et al. 2008), and while I focused on a small spatial scale (2 × 2 m plots), satellite remote sensing can also be a valuable tool for phenological studies (Rich et al. 2008). I focused on flowering phenology, but as Post et al. (2008) point out, not all life history events respond similarly to environmental variation. No matter the scale at which it is measured, or who is collecting the data, it is likely that phenology will become a more common element of scientific studies of the effects of future climate change.

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Flowering phenology, fruiting success and progressive deterioration of pollination in an early-flowering geophyte

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Spatio-temporal patterns of snowmelt and flowering times affect fruiting success in *Erythronium grandiflorum* Pursh (Liliaceae) in subalpine western Colorado, USA. From 1990 to 1995, I measured the consistency across years of snowmelt patterns and flowering times along a permanent transect. In most years since 1993, I have monitored fruit set in temporal cohorts (early- to late-flowering groups of plants) at one site. To assess 'pollination limitation', I have also conducted supplemental hand-pollination experiments at various times through the blooming season. The onset of blooming is determined by snowmelt, with the earliest years starting a month before the latest years owing to variation in winter snowpack accumulation. Fruit set is diminished or prevented entirely by killing frosts in some years, most frequently but not exclusively for the earlier cohorts. When frosts do not limit fruit set, pollination limitation is frequent, especially in the earlier cohorts. Pollination limitation is strongest for middle cohorts: it tends to be negated by frost in early cohorts and ameliorated by continuing emergence of bumble-bee queens in later cohorts. This lily appears to be poorly synchronized with its pollinators. Across the years of the study, pollination limitation appears to be increasing, perhaps because the synchronization is getting worse.

Keywords: *Bombus*; climate change; *Erythronium*; frost; phenological synchrony; pollinator decline

1. SNOW, PHENOLOGY, POLLINATION AND FRUITING SUCCESS

Studies of flowering phenology often focus on variation across years, but the reproductive success of plants can vary within populations depending on when they bloom (e.g. Augspurger 1981; Schmitt 1983; Dieringer 1991; Ehrlen & Munzbergova 2009). This variation is particularly striking in alpine and subalpine habitats, where the weather is harsh, the growing season short and where heterogeneous melting of the winter snowpack determines the onset of early growth (Billings & Mooney 1968; Inouye & McGuire 1991). Patchiness in snowmelt produces a spatio-temporal mosaic in blooming phenology (Kudo 1993; Stanton *et al.* 1994; Kudo & Hirao 2006) such that different patches of spring flowers are exposed to different pollinator availabilities and different abiotic stresses, particularly late storms and frosts. This variation, therefore, may exert selection on plant characteristics (Widén 1991) and influence plant responses and adjustments to changing environments. For example, climate change has been suggested to increase plants' susceptibility to late frosts (Inouye 2000, 2008) and pollination deficits (Saavedra *et al.* 2003; deficits are postulated to arise from phenological mismatches between plants and pollinators; cf. Kudo *et al.* 2004; Memmott *et al.* 2007; Williams & Jackson 2007). These consequences, however, depend on whether the same phenological patterns are observed consistently across years (among other things; see Ollerton & Lack 1992). This makes multiyear studies essential, but few such studies exist. Here, I present a set of long-running observations and experiments focused intensively on the vagaries of fruit set and pollination limitation in a small subpopulation of an abundant subalpine lily. Although the original intention was to study within-year variation, the data suggest a noteworthy increase in pollination deficits from 1993 to 2009. Such deterioration of pollination service is relevant to concerns about declining pollinators (Ghazoul 2005; National Research Council 2006), and these results appear to be unique. A review by Knight *et al.* (2005) found only one study (Primack & Stacy 1998) that assessed pollination limitation in 10 or more years (T. Knight 2009, personal communication), and that study was designed to assess costs of reproduction in repeatedly stressed plants, not natural variation.

2. MATERIAL AND METHODS

(a) *Study site*

I conducted observations and experiments on and immediately adjacent to private property (Block 28, Lots 7–14) in the town site of Irwin, Colorado

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One contribution of 11 to a Theme Issue 'The role of phenology in ecology and evolution'.

(elevation 3170 m), in a roughly triangular area (coordinates 38°52.566′ N 107°06.050′ W, 38°52.553′ N 107°05.988° W, 38°52.625′ N 107°05.997′ W) of about 3 ha. The site is 11.7 km southwest of the Rocky Mountain Biological Laboratory, a centre for much pollination research and reference weather data (www.rmbl.org). The area receives locally heavy snowfall; snow cover frequently persists into early June on open ground and into July in forested areas. Irwin received some intermittent cattle grazing in the late twentieth century, but there has been none since at least 1987. The study site is nearly level, with a slightly southern exposure. Because other parts of the surrounding habitat have steeper southern exposures, flowering is well advanced in those areas before it begins in the study site proper. My characterization of plants as 'early' or 'late' applies only within my study area; none of these plants are truly early within the larger meadow system.

The usual progression of spring at the site includes unpredictable wintry weather through May and into early June, with hard frosts and snowstorms interspersed with brief breaks of sun. This unsettled pattern then yields to 'the June drought', which usually brings several weeks of long sunny days before yielding in turn to a monsoonal pattern of afternoon thunderstorms in early July.

From field notes, I have extracted two phenological indicators relating to the earliness of different springs since 1990. The first is the day on which the last winter snow disappears from the property referenced above; the second is the date of winter ice breakup on Lake Irwin, approximately 100 m from the study area.

(b) *Study plant*

The glacier lily *Erythronium grandiflorum* Pursh (Liliaceae) is a long-lived, spring-ephemeral geophyte, abundant in the meadows at Irwin. Seedlings comprise a single, grass-like cotyledonary leaf in their first growing season; older plants produce broader bladed leaves. A non-flowering plant makes a single leaf and a flowering plant makes a pair. (Occasionally, a two-leafed plant does not produce a flowering scape, but this seems to represent a developmental aberration: remnants of the aborted scape persist between the leaf bases.) Shoot emergence coincides with the recession of snowpack, and flowers bloom within a few days. The blooming period is about four weeks, typically spanning the end of the bad 'May' weather and extending into the 'June drought'.

