

## Seasonal change in a pollinator community and the maintenance of style length variation in *Mertensia fusiformis* (Boraginaceae)

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• **Background and Aims** In sub-alpine habitats, patchiness in snowpack produces marked, small-scale variation in flowering phenology. Plants in early- and late-melting patches are therefore likely to experience very different conditions during their flowering periods. *Mertensia fusiformis* is an early-flowering perennial that varies conspicuously in style length within and among populations. The hypothesis that style length represents an adaptation to local flowering time was tested. Specifically, it was hypothesized that lower air temperatures and higher frost risk would favour short-styled plants (with stigmas more shielded by corollas) in early-flowering patches, but that the pollen-collecting behaviour of flower visitors in late-flowering patches would favour long-styled plants.

• **Methods** Floral morphology was measured, temperatures were monitored and pollinators were observed in several matched pairs of early and late populations. To evaluate effects of cold temperatures on plants of different style lengths, experimental pollinations were conducted during mornings (warm) and evenings (cool), and on flowers that either had or had not experienced a prior frost. The effectiveness of different pollinators was quantified as seed set following single visits to plants with relatively short or long styles.

• **Key Results** Late-flowering populations experienced warmer temperatures than early-flowering populations and a different suite of pollinators. Nectar-foraging bumble-bee queens and male solitary bees predominated in early populations, whereas pollen-collecting female solitary bees were more numerous in later sites. Pollinators differed significantly in their abilities to transfer pollen to stigmas at different heights, in accordance with our prediction. However, temperature and frost sensitivity did not differ between long- and short-styled plants. Although plants in late-flowering patches tended to have longer styles than those in early patches, this difference was not consistent.

• **Conclusions** Seasonal change in pollinator-mediated selection on style length may help maintain variation in this trait in *M. fusiformis*, but adaptation to local flowering time is not apparent. The prevalence of short styles in these populations requires further explanation.

**Key words:** Boraginaceae, floral morphology, frost, herkogamy, local adaptation, *Mertensia fusiformis* Greene, *Osmia*, phenology, pollination, Rocky Mountain Biological Laboratory, temperature.

### INTRODUCTION

Interspecific interactions vary over a species' geographic range, potentially resulting in a 'geographic mosaic' of co-evolution (Thompson, 2005) – or, at least, in a mosaic of selective forces acting on one of the interaction partners. The importance of spatial variation in interactions, and resulting selection, is widely recognized (e.g. Herrera *et al.*, 2006; Andrew *et al.*, 2007; Siepielski and Benkman, 2007; Gómez *et al.*, 2008). If gene flow between localities is limited, local adaptation is possible. Local adaptation combined with limited migration is one possible mechanism for the maintenance of genetic variation within individual populations (Mitchell-Olds *et al.*, 2007).

There is a temporal analogy to spatial variation in selection. If a species exhibits variation in reproductive phenology, even within a small area, early-reproducing individuals may never

mate with late individuals. Limited gene flow between early and late sub-populations can result in 'isolation by time', analogous to the more widely appreciated isolation by distance concept of population genetics (Hendry and Day, 2005). Such isolation may be accompanied by divergent selection pressures acting on early and late sub-populations (Fox, 2003; Hendry and Day, 2005) and, in theory at least, can lead to allochronic speciation (Devaux and Lande, 2008).

Alpine plants often show marked variation in flowering phenology even over small geographic areas due to patchiness in snow accumulation and melt date (Galen and Stanton, 1995; Yamagishi *et al.*, 2005). The locations of late and early patches can be remarkably stable over multiple years (Stanton *et al.*, 1994; Thomson, 2010), such that individual plants of polycarpic species are fairly consistent in their rank order of flowering across years. If seed dispersal is limited, and pollen transfer occurs only between individuals flowering at the same time,

this consistency in relative flowering time can extend across generations – raising the possibility of ‘adaptation by time’ (cf. Hendry and Day, 2005). In particular, flowering earlier or later in the season could mean exposure to different weather conditions as well as a different biotic environment: patchiness in snowmelt does not greatly affect the phenology of mobile animals in the community, which is likely to be more affected by other factors (i.e. above-ground temperature). Early and late plants are therefore likely to come into contact with different herbivores and pollinators (e.g. Kameyama and Kudo, 2009). This small-scale heterogeneity in selection could play a role in the maintenance of phenotypic variation in plant populations.