The principal pollinators are bumble-bee queens of the early-emerging species *Bombus bifarius and Bombus occidentalis* (Hymenoptera: Apidae); the latter species is larger and, therefore, more likely to contact the stigma and deposit pollen (Thomson 1986). Queens seek nectar. Although they become dusted with pollen, they typically groom it off their bodies rather than packing it into corbicular pellets. *Salix* is the preferred pollen source, and is abundant at the site. I have observed active pollen collection from *E. grandiflorum* only once, in the unusually late spring of 1995. In open meadow habitats, flowering is finished before *Bombus* workers emerge. Workers may visit very late-flowering patches in the forests, but I have not observed this. Broad-tailed hummingbirds (*Selasphorus platycercus*) take nectar by visiting the pendent flowers from the side, and smaller solitary bees harvest pollen by alighting on the large anthers, but neither transfers much pollen to stigmas. By midsummer, leaves and the scapes of unfertilized flowers wither and the plant withdraws into a discrete underground corm. If flowers are pollinated, the scape elongates to *ca* 25 cm and bears capsules that dehisce terminally, spreading seeds by a salt-shaker mechanism in late July–August. Plants grown from seed on site take at least 6 years to reach flowering size. Mature plants make one to three flowers, rarely more, depending on corm size; successful fruit production diminishes corm size, so flower production is partially regulated by the cost of fruiting (J. Thomson 1990–2009, unpublished data, available at http://rmbl.info/jthomson). Unlike some congeners, *E. grandiflorum* does not form clonal patches through the lateral spread of rhizomes, but some vegetative reproduction occurs by the splitting of corms, especially larger ones. Seeds lack elaiosomes. Flowering plants tend to be more abundant in areas of shallow soil on rock outcrops, a pattern hypothetically driven by predation by pocket gophers (*Thomomys talpoides*; Thomson *et al.* 1996).

(c) *Patterns of snowmelt and flowering: permanent transect*

I established a permanently marked 2 × 200 m belt transect that ran from the open meadow, past several trees and into a matrix of forest and forest gaps. I placed the transect subjectively to include very early-flowering areas and very late-flowering areas. The transect was divided into 40 quadrats of 2 × 5 m each, with the edges marked by a nylon cord. The positions of trees or clumps of trees (all *Picea engelmannii* or *Abies lasiocarpa*) near the transect were also noted. In 1990–1995, I counted open flowers in each quadrat at 2 day intervals. To determine whether flowering patterns along the transect were consistent across years, within each year's dataset, I ranked the 40 quadrats according to their median date of flower production, and then correlated the ranks across years.

In 1992–1995, I also made sketch maps (by eye) of the course of snowmelt along the transect by recording the edges of bare ground. It was evident from comparing these sketch maps visually that snowmelt patterns were highly consistent from year to year, even though this sample included an early year (1992, first open ground 28 May) and a very late one (1995, first open ground 4 July). To quantify this consistency more objectively, I began with the sketch map for 1994, which was an intermediate year (first open ground 4 June). There were nine snow-mapping dates in 1994. On the map, I arbitrarily chose one spatial point for which the first open ground appeared on each of the nine census dates. I then transferred these nine locations to the maps for the other years and determined the dates on which snow disappeared at those locations in the other years. If sections along the transect melt in the same relative sequence in different years, despite large differences in absolute dates of melting, the dates of the first open ground at the nine reference locations will be correlated across years.

On 22 June of the extremely heavy snow year 1995, I also measured snow depth directly at 2 m intervals along the midline of the transect. At that date, the first open ground in the transect had just appeared and was limited to one 4 m patch.

As part of the transect studies, I also counted fruits of *E. grandiflorum* in the quadrats along the transect. Those data, not presented here, suggested interesting temporal patterns in fruiting success, possibly attributable to lack of early pollination. However, flowering time was strongly confounded with spatial position in those data sets. Because the spatial patterns of snowmelt and flowering times along the transect were virtually identical across years (see §3), I discontinued monitoring the transect after 1995 and concentrated instead on more spatially distributed observations of fruiting success. As explained below, the protocols for these experiments evolved slightly over the years.

(d) *Observational study of fruit set patterns: phenological cohorts*

Beginning in 1993, I marked successive flowering cohorts of approximately 100 single-flowering plants each at intervals through the flowering period. In the first year, I used 5 day intervals; in later years, I increased the sampling frequency to 4 day intervals, and later to 3 or 4 day intervals, depending on weather. A 3 day interval approximates the length of anthesis of individual flowers in the dry weather that is characteristic for June at this site. In cooler or rainy weather, flowers last longer, so a 4 day interval is more appropriate for separating non-overlapping cohorts. Because flowering lasts approximately a month, the total number of cohorts in a year typically ranges from eight to 10, depending on whether that year's blooming period was extended or compressed. Plants were marked with surveyor's pin flags during flowering, and capsules were collected when fully developed but not yet dehisced. I selected plants haphazardly, subject to the constraints that (i) they were fully open, with all six anthers dehisced and (ii) they were neither especially small nor large. (In this population, the smallest flowers frequently have poorly developed ovaries, are unlikely to set fruit even if pollinated and seem to be acting effectively as males.) I have repeated this procedure in most years since 1993. Plants for the earliest cohort are always in the same locations, on two south-facing slopes, and the latest cohort is always in another particular site that accumulates drifting snow and is partly shaded by conifers. The middle cohorts are less tightly associated with particular, extreme microsites. Therefore, they are larger in extent and more variable in location from year to year. I have not mapped the positions of the cohorts; nevertheless, there has been considerable consistency across years in the spatial locations of the cohorts.

For the first years of the study, I marked cohorts using red pin flags. Flags for different cohorts were distinguishable by spots of different colours of paint applied inconspicuously to the steel shafts. In 2003, complaints about the gaudiness of the red flags impelled me to switch to brown, still with colour-marked shafts. I do not think that pollinators reacted to the colour spots, which were *ca* 30 cm above the associated flowers, but I randomized the order of shaft colours across years as a precaution against confounding. Sample sizes approximate 100 plants per cohort, but vary somewhat. I used bundles that nominally contained 100 flags but often included a few extras; also, I did not always find and recover all of the flags set out each spring.

(e) *Experimental study of fruit set patterns: supplemental pollinations*

In these experiments, I selected adjacent pairs of single-flowered plants, using the same criteria as in the cohort study but also matching the members of the pair for stature. These plants were marked with green flags, alternating between two shaft colours. Plants with one colour of flag were supplementally pollinated by hand, while those with the other colour were left as unmanipulated controls. I prepared pollen mixtures by harvesting anthers from at least 12 different donor plants into a polystyrene vial. *Erythronium* anthers are *ca* 15 mm long and dehisce when mature by everting along a longitudinal suture ('unzipping') from the distal to the proximal end. I collected half-dehisced anthers as a way of standardizing pollen freshness (Thomson *et al.* 1994). I let dehiscence go to completion in the vial, and mixed the pollens by shaking the vial. As the mixture became depleted during the pollination process, I occasionally added fresh anthers. Until 2001, I applied pollen with applicators made of nylon fishing line; after that, I switched to MFH10 microbrush applicators (Microbrush International). I applied pollen over all three stigma lobes until a dense coating was visible by eye. Both tools applied equivalent coatings, but the microbrush was faster to load with pollen. The sample size for the supplementation experiment was about 150 single-flowered plants for each of the treatments, supplementation and control. Although plants were selected as pairs, I did not analyse the experiment as a paired design. It would have been logistically burdensome to keep paired fruits associated through harvest, and many pairs would be broken up by frost or herbivory.

In 1993–1995, I did a single supplementation experiment at approximately the middle of the flowering period. In 1997, I added a second (smaller, $N = 50$) experiment late in flowering. In all later years, I expanded to three experiments corresponding to the early, middle and late portions of flowering. The earliest and latest cohort dates always fell outside the period during which supplementations were done. The supplements can be viewed as representing the first third, middle third and last third of the flowering period.

For both the cohort and supplementation studies, I missed some years, as shown in subsequent tables.

(f) *Harvesting and scoring fruit and seed set*

I harvested capsules when they were dry and straw-coloured but not yet dehisced. In all years, I classified scapes into three categories: fruiting, failed or grazed. Failed scapes were thread-like, unelongated and retained remnants of the flowers. Failure was usually caused by lack of pollination or by frost; the causes were not distinguishable by the condition of the scape. The condition of grazed scapes depended on the stage at which herbivores attacked. Those that were attacked while flowering were thread-like and unelongated but retained no trace of flower tissue, having instead a cleanly snipped apex. This damage was typically caused by flower-eating chipmunks, which tended to attack only the earliest blooming cohorts. Later, grazing of developing fruits by deer left stiff, straight scapes with roughly torn ends. Neither form of grazing took more than a few per cent of flowers, and numerous harvests lost no flowers to grazers. Very rarely, developing fruits were attacked internally by caterpillars that destroyed some or all of the ovules. These were counted as successful fruits in tabulations for fruit set, but were eliminated from tabulations of seed set (below).

In some years, I also scored seed set by dissecting fruits that had been preserved in 70 per cent ethanol. At the stage at which I harvested, *Erythronium* ovules appear as small and white, medium and shrivelled brown or large, turgid and green; these correspond to unfertilized, fertilized but aborted and successfully matured seeds, respectively (Rigney *et al.* 1993). I calculated the fractional seed set as the number of successful seeds divided by the total number of ovules.

(g) *Dependence on pollinators*

Although 'pollinator limitation' of fruit and seed set is widely assessed by conducting supplemental hand pollinations with outcross pollen (reviews by Burd 1994; Knight *et al.* 2005), the procedure does not truly mimic the improvement in pollination service that could be achieved by an increase in visitation: animal visitors will typically deposit a mixture of self- and outcross pollen that may be inferior to the pure outcross pollen usually used in supplementation experiments (Thomson 2001; Aizen & Harder 2007). To examine the response of *E. grandiflorum* fruit and seed set to direct manipulation of bumble-bee visitation, on 6 June 1991, I set up a small exclusion/enrichment experiment as follows. Through a dense stand of plants in bud, I laid out three contiguous, parallel belt transects of 0.6 × 3.7 m, and tagged all flower buds in each strip. Two of the transects were caged with side walls of 20 cm lumber and tops of fibreglass mosquito screen. One of them was kept closed to exclude bees; to the other, I added one queen bumble-bee (*B. occidentalis*, *B. bifarius* or *Bombus flavifrons*) per day until all buds had opened. The third strip was left uncaged as an open-pollinated control. All flowers had wilted by 20 June, when I removed the cages and let fruits develop without further intervention. When fruits were mature, I determined the fates of the flowers (aborted or successfully fruited) and counted the seeds produced by the successful fruits. This study plot was in a late-melting area, equivalent to the last or penultimate cohorts.

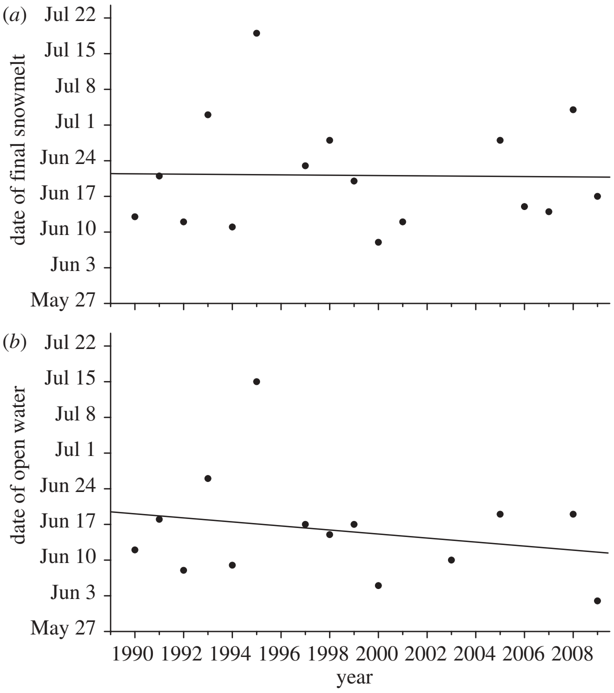

Figure 1. Phenological indicators at the study site during the period studied. Data are missing for some years. (*a*) The date on which the last winter snowpack disappeared in the property that formed the core of the study area. The property is mostly an open meadow with scattered trees. Snow remained beyond this date in nearby forests. (*b*) The day on which ice broke up on Lake Irwin, adjacent to the study area. Lines are simple linear regressions; neither indicator shows a significant trend over the years of the study. The slight negative trend for ice breakup disappears if the abnormally snowy year of 1995 is ignored.

3. RESULTS

(a) *Snowmelt and flowering*

The study period included early and late years, with the greatest contrast between 2006 and the very snowy 1995, in which local winter residents estimated over 3 m of new snow during the month of May. Indeed, the last cohort of 2006 was marked on 21 June, whereas the first cohort of 1995 was marked on 28 June. The *Erythronium* bloom was offset by a full month in these 2 years, a noteworthy displacement in a habitat where the entire growing season is considered to last only about three months. Although local summer high temperatures increased over the study period, this warming did not translate to earlier springs at the Irwin site: neither local indicator showed a trend (figure 1; cf. Inouye *et al.* 2000.) In a longer time series of snowmelt data from the RMBL, Forrest *et al.* (2010) do show a significant trend towards earlier springs since 1973. That trend may be unapparent at Irwin because of the shorter time series; Irwin's snows are also heavier and may therefore show higher variance.

Despite large timing differences in the onset of snowmelt, the spatial patterns of snowmelt across the study area were strikingly consistent across years, as shown in table 1 for the 4 years for which I compiled maps of the snow's recession. Beyond those 4 years, qualitative observations confirmed this consistency. It was obvious that trees and clumps of trees strongly influenced the pattern (figure 2): bare ground

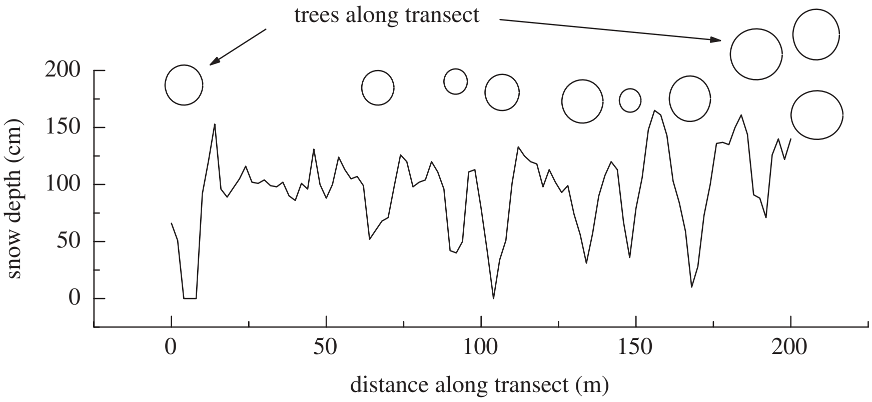

Figure 2. Patterns of snow depth along the permanent transect for 1995. The circles (not to scale) diagrammatically suggest the approximate positions and relative sizes of large trees near the transect. The trees were all to the northwest of the transect, and strongly influenced snowmelt patterns.