*Mertensia fusiformis* (Boraginaceae) is a herbaceous perennial that flowers shortly after snowmelt in sub-alpine meadows of western North America. It is self-incompatible, insect pollinated and lacking mechanisms for long-distance seed dispersal (although its seeds possess an elaiosome attractive to ants; Turnbull *et al.*, 1983). Timing of flowering is strongly influenced by local snowmelt date: plants in sheltered, low-lying or north-facing patches may flower up to a month later than plants growing in more exposed, south-facing patches nearby. Intriguingly, the flowers of this species show pronounced, continuous variation in style length (Fig. 1): some



FIG. 1. Inflorescences of *Mertensia fusiformis*, showing variation in style length. (A) Approach herkogamy; (B) reverse herkogamy.

individuals have stigmas projecting well beyond the anthers, a phenomenon known as approach herkogamy (Webb and Lloyd, 1986), while others have stigmas concealed beneath the ring of anthers (reverse herkogamy). Style length ranges from 2 to 10 mm in flowers that are approx. 1 cm long.

In addition to the variation among individuals, there also appears to be variation among *M. fusiformis* populations in the distribution of style lengths. This could be due to differences in selection between populations. Specifically, we hypothesized that, over the course of the season, changes in weather (including, for instance, the frequency of damaging frosts) or in the pollinator fauna could impose temporally varying selection on style length. This could generate consistent differences in floral morphology between relatively early- and late-flowering populations, i.e. adaptation by time. This hypothesis depends on style length being heritable in *M. fusiformis*, something for which we currently lack evidence. However, the existence of fixed (and taxonomically important) differences in style length among other *Mertensia* species suggests that the trait has some genetic basis in this genus. For example, the early-flowering, high-elevation *M. brevistyla* has short styles (i.e. reverse herkogamy), while the later flowering *M. ciliata* has long styles (i.e. approach herkogamy).

The following two complementary hypotheses were investigated to explain existing patterns of style length variation in *M. fusiformis*.

*Hypothesis 1: variation in temperature and risk of frost favours different style lengths at different times of the season.* There are two elements of this hypothesis. (1) Early-flowering populations may be more likely than late ones to experience frost, which can damage open flowers, preventing seed set (i.e. destroying female function). Frost due to radiative heat loss may pose a greater threat to long styles than to short styles, the stigmas of which are shielded by the anthers and corolla tubes. Therefore, if frost damage is indeed more likely early in the season, we expect short-styled plants to be favoured in early-flowering populations. (2) Even in the absence of tissue damage by frost, cold temperatures could affect chances of ovule fertilization by slowing or preventing pollen tube growth in styles (Lankinen, 2001; Hedhly *et al.*, 2005; Seymour *et al.*, 2009). If pollen tubes fail to reach the relatively distant bases of long styles in cool conditions, this might favour short styles in early-flowering populations.

*Hypothesis 2: variation in the suite of available pollinators favours plants with different style lengths at different times of the season.* In our study area, *Bombus* queens are the most conspicuous potential pollinators for early-spring flowers. When visiting *M. fusiformis*, these insects appear to focus mainly on nectar collection. Males of certain solitary bee species, most of which are protandrous, also appear in early spring; those with long tongues (e.g. *Osmia* spp.) can reach the nectar in *M. fusiformis* flowers. Female solitary bees emerge later in the season, and may visit *M. fusiformis* for nectar (if long tongued), for pollen (regardless of tongue length) or both. Hence, relatively late-flowering *M. fusiformis* may receive more visits from smaller, pollen-collecting bees. It is reasonable to expect different selective pressures to be exerted by the different types of pollinators, which differ markedly in size and foraging behaviour. In particular,

compared with nectar-collecting bees, which must force their mouthparts to the base of the flower, pollen-collecting solitary bees would be less likely to contact the stigmas of short-styled plants. If this is the case, and if female solitary bees are in fact more important pollinators late in the season, we expect long-styled plants to have more pollen deposited on stigmas than short-styled plants in later flowering populations. If greater pollen deposition results in increased fitness through seed set, long styles should be favoured in late populations.

Here, we present tests of these two hypotheses using field experiments and observations. Data were collected from multiple populations on style length, ambient temperatures during flowering and flower visitors in order to determine whether differences consistent with our hypotheses exist between early- and late-flowering sites. We then experimentally tested whether particular weather conditions or pollinators could differentially affect the female reproductive success of plants with different style lengths. The results provide a partial explanation for the existing style length variation in *M. fusiformis*.

## MATERIALS AND METHODS

### *Study organism*

*Mertensia fusiformis* Greene ('alpine', 'dwarf' or 'spindle-root bluebells') grows abundantly in sub-alpine meadows around the Rocky Mountain Biological Laboratory (RMBL), near Crested Butte, Colorado, USA, where it typically begins flowering 7–14 d after snowmelt (Inouye *et al.*, 2000). Individual flowering plants are approx. 10 cm tall and commonly produce approx. 50 flowers (although >900 flowers, on multiple stems, are possible). Flowers are approx. 1 cm long, blue, pendent and campanulate. Stamens have short, flattened filaments arising from the junction of the corolla tube and limb, and the filaments and anthers together form a cone around the style, making the nectaries at the base of the corolla tube inaccessible to short-tongued insects. The plants are self-incompatible (Forrest and Thomson, 2010) but compatible with other plants of similar style length (i.e. there is no heteromorphic incompatibility; J. Ogilvie, unpubl. res.).