Table 1. Consistency of spatial pattern of snowmelt across 4 years ranging from early to late. (Nine points along the transect were chosen, each corresponding to open ground on each of the nine snow-mapping dates in the intermediate year 1994 (first open ground 4 June). The date of open ground at each of the nine points was determined for the maps from each of the other years, and the sets of open-ground dates correlated for all pairs of years. Values in the table are Pearson correlation coefficients. All correlations are highly significant, 7 d.f., $p < 10^{-5}$.)

	1992	1993	1994
1993	0.977		
1994	0.996	0.979	
1995	0.969	0.964	0.980

appeared first through sublimation driven by long-wave radiation from these conifers (Marchand 1991), especially on the south–southeast sides. For example, the spot where snow last disappeared from the property was always at the same place each year from 1991 to 2009, within a metre. This high-drifting spot is just north of a 70 cm diameter spruce. Interestingly, the melt hole to the south of the same tree is consistently one of the earliest patches to melt. Although only 7 m apart, these two patches have no temporal overlap in flowering. In parts of the meadow farther away from trees, the depths to which snow drifts are important in determining the sequence of melting out, and these drifting patterns are also consistent from year to year.

Shoots of *E. grandiflorum* typically emerged through the last few centimetres of snow at the receding edges, and plants frequently opened their flowers within a metre of the snow's edge. This visually obvious tight linkage between snowmelt and lily bloom, coupled with the across-year consistency of snowmelt patterns (table 1), yields across-years consistency in the timing of flowering along the transect (table 2).

(b) *Role of pollination in fruit and seed set*

Flowers caged to exclude animals showed negligible capacity for autogamous fruit production (table 3), although *E. grandiflorum* is partially self-compatible (Rigney *et al.* 1993). The 1991 cage experiment did not include a hand-outcross treatment, which would have been necessary to assess rigorously whether visitation by bumble-bees could approach the success of pure outcross pollination. A rough comparison can be made, however, by comparing the fruit set and seed numbers from the 1991 bee-addition experiment to comparable late hand pollinations from other years of the supplementation experiments (table 4). The mean seed number (26.9) from the 1991 bee-addition experiment is comparable to the late supplementation experiments in 1997, 1999, 2003 and 2008 (13.6, 28.5, 23.7 and 20.5, respectively). The 1991 fruit set (0.52) is lower than that for the late supplementation experiments in

Table 2. Consistency across 6 years of flowering in 40 2×5 m quadrats along the permanent transect. (Values in the table are Pearson correlation coefficients of the ranks of quadrats by their date of median bloom. All correlations are highly significant, 38 d.f., $p < 10^{-12}$.)

	1990	1991	1992	1993	1994
1991	0.932				
1992	0.913	0.956			
1993	0.880	0.910	0.900		
1994	0.887	0.954	0.942	0.959	
1995	0.863	0.933	0.902	0.945	0.943

Table 3. Response of fruit and seed set to experimental exclusion or enrichment of bumble-bee queens in caged groups of plants, late 1991. (Fruit set did not differ between the control and bees-added treatments (2×2 contingency $\chi^2 = 0.36$, $p = 0.55$), although both differed sharply from the bees-excluded treatment. Control and bees-added produced equivalent numbers of seeds per fruit (two-tailed t-test, $p = 0.34$).)

	control	bees added	bees excluded
flowers that successfully fruited	31	30	0
flowers that aborted	23	28	27
seeds per successful fruit (mean, s.d.)	23.7, 11.2	26.9, 13.8	undefined

Table 4. Fruit set and seed set in phenological cohorts of supplementally pollinated flowers and matched open-pollinated controls. (In 1993–1995, a single experiment was done near the peak date of flowering. In 1995, a second, smaller experiment was added after the peak. In subsequent years, three experiments of *ca* 300 flowers were done, before, at and after the peak. 'Failed fruits' include those lost to frost and to abortion. Fruit set is calculated as the number of fruits divided by the total number of successful and failed fruits. The index of 'fruit set limitation' equals the fruit set for the supplemented flowers divided by the sum of the fruit sets for supplemented and control flowers; values of 0.5 indicate equal success of supplemented and control flowers, whereas higher values indicate greater success of supplemented flowers, i.e. pollination limitation, with significant values in boldface. The significance of pollination limitation (p-value (fruits)) reflects the χ^2-squared statistic (not shown) for a 2 × 2 contingency table of (successful versus failed fruits) × (supplemented versus control). For years in which ovule fates were determined for successful fruits, 'seed set' is the number of matured seeds divided by the total number of ovules; the 'seed set limitation' index is calculated analogously to 'fruit set limitation', and the significance of seed set limitation is determined by a t-test (for unequal sample variances) of seed set, after arcsine-square-root transformation.)

year	date	treatment	total sample	eaten	fruits	failed fruits	fruit set	fruit set limitation	p-value (fruits)	seed set	seed set limitation	p-value (seeds)
1993	28 Jun	supp.	150	1	5	144	0.034	0.386	0.4018			
		control	150	0	8	142	0.053					
1994	16 Jun	supp.	150	12	121	17	0.877	0.498	0.8879			
		control	150	14	120	16	0.882					
1995	11 Jul	supp.	150	0	85	65	0.567	0.521	0.4172			
		control	150	0	78	72	0.520					
1997	20 Jun	supp.	175	5	105	65	0.618	**0.582**	0.0012	0.435	**0.520**	0.0031
		control	180	2	79	99	0.444			0.402		
	30 Jun	supp.	50	1	30	19	0.612	0.586	0.0819	0.356	0.480	0.517
		control	50	6	19	25	0.432			0.385		
1998	12 Jun	supp.	150	2	0	148	0	undefined	n.s.			
		control	150	7	0	143	0					
	20 Jun	supp.	149	0	0	149	0	undefined	n.s.			
		control	151	0	0	151	0					
	28 Jun	supp.	149	0	121	28	0.812	0.503	0.8135			
		control	151	0	121	30	0.801					
1999	12 Jun	supp.	150	0	39	111	0.260	**0.886**	<0.0001	0.411	**0.753**	<0.0001
		control	150	0	5	145	0.033			0.135		
	20 Jun	supp.	150	0	115	35	0.767	**0.714**	<0.0001	0.489	**0.645**	<0.0001
		control	150	0	46	104	0.307			0.269		
	28 Jun	supp.	150	0	82	68	0.547	**0.617**	0.0003	0.568	**0.559**	<0.0001
		control	150	0	51	99	0.340			0.449		
2003	7 Jun	supp.	147	3	114	30	0.792	**0.736**	<0.0001	0.582	**0.650**	<0.0001
		control	149	1	42	106	0.284			0.313		
	15 Jun	supp.	118	1	64	53	0.547	**0.660**	<0.0001	0.540	**0.675**	<0.0001
		control	124	0	35	89	0.282			0.260		
	23 Jun	supp.	150	0	69	81	0.460	**0.620**	0.0014	0.434	0.529	0.26
		control	149	0	42	107	0.282			0.386		
2005	18 Jun	supp.	144	10	84	50	0.627	**0.558**	0.0294			
		control	149	8	70	71	0.496					
	22 Jun	supp.	106	4	85	17	0.833	**0.611**	<0.0001			
		control	103	3	53	47	0.530					
	27 Jun	supp.	159	12	128	19	0.871	**0.566**	<0.0001			
		control	150	8	95	47	0.669					
2006	1 Jun	supp.	148	7	81	60	0.574	**0.673**	<0.0001			