### *Study sites*

Fourteen sites were selected in the vicinity of the RMBL, comprising seven pairs of 'early' and 'late' sites (details in Supplementary Data Table S1, available online). Paired sites were closer to each other than to other sites, but were separated from each other by up to 2.8 km and 187 m in elevation (on average, 1.0 km and 40 m). Our intention was to find sites that differed in snowpack but otherwise experienced similar conditions. At each site, we set up an observation plot of approx. 300 m<sup>2</sup> within which we monitored flowering phenology and observed pollinators (see below). To obtain an unbiased sample of individual plants, 4–6 belt transects bisecting each plot were established, and *M. fusiformis* plants with  $\geq 5$  buds growing inside the transects were flagged; transect widths were adjusted according to plant density so that the number of plants would total approx. 30. These 30 plants were used to characterize flowering phenology

and style length distribution within the plot. Peak *M. fusiformis* flowering, defined as the first date on which the maximum number of flagged plants were in flower, occurred from 11 to 27 d (mean = 16 d) later at late sites compared with their early counterparts. A data-logger [either a Hobo (Onset Computer Corp., Bourne, MA, USA) or a LogTag (MicroDAQ.com, Contoocook, NH, USA)] was placed in a shaded location within 100 m of each plot to record air temperatures during the flowering period. At a sub-set of these sites, plants growing up to 50 m outside the observation plots were used for the experiments described below (Supplementary Table S1).

### *Floral morphology*

To verify that there is less variation in style length within than among individuals, 30 plants were haphazardly selected outside of the study plots for repeated flower sampling. Near each of three 'early' study plots (Supplementary Table S1) (without regard to style length), ten plants that had at least 20 flower buds each were haphazardly chosen. Two open flowers per plant were collected (if possible) on each of four separate occasions throughout the flowering period of each plant, spanning a period of approx. 2 weeks. If plants had multiple stems, the two flowers were chosen from different stems. To characterize floral morphology at each study site, one haphazardly selected open flower was also collected from each of the 30 flagged plants in each observation plot. For each flower, corolla length, stigma height (from the base of the superior ovary to the top of the stigma) and anther height (from the base of the ovary to the top of the highest anther) were measured; from these measurements herkogamy was calculated as stigma height – anther height. Total stem length (the summed lengths of all the plant's stems) was also measured as an index of plant size.

Although *M. fusiformis* shows continuous variation in stigma height and stigma–anther separation rather than discrete style length morphs, for all experiments described below plants were categorized as either 'long styled' (i.e. stigma exerted beyond the tips of anthers) or 'short styled' (stigmas below the base of anthers), based on examination of at least two flowers per plant. When necessary, flowers were dissected in the field to determine the positions of concealed stigmas. Plants falling between these two categories (i.e. with stigmas overlapping anthers in one or more of the examined flowers) were omitted from experiments because we were primarily interested in detecting functional differences between the extreme phenotypes, rather than in characterizing the shape of selection on style length.

### *Floral temperature measurements*

To determine whether stigmas of long- and short-styled plants experience different temperature regimes, thermocouples (type T, 36-gauge copper–constantan; limit of error  $\pm 1.0^\circ\text{C}$ ) attached to flowers were used during several clear nights. To minimize microsite variation in temperature associated with different plants, only long-styled plants were selected for these measurements, using a paired design in which one flower per plant was assigned to be 'long' and a matched flower on the

same plant was assigned to be ‘short’. (Taking both ‘long’ and ‘short’ measurements on the same flower would have been preferable but was logistically impossible.) A thermocouple was looped around the end of the style of the ‘long’ flower to obtain temperatures close to the stigma, and a second thermocouple was inserted into the ‘short’ flower to measure temperatures within the corolla tube, where the stigma of a short-styled plant would be situated. Data-loggers (OM-CP-QuadTemp, Omega Engineering Inc., Stamford, CT, USA) recorded overnight temperatures every 10 min between 1900 h and 0900 h. A total of 15 plants were used at three different sites (Supplementary Table S1), and temperatures were recorded over five nights (2–4 plants per night).