(*Continued.*)

Table 4. (*Continued*.)

year	date	treatment	total sample	eaten	fruits	failed fruits	fruit set	fruit set limitation	*p*-value (fruits)	seed set	seed set limitation	*p*-value (seeds)
		control	149	9	39	101	0.279					
	9 Jun	supp.	145	4	46	95	0.326	0.563	0.1776			
		control	145	3	36	106	0.254					
	15 Jun	supp.	137	2	92	43	0.681	0.535	0.1311			
		control	134	4	77	53	0.592					
2008	18 Jun	supp.	144	11	89	44	0.669	**0.643**	<0.0001	0.475	**0.572**	0.0011
		control	142	10	49	83	0.371			0.356		
	22 Jun	supp.	148	9	100	39	0.719	**0.676**	<0.0001	0.351	**0.589**	0.0004
		control	149	10	48	91	0.345			0.245		
	26 Jun	supp.	145	6	93	46	0.669	**0.649**	<0.0001	0.370	0.535	0.0859
		control	148	10	50	88	0.362			0.322		
2009	4 Jun	supp.	145	0	10	135	0.069	**0.909**	0.0059			
		control	146	2	1	143	0.007					
	12 Jun	supp.	132	1	27	104	0.206	**0.904**	<0.0001			
		control	139	2	3	134	0.022					
	17 Jun	supp.	145	0	75	70	0.517	**0.640**	0.0001			
		control	148	0	43	105	0.291					

7 of 9 years, but is higher in 2003 and 2008. Although comparing different years is unwise because of different environmental conditions, we can tentatively conclude that the pollination efficacy of bumble-bees can approach that of pure outcross pollen, despite the fact that bees deliver substantial fractions of self-pollen (Thomson & Stratton 1985).

(c) ***Fruit set within and across year: cohorts and supplementations***

The 'cohort' data in figure 3 reveal sharp differences among years in the temporal pattern of fruit set. Herbivory was usually low and restricted to chipmunks eating early flowers, although deer grazing became more important in 2005–2008, perhaps owing to fewer domestic dogs near the study site. Fruiting failures reflect both frost damage and pollination deficits, so it is best to consider the among-year variation in conjunction with the supplementation experiments (table 4 and figure 4), and with observations on frost effects. *Erythronium grandiflorum* flowers can withstand mild frosts, but harder freezes kill flowers. Although frozen flowers appear superficially normal the day after a killing frost, the style and ovary lose turgor and become wrinkled. Such flowers never recover to set fruits. Buds and developing fruits are more resistant to frost than flowers in anthesis. Therefore, frosts of intermediate severity can kill later-opening flowers while sparing earlier ones that have started maturing fruits. Extremely hard frosts kill a wider range of developmental stages. For example, a hard frost and snowstorm on 5 June 2007 killed virtually all flowers in the study area, leading me to cancel the regular experiments in that year. The destruction in 1993 was almost as complete, although some flowers survived in two cohorts, probably by being somewhat sheltered from longwave radiative cooling to cold night skies (Leuning & Cremer 1988; Inouye 2000). In contrast, plants in 1994, 2003 and 2008 escaped strong frosts and showed roughly similar fruit set in all cohorts; in 1998, 1999 and 2005, earlier cohorts failed completely but later ones succeeded. In 1995, 1999 and 2009, fruit set increased more gradually over time. Pollinator availability probably interacted with frost damage in these years. The year 1995 was quite anomalous because of the greatly delayed bloom; 2009 was anomalous because the June drought pattern never materialized: after warm weather in May; June 2009 was uncharacteristically cold and stormy. Therefore, flowering started early, but the date of snow disappearance was pushed back (figure 1*b*).

Considered only by themselves, patterns of early failure and later success are consistent with either frost damage or insufficient pollination. Unfortunately, I have no direct measures of frost damage, so I cannot formally deconfound these two sources of failure. Their effects can be partially disentangled, however, by considering the temporal patterns of the pollen supplementation experiments, at least in the later years in which I conducted early, middle and late supplementations (figure 4). If supplemented and control flowers both fail heavily, as in the early experiments from 1999 and 2009, pollination deficits can be ruled out. On the other hand, substantial differences between control and supplemented treatments

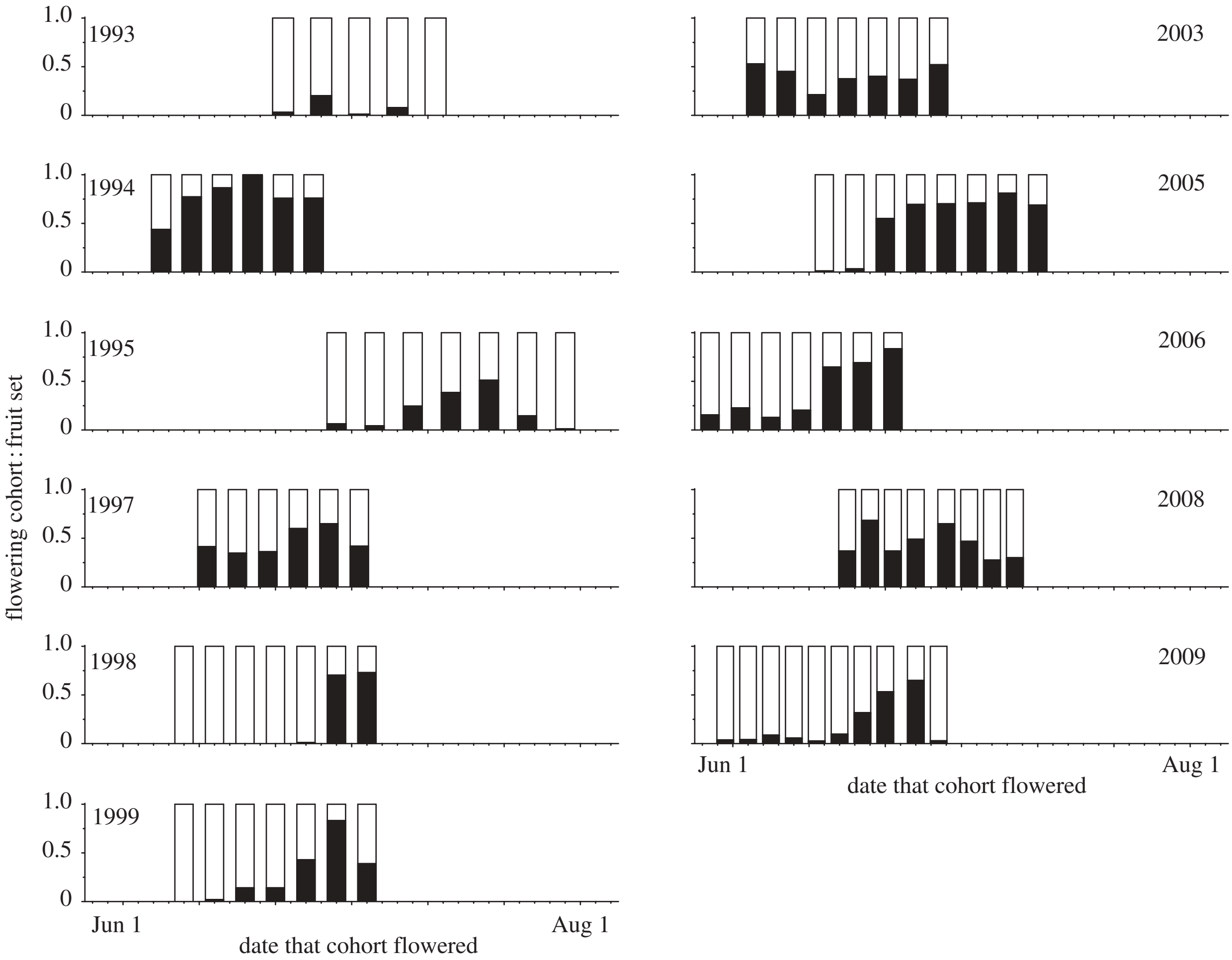

Figure 3. Patterns of fruit set over 11 years of observation of numerous temporal cohorts per year of unmanipulated, open-pollinated, single-flowered plants of *E. grandiflorum*. (Sample sizes approximate 100 plants for each cohort. The black portions of bars indicate the fraction of flowers that successfully set fruits, and the white portions indicate flowers that failed for any cause other than grazing. A few flowers were typically lost to herbivory (see table 4 for the magnitude of herbivory in different years); these were removed from this tabulation.)

suggest pollination deficits. Significant limitation of fruit set by pollination is evident in most but not all years and cohorts. In particular, cases in which pollination limitation—the gap between control and supplemented flowers—decreases from the middle to the late experiment suggest that pollinators were emerging too late to service the earlier flowers fully (figure 4, 1999, 2003, 2006).

In aggregate, the data suggest a hierarchy of effects. First, frost can reduce or prevent fruit set in any cohort, but is more likely to affect earlier cohorts. By killing flowers regardless of their pollination status, severe frosts render pollination limitation moot. In years when frost spares earlier cohorts, those flowers may suffer pollination deficits. Like frost, pollination deficits are more probable early in bloom. Therefore, both abiotic and biotic factors conspire to produce the pattern of greater fruiting success in later cohorts, which is evident in figure 3.

(d) *Ovule fates and limitation of seed set*

Erythronium grandiflorum flowers typically mature only about half of their ovules, even when supplementally pollinated (table 4). In all 4 years for which ovule fates were scored, however, supplemented flowers in earlier experiments set significantly more seeds than open-pollinated controls, i.e. there was significant pollination limitation of seed set (always in addition to limitation of fruit set). In all years, moreover, seed set limitation declined in the late experiments; in 3 of the 4 years, it declined to statistical insignificance. This pattern is consistent with the late emergence of bumble-bee pollinators.

(e) *Trends across years in pollination limitation*

Given that pollination limitation is an important factor for *E. grandiflorum* at this study site, one can ask whether it is changing in importance with time. For each set of experiments, the pollination limitation index tends to increase over the period of the study (figure 5), suggesting a deterioration of pollination service. Following a suggestion from an anonymous referee, I have examined the significance of these trends by calculating the log-response ratio measure of effect size (Knight *et al.* 2005), which has better statistical properties than the bounded proportional index I use in table 4 and the figures. I assessed trends by calculating Pearson correlations between log-response ratios (ln (supplemented proportional fruit set/control proportional fruit set)) and the year of sampling. Data from early and middle experiments