#### Post-pollination temperature experiment

Natural temperature variation was used to test whether low temperatures, perhaps by slowing the rate of pollen tube growth down styles, would negatively affect seed set in long-styled plants. For this experiment, which took place at a single site (Supplementary Table S1), all wilted and open flowers were first removed from 20 long and 20 short plants to prevent resource pre-emption by early fruits. Plants were then covered with pollinator-exclusion bags (1 mm mesh). As new flowers opened over the following 9 d, matched pairs of flowers on each plant were assigned to morning and evening pollination treatments and marked accordingly. ‘Morning’ flowers were cross-pollinated by hand between 1000 h and 1130 h, when air temperatures were expected to continue to rise. Two different plants were used as pollen donors for each recipient flower. ‘Evening’ flowers were pollinated in the same way between 1825 h and 1930 h, to correspond with sunset at our site. All hand pollinations were conducted during conditions in which bumble-bees normally forage. In total, a minimum of four ‘morning’ and four ‘evening’ flowers were pollinated on each plant (one of each treatment per day on at least 4 d) between 23 and 31 May. Plants remained bagged until seed set of all marked flowers had been recorded.

#### Frost experiment

A natural frost event on the night of 24 May 2010, when air temperature dropped to approx.  $-5^{\circ}\text{C}$ , was used to test whether freezing temperatures differentially impair the functioning of long and short styles. On the day before the frost, 20 short and 20 long plants (ten each at each of two sites; Supplementary Table S1) were selected and all wilted flowers were clipped. Up to five open flowers per plant were then marked as a ‘frost’ treatment. On the morning after the frost, as many of the marked flowers as possible were cross-pollinated to ensure that pollen supply was not limiting fruit set. Flowers that were visibly damaged by frost were not pollinated and were omitted from analysis. Up to five newly opened flowers (that had not been open during the frost) or unopened buds on the same plants were then marked to serve as paired controls; these were cross-pollinated in the same way over the following days as they opened. Minimum temperatures on subsequent nights stayed above  $-2^{\circ}\text{C}$ . Seed set of all marked flowers was recorded.

Both the temperature and frost experiments were set up in a paired design, with both treatments applied to each individual plant. However, extensive herbivore damage to developing fruits meant that many plants were missing one treatment or the other. Therefore both experiments were analysed as simple two-factor analyses of variance (ANOVAs) to maximize the sample size. Evaluating the effect of style length on within-plant differences between treatments with *t*-tests (i.e. taking advantage of the paired design, but with reduced sample sizes) gave qualitatively identical results.

#### Pollinator surveys

The pollinator community at each site was surveyed by spending a minimum of five observer-hours (up to eight, if pollinators were scarce) observing insect visits to *M. fusiformis* within the observation plots. Surveys took place in good weather on at least five separate days, during an interval spanning 4 d before peak flowering to 9 d after peak flowering in the patch. During this time, a minimum of 60 % (mean = 96 %) of all flagged plants in the plot were simultaneously in flower. All insects observed entering flowers to obtain either pollen or nectar were considered potential pollinators. We attempted to follow each insect for its entire foraging bout. To characterize the pollinator fauna, the number of foraging bouts by each taxon of flower visitor was counted. Most insects were identified to family or genus in the field, and sex or caste of all bees was recorded. Most *Bombus* individuals could be identified to species. A small number of flower visitors were collected for more precise identification.

Visitors were categorized as pollen collectors or nectar collectors. All *Bombus* queens visiting *M. fusiformis* appeared to be collecting nectar exclusively (most did not have pollen loads and showed no sign of active pollen collection). Male bees, all butterflies and all long-tongued bombyliid flies were assumed to be visiting for nectar. In contrast, short-tongued bees (Halictidae and Colletidae) and syrphid flies were unable to reach the nectaries, but were clearly collecting or consuming pollen. Female *Osmia* can reach *M. fusiformis* nectaries and often enter flowers for nectar. However, because the majority of female *Osmia* were carrying pollen loads, or were actively collecting pollen, all visits by female *Osmia* were categorized as pollen visits.

#### Pollinator effectiveness

To test the effectiveness of different types of pollinators at transferring pollen to stigmas of short- and long-styled plants, freely foraging insects were allowed to make single visits to unvisited plants. These plants had previously been covered with pollinator-exclusion bags and categorized as short or long (again excluding plants of intermediate style length). The bags were removed and plants were observed until they were visited. The visitor’s identity and the number of visited flowers were noted, and the visited flowers on each inflorescence were marked. The plant was then re-bagged until all seed set data had been collected. In some cases, it was not possible to tell which individual

flowers within a dense inflorescence had been visited, so all open flowers were marked. Seed set was calculated as total number of seeds produced by marked flowers  $\div$  (number of visited flowers  $\times$  ovule number per flower), where per-flower ovule number was typically four. Mean seed mass (an average across all mature seeds resulting from a single pollinator visit) was obtained as an additional measure of pollinator quality. The individual plant was considered the unit of replication.