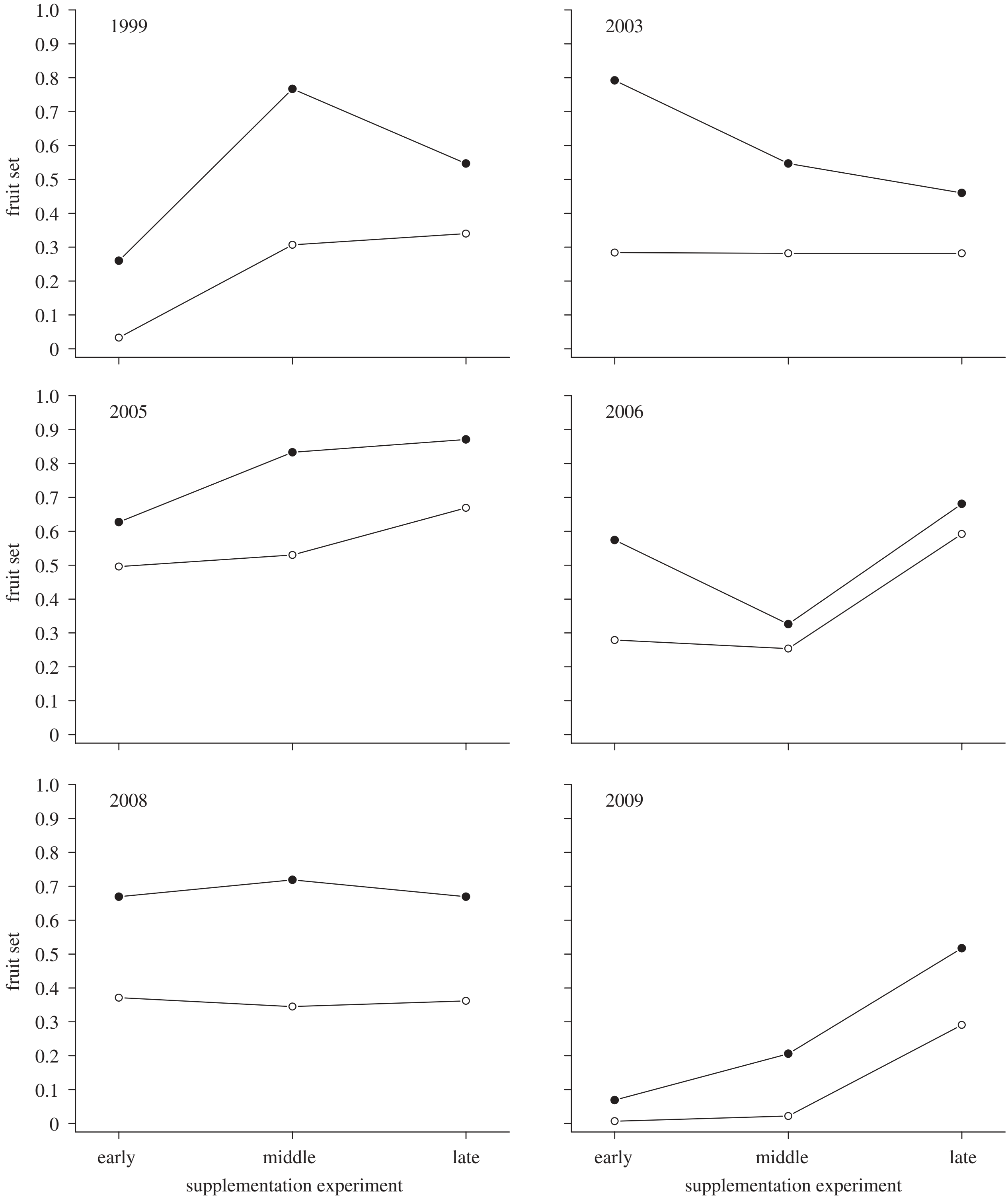

Figure 4. Temporal patterns of response to supplemental hand-pollination experiments for the 6 years in which three experiments were done in the early, middle and late portions of the flowering period (table 4). (Filled circles represent fractional fruit set of flowers receiving supplemental outcross pollen; open circles represent open-pollinated controls.)

in 1998 could not be included because frost damage eliminates all fruit set in both treatments, rendering the log–response ratio undefined. The single 1993 datum is also problematic, being based on very few surviving fruits in either treatment. It is true to say that there was no pollination limitation in those years, but those data contribute little information about pollination service.

The trends for early and late supplementation experiments do not approach significance. The trend for mid-bloom supplementations is significant if the 1993 datum is included ($r = 0.714$, $n = 10$, $p = 0.020$), but not if it is discarded ($r = 0.633$, $n = 9$, $p = 0.067$). Doing a single test that combines early, middle and late datasets requires correcting for the tendency of pollination limitation to decline within a season. Averaged across all years for which all three measures are available, the log-response ratios for early and late experiments differ from that of the middle experiments by factors of 1.50 and 0.58,

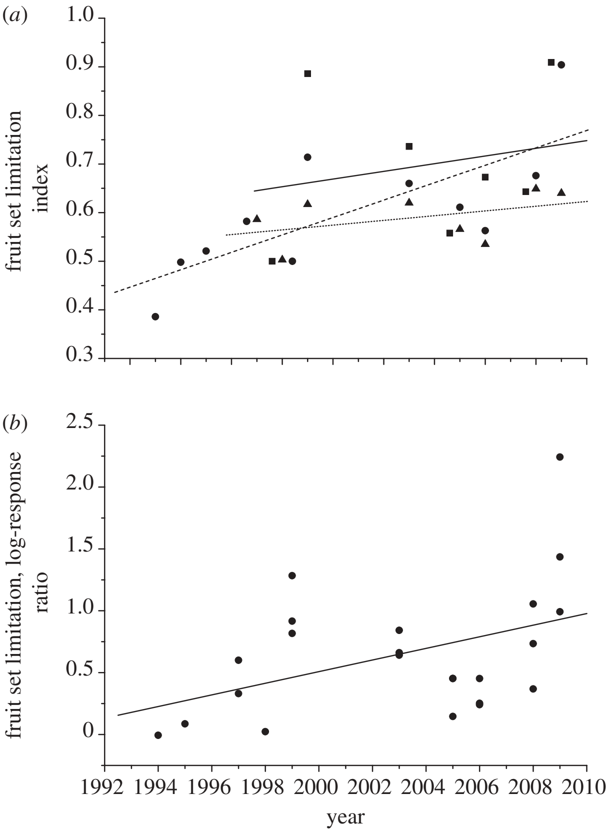

Figure 5. Temporal trends in the degree of pollination limitation over the course of the study. (*a*) Indices of pollen limitation for supplementation experiments done in the early, middle or late portions of the flowering period are indicated by squares, circles and triangles, respectively. Symbols that overlapped have been offset horizontally. Simple linear regression lines are presented to indicate trends within each dataset (early, solid; middle, long dash; late, short dash). These graphs include points from 1993 and 1998 experiments with such heavy frost damage that few or no fruits were produced in either treatment. These points are excluded from the analyses of significance (see text and table 4). (*b*) As above, but the data have been transformed to log-response ratios, the problematic 1993 and 1998 data removed and the early and late datasets rescaled by factors representing the average differences among the three temporal supplementation experiments within years. This allows an overall test of the hypothesis that pollination service has deteriorated over the study period ($r = 0.434$, $n = 23$, $p = 0.039$).

respectively. Therefore, I rescaled all early and late data by these factors to examine an overall temporal trend. For the rescaled data including 1993, $r = 0.526$, $n = 24$, $p = 0.008$; eliminating the dubious datum for 1993, $r = 0.434$, $n = 23$, $p = 0.039$. Therefore, the data strongly suggest a deterioration of pollination service, although the next several years' data will probably determine how robust it is. Why is the trend more significant for middle cohorts than for early or late cohorts? In part, there are more data points for the middle cohorts, but the differences may well reflect ecology, too: in early-flowering cohorts, pollination limitation will more frequently be negated by frost damage; in late cohorts, it may be ameliorated by the continuing emergence of more pollinators. The index of pollination limitation was uncorrelated with the date of last snowmelt ($r = -0.30$, $n = 10$, $p = 0.4$).