To increase our sample size of plants visited by *Bombus* queens (which were scarce at these sites at the time of our experiments), we used five *B. bifarius* queens that had been kept in captivity for 4 weeks on a diet of artificial nectar and prepared pollen for use in an unrelated experiment. These bees were chilled and then released individually into a portable flight cage (approx. 1 m diameter  $\times$  1 m height) placed over a small number of previously bagged plants. Each bee was allowed to probe at least one virgin flower before visiting the experimental plants to ensure the possibility of cross-pollination. Each bee visited between two and six plants, each of which was treated as an independent replicate.

Visitors were divided into four categories for analysis: (1) *Bombus* queens (specifically, *B. bifarius*, *B. melanopygus* and *B. sylvicola*; all are ‘short-tongued’ bumble-bees); (2) male *Osmia*; (3) female *Osmia*; and (4) female Halictidae [*Halictus virgatellus* and *Lasioglossum (Evyllaesus)* sp.]. Visits by the first two were considered nectar visits, and those by the latter two pollen visits (as above). Rarer visitors (*Colletes*, syrphids) were not included because of low sample sizes ( $\leq 3$ ). This gave us a fully crossed design (4 flower visitors, or 2 visit types,  $\times$  2 style lengths).

### Analysis

All analyses were conducted in R v. 2.7.2 (R Development Core Team 2008). Type III sums of squares (in the *car* package) were used for all ANOVAs. Response variables that were proportions were arcsine transformed, and other variables were fourth root- or log-transformed, to achieve normality whenever possible.

Among-site differences in herkogamy were tested first using a single-factor ANOVA with site as the sole predictor. To test for consistent differences between early and late sites, a general linear mixed model (package *lme4*) was used, with ‘early/late’ as a fixed predictor and ‘site’ as a random predictor. Significance of the early/late factor was evaluated using a likelihood ratio test of nested models.

Our pollinator effectiveness experiment was set up as a two-factor ANOVA, but the large number of zero values for seed set meant that parametric analysis was potentially inappropriate. (Seed masses, however, were normally distributed.) We therefore tested for effects of pollinator type and style length on seed set using randomization tests, in which all observations were reshuffled and the *F* statistics were recalculated 9999 times. The *P*-value for a given test was the number of *F* statistics that matched or exceeded the observed statistic, divided by 10 000.

## RESULTS

### Floral morphology

Across a sample of 30 plants, mean corolla length, stigma height and anther height were all positively correlated (Pearson’s  $r > 0.65$ ,  $P < 0.0001$ ). Mean stigma height was more variable than the other traits (among-plant coefficient of variation = 14.5%, vs. 8.5 and 9.8% for corollas and anthers). Herkogamy was correlated with stigma height ( $r = 0.68$ ,  $P < 0.0001$ ) but not with anther height ( $r = 0.02$ ,  $P = 0.91$ ), indicating that among-plant variation in herkogamy was primarily due to variation in stigma height. None of the floral measurements was correlated with plant size [measured as  $\log_e(\text{total stem length})$ ; all  $|r| < 0.12$ ,  $P > 0.55$ ]. There were significant differences among individual plants in the degree of herkogamy ( $F_{29,204} = 24.4$ ,  $P < 0.0001$ ), but the sample as a whole showed a fairly continuous, close-to-normal distribution (Fig. 2).

Floral morphology differed significantly among study plots (one-way ANOVA,  $F_{13,401} = 5.3$ ,  $P < 0.0001$ ; Fig. 3). Plants in late-flowering plots tended to have more exerted stigmas, but this trend was not significant (likelihood ratio  $\chi^2 = 5.7$ , d.f. = 3,  $P = 0.13$ ; Fig. 3), and the trend was reversed at two site pairs.

### Environmental conditions at early and late sites

During the period spanning our pollinator observations at each site, early sites were significantly cooler, on average,

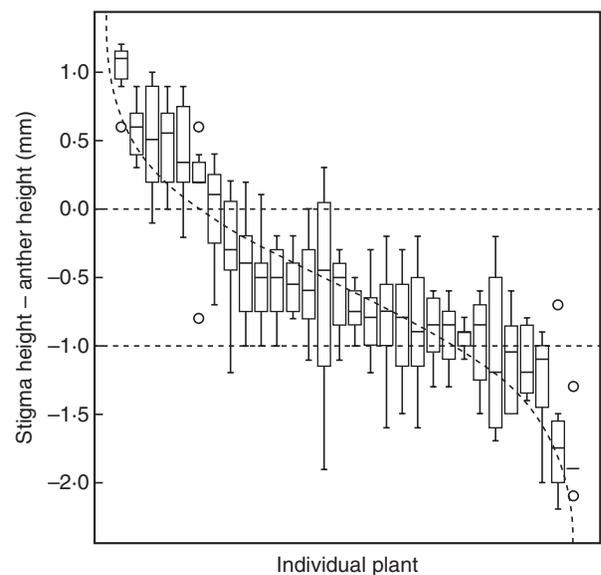


FIG. 2. Tukey boxplots showing variation in herkogamy within and among 30 individual *Mertensia fusiformis* plants. Plants are plotted in order of decreasing mean stigma exertion. Boxes show within-plant medians and interquartile ranges. The dashed line was generated by drawing many random values from a normal distribution with mean =  $-0.56$  and s.d. =  $0.67$ , and plotting the sampled values in descending order. Plotted values were connected with a spline. Values between 0 and  $-1$ , demarcated by the two horizontal dotted lines, indicate overlap between stigmas and anthers (i.e. no herkogamy), assuming anthers are 1 mm long (an approximate value; not measured in all flowers). Differences in herkogamy among individual plants are significant.  $n = 5\text{--}8$  flowers per plant.

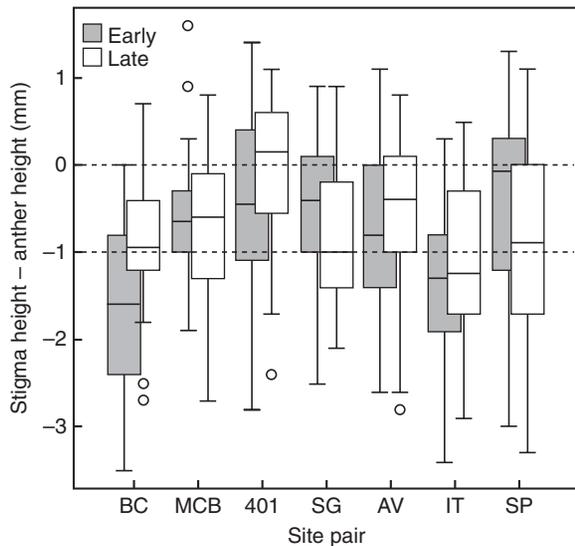


FIG. 3. Boxplots of herkogamy for plants in the seven pairs of study plots; early-flowering and late-flowering plots are as indicated. Site pairs are plotted in order of increasing peak flowering date of early plots. Dotted lines are as in Fig. 2. Early and late plots do not differ significantly.  $n = 29\text{--}30$  plants per plot.

than late sites (mean temperature  $9.7$  vs.  $12.1$  °C; paired  $t$ -test,  $t_6 = 6.9$ ,  $P = 0.00046$ ). Early sites were also more likely to experience a severe frost: three of seven early sites had minimum temperatures less than  $-4$  °C, whereas no late site experienced temperatures below  $-1$  °C.

A total of 117 visitors to *M. fusiformis* flowers were observed in 48 observer-hours (on average, 2.4 per observer-hour) at early sites and 452 visitors in 45 h (10.0 per observer-hour) at late sites (Table 1). This almost certainly represents a higher per-flower visitation rate at late sites, but we cannot be sure without estimates of floral density at each site. Early sites had a higher proportion of visitors foraging for nectar than late sites (70 % vs. 39 %; paired  $t$ -test,  $t_6 = 2.7$ ,  $P = 0.034$ ). Late sites were instead dominated by pollen-collecting bees (Fig. 4). This change was mainly driven by the greater number of visits by pollen-collecting female *Osmia* and halictids in late sites (mean 0.62 *Osmia* and 0.36 halictid visits per site per observer-hour in late sites, vs. 0.035 and 0.077 visits per observer-hour in early sites), rather than by a decline in the number of visits by *Bombus* queens or male *Osmia*.

#### Temperature and its effects in long and short styles

Contrary to expectations, no difference in minimum or mean overnight (2000–0600 h) temperatures was found between ‘long’ (exposed) and ‘short’ (shielded) stigmas (paired  $t$ -tests,  $t_{14} < 1.5$ ,  $P > 0.15$ ); in fact, long stigmas had slightly higher mean and minimum temperatures than short stigmas ( $0.7$  and  $-2.7$  °C for long, vs.  $0.6$  and  $-2.9$  °C for short). Evening temperatures (1900–2100 h) also did not differ between long and short stigmas (paired  $t_{14} < 0.1$ ,  $P > 0.9$ ).