4. DISCUSSION

(a) *Timing of snowmelt and flowering*

The *E. grandiflorum* plants in the study area do not comprise an idealized panmictic population but rather a snowpack-driven mosaic of different patches that bloom at different times and face different stresses (Yamagishi *et al.* 2005). Given the potential variability in such factors as the timing and character of snow storms, and the wind-driven drifting of snow, it seems somewhat surprising that the spatial pattern of spring snowmelt should be as repeatable as it is. However, similar repeatability has been reported from alpine regions (e.g. Kudo 1993, p. 1304; Stanton *et al.* 1994, p. 364, and references therein). It is also commonplace that the timing of flowering is correlated with the depth of snowpack (Billings & Mooney 1968; Inouye & McGuire 1991). What distinguishes the subalpine study population of *E. grandiflorum* from the tundra habitats studied by Kudo and Stanton *et al.* is the important role of large trees that are scattered through the Irwin meadows. By influencing drift patterns and creating early melt holes in spring, these trees contribute much to the spatio-temporal mosaic of lily bloom, with effects on both population-genetic structure and the exposure of different patches to different conditions of weather and pollinator availability. Early flowering plants growing in tree melt holes may be able to mate with each other, as early bees fly from one hole to another, but unable to exchange gametes with much closer neighbours. In treeless tundra, by contrast, the major structuring template would be the microtopography of the terrain itself. Of course, tree-based phenological structure will shift when the trees die, but the large trees at Irwin are old (e.g. one human-felled Engelmann spruce stump of 85 cm diameter had 349 annual rings), and they frequently grow with apparently self-perpetuating clusters of daughter stems around their original trunks. Recruitment of new trees is rare, and seedlings grow slowly (e.g. one spruce at the centre of the study area is at least 20 years old but is only 75 cm tall). Therefore, trees are a rather stable structuring element in these subalpine meadows.

Under present conditions, early flowering cohorts are less likely to set fruit in most years, which suggests a non-equilibrial situation that might induce selection against early flowering time if unopposed. To explain a similar situation in *Rhododendron aureum*, Kudo (1993) proposed that the pollination advantage of late flowering was countered by the disadvantage of maturing fruits in the face of autumn frosts and snow. This seems inapplicable to *E. grandiflorum*, which fruits in midsummer, with no obvious penalty for flowering later. However, response to selection on flowering time would be attenuated because any genetic basis for flowering time would tend to be swamped by non-genetic variation arising from snowpack effects. Where a seed lands is likely to affect the resulting plant's flowering time more than its genetic heritage.

(**b**) *Pollination, frost and fruit failure*

Reviews suggest that alpine tundra plants frequently suffer from pollination limitation (García-Camacho & Totland 2009) and that their reproductive success tends to increase as the season progresses (Molau 1993). The subalpine *E. grandiflorum* exemplifies both tendencies. Pollinating bees are critical to seed reproduction in *E. grandiflorum*. The 1991 cage experiment showed that autogamous fruit set is minimal, and that bumble-bee queens by themselves can produce fruit and seed set equivalent to open pollination late in flowering (when pollination is characteristically more sufficient). Pollination limitation of both fruit and seed set prevailed in most but not all years of the study, highlighting again the importance of replicating limitation studies, first stressed by Campbell (1987). Pollination limitation is clearly a characteristic of an ecological situation rather than a constitutional attribute of a plant species (Wilson *et al*. 1994). More precisely, *E. grandiflorum* tends to receive poor pollination service early in its blooming period, but pollination typically improves through the blooming period. That improvement correlates with increasing numbers of bumble-bees seen while conducting experiments, and is probably monotonic (although the latest lily flowers did poorly in the unusual years of 1999 and 2009, possibly because bees switched to other forage). Because early flowers are more frequently killed by frost, however, *pollination limitation* (as defined by the difference between control and supplemented flowers) is not monotonic, tending instead to be strongest in mid-bloom.

Has recent climate change affected this system? Specifically, do we see earlier spring melts, more killing frosts (as proposed by Inouye 2000) or more pollination deficits (as proposed by Price & Waser 1998; Dunne *et al*. 2003; Saavedra *et al*. 2003; Memmott *et al*. 2007)? No, no and yes. Considering only data from the Irwin study site, neither abiotic (figure 1) nor biotic (figure 2) events have advanced. It may be that the effects of warming have been counteracted by concomitant increases in snowpack depth, as suggested by Inouye *et al*. (2000). On the other hand, Miller-Rushing & Inouye (2009) have documented a longer term trend towards earlier snowmelt in the broader area around the Irwin study area since 1973. This trend has not registered at Irwin over the course of my study, however. Nor is it apparent that killing frosts have increased, at least insofar as these have affected the reproductive success of *E. grandiflorum*. Admittedly, the recent years of 2007 and 2009 delivered killing frosts, but 2003, 2006 and 2008 were relatively benign.

On the other hand, pollination service has apparently deteriorated, especially for plants that flower during the middle of the bloom (figure 5). Because such deterioration has been anticipated on the grounds of pollinator declines (National Research Council 2006) or climate-driven phenological shifts (Memmott *et al*. 2007), this first documentation of a progressive decline may warrant further discussion despite its borderline significance. The most likely possibilities are that (i) pollinator populations have declined, (ii) lily flowering and pollinator emergence have become less synchronous, or (iii) pollinators have shifted their activity away from the lilies, presumably by visiting other plant species. Rigorously evaluating these possibilities would require direct estimates of pollinator abundance, which are utterly lacking. Nevertheless, I speculate that the third explanation is unimportant. The only other significant native floral resources for bumble-bees during the lily peak are *Mertensia fusiformis* Greene and *Salix* spp., and neither of these appear to have changed in density or timing. Turning to pollinator declines, the best pollinator of *E. grandiflorum*, *B. occidentalis*, has declined over much of its range (Williams & Osborne 2009), and some RMBL researchers believe that it may have become rarer in the study area. No estimates of absolute abundance are available. However, replicated quantitative surveys near the RMBL show no decline in the relative abundance of *B. occidentalis* as a fraction of all bumble-bees: Pyke (1982) found 3.8 per cent (502/13 136) in 1974; J. Thomson & E. Long (unpublished data) found 6.9 per cent (42/611) in 1998; and J. Thomson & B. Thomson (unpublished data) found 5.2 per cent (30/579) in a late-season sample from 2007. Because this species has not declined relative to other *Bombus* spp., the increasing pollination deficits are more probably attributable to a phenological mismatch or to a general decline in bumble-bee species. Casual observations are inconsistent with an overall decline in bumble-bees. Essentially, all flowers of *Corydalis caseana* Gray in a large stand near the Irwin study area show holes from nectar robbing, almost certainly done by *B. occidentalis* workers, with no decline in attack rates evident from 2007 to 2009 (J. Thomson, unpublished data). It appears that healthy populations of effective pollinators remain in the area.

Therefore, weak inference suggests a growing phenological mismatch between the blooming of *E. grandiflorum* and the emergence of its best pollinators. Hegland *et al*. (2009, p. 184) argue that such mismatches ought to be rare because the ‘onset of flowering in plants and first appearance dates of pollinators in several cases appear to advance linearly in response to recent temperature increases’, but there could certainly be exceptions. Kudo *et al*. (2004) reported that one particularly early spring in Hokkaido depressed seed set in two bee-pollinated spring ephemerals but not in two fly-pollinated ones. Even if emergence times remain in step, however, both the activity levels of queen bumble-bees and the longevity of flowers may be very sensitive to air temperatures, insolation, precipitation and wind. If these factors are changing, subtle dislocations of bees and flowers seem plausible. Further research is needed to see whether the trend continues, and what might be driving it.

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