Daytime (1200–1600 h) air temperatures were consistently higher than night-time (2000–0000 h) temperatures at our study site during the post-pollination temperature experiment (mean difference =  $9.8$  °C). However, no effect of the timing

TABLE 1. Flower visitors recorded in *Mertensia fusiformis* observation plots

Visitor	Sex/caste*	Total in early plots	Total in late plots
<b>DIPTERA</b>			
Bombyliidae		2	0
Muscoidea		0	2
Syrphidae		3	20
<b>HYMENOPTERA</b>			
Apidae			
<i>Bombus appositus</i>	Q	1	0
<i>Bombus bifarius</i>	Q	28	10
<i>Bombus bifarius</i>	W	0	1
<i>Bombus californicus</i>	Q	2	1
<i>Bombus centralis</i>	Q	0	1
<i>Bombus flavifrons</i>	Q	6	30
<i>Bombus frigidus</i>	Q	1	0
<i>Bombus mixtus</i>	Q	0	1
<i>Bombus sylvicola</i>	Q	0	3
<i>Bombus</i> sp.	Q	6	2
Unknown	M	1	0
Colletidae			
<i>Colletes</i> sp.	F	0	1
Halictidae	F	26	114
Megachilidae			
<i>Osmia</i> spp.	M	28	67
<i>Osmia</i> spp.	F	12	198
<b>LEPIDOPTERA</b>			
Hesperiidae		1	0
Pieridae		0	1

Observations took place on a total of 48 h over 22 d between 16 May and 15 June 2010 in early plots, and on 45 h over 21 d between 2 and 26 June 2010 in late plots. See Supplementary Data Table S2 (available online) for the full species list.

\*Sex/caste recorded only for Hymenoptera. W, worker; Q, queen; M, male; F, female.

of pollination (i.e. morning or evening) on seed set (two-factor ANOVA,  $F_{1,76} = 0.21$ ,  $P = 0.65$ ) was found; nor was any interaction found between style length and post-pollination temperature ( $F_{1,76} = 0.18$ ,  $P = 0.67$ ).

Freezing temperatures before pollination did have a negative effect on ovule fertilization, indicating that our ‘frost’ treatment was effective: seed set of even apparently undamaged flowers was reduced from 55 % to 37 % on average ( $F_{1,72} = 4.64$ ,  $P = 0.035$ ). This was due to fewer frosted flowers setting fruit compared with unfrosted controls ( $F_{1,72} = 3.98$ ,  $P = 0.050$ ; Fig. 5), and not to any reduction in the number of seeds per flower among those that did set fruit ( $F_{1,65} = 0.40$ ,  $P = 0.53$ ; Fig. 5). Short-styled plants set significantly fewer seeds than long-styled plants ( $F_{1,72} = 4.28$ ,  $P = 0.042$ ), suggesting that we or the plants’ natural pollinators were less effective at pollinating short styles. Again, however, there was no style  $\times$  treatment interaction ( $F_{1,72} = 0.06$ ,  $P = 0.80$ ) – a result that is consistent with the lack of detectable temperature difference between long and short stigmas.

#### Pollinator effectiveness on long and short styles

Interestingly, seed set from single pollinator visits depended on the interaction between flower visitor, or visit type, and style length [ $F_{3,83} = 3.25$ ,  $P = 0.023$  (by randomization)] for

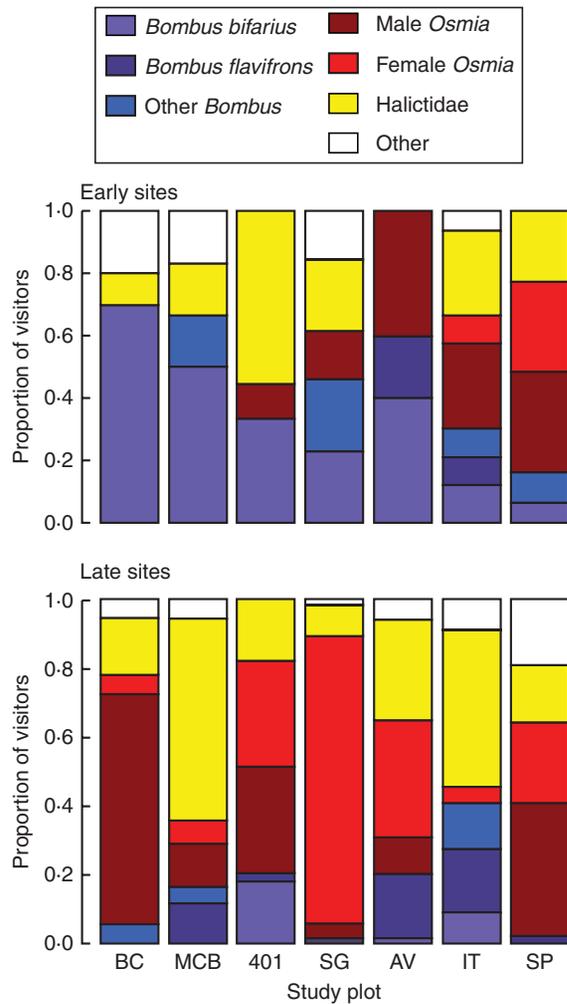


FIG. 4. Flower-visitor community composition at each of the 14 study plots. Bumble-bees (blue bar segments) are responsible for a larger proportion of visits in early sites. Solitary bees (red and yellow bar segments) make up the majority of visits in late sites.

flower visitor;  $F_{1,87} = 7.38$ ,  $P = 0.0082$  for visit type; Fig. 6). Female *Osmia* and halictids were significantly less effective at transferring compatible pollen to short- than to long-styled plants (*Osmia*:  $F_{1,33} = 12.82$ ,  $P = 0.0021$ ; halictids:  $F_{1,13} = 7.59$ ,  $P = 0.015$ ). In contrast, *Bombus* queens were equally effective at pollinating both types of plants ( $F_{1,22} = 0.85$ ,  $P = 0.36$ ). Male *Osmia* tended to produce higher seed set on short- than long-styled plants (44 % on average vs. 29 %; Fig. 6), but this trend was not significant ( $F_{1,15} = 0.65$ ,  $P = 0.45$ ).

Because of low seed set in certain pollinator  $\times$  style length combinations (specifically, male *Osmia* on long styles and halictids on short), we have low confidence in some of our seed mass estimates, and low power to detect treatment effects. Trends in mean seed mass were nevertheless similar to those for seed set (Fig. 6); however, there was no detectable effect of style length on seed mass for any pollinator or visit type ( $P > 0.3$ ).

## DISCUSSION

Our results provide only partial support for the hypothesis that variation in style length in *M. fusiformis* reflects adaptation to

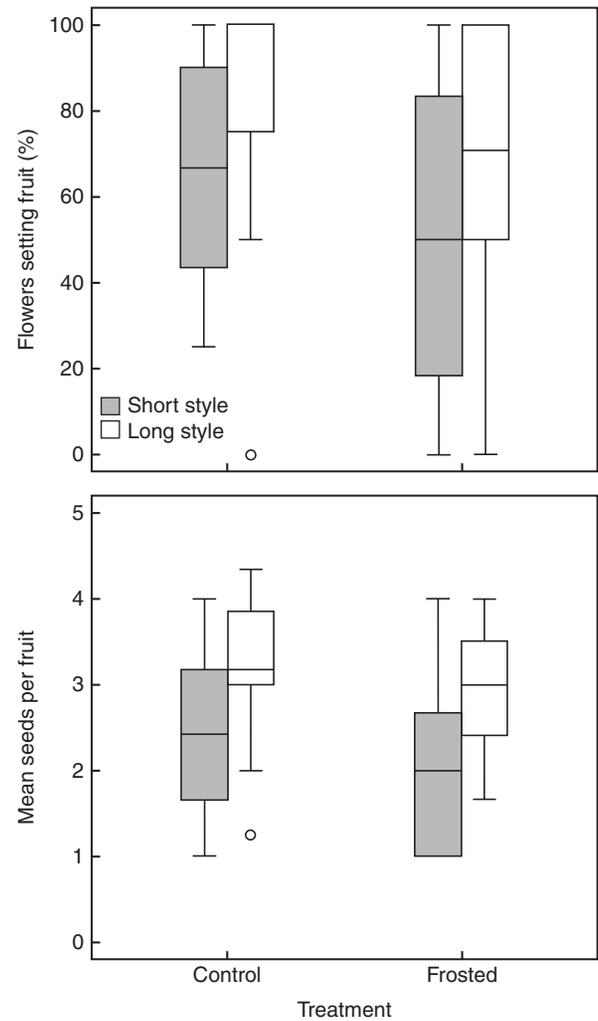


FIG. 5. Boxplots showing the percentage of flowers setting seed, and the mean number of seeds per flower (excluding those that set no seed), for flowers that had either experienced a severe frost the night before ('frosted') or not ('control'). There are significant main effects of treatment and style length, but there is no significant interaction.  $n = 15$ – $20$  plants per treatment combination.

conditions at the time of flowering. Although both ambient temperatures and pollinators differed between early- and late-flowering sites, no evidence was found that cold temperatures or frost differentially affected long- and short-styled plants; our results therefore do not support our Hypothesis 1. We did find that late-season pollinators were more effective at pollinating long-styled flowers, supporting our Hypothesis 2. However, although plants at late sites tend to have longer styles – the pattern that would be expected under pollinator-mediated selection via female function – the tendency is not significant. Below, we attempt to reconcile these findings, and we discuss other possible explanations for the maintenance of pronounced style length variation in *M. fusiformis*.

### Patterns of variation in floral morphology

In *M. fusiformis*, variation in herkogamy is a result of continuous variation in style length, without the discrete morphs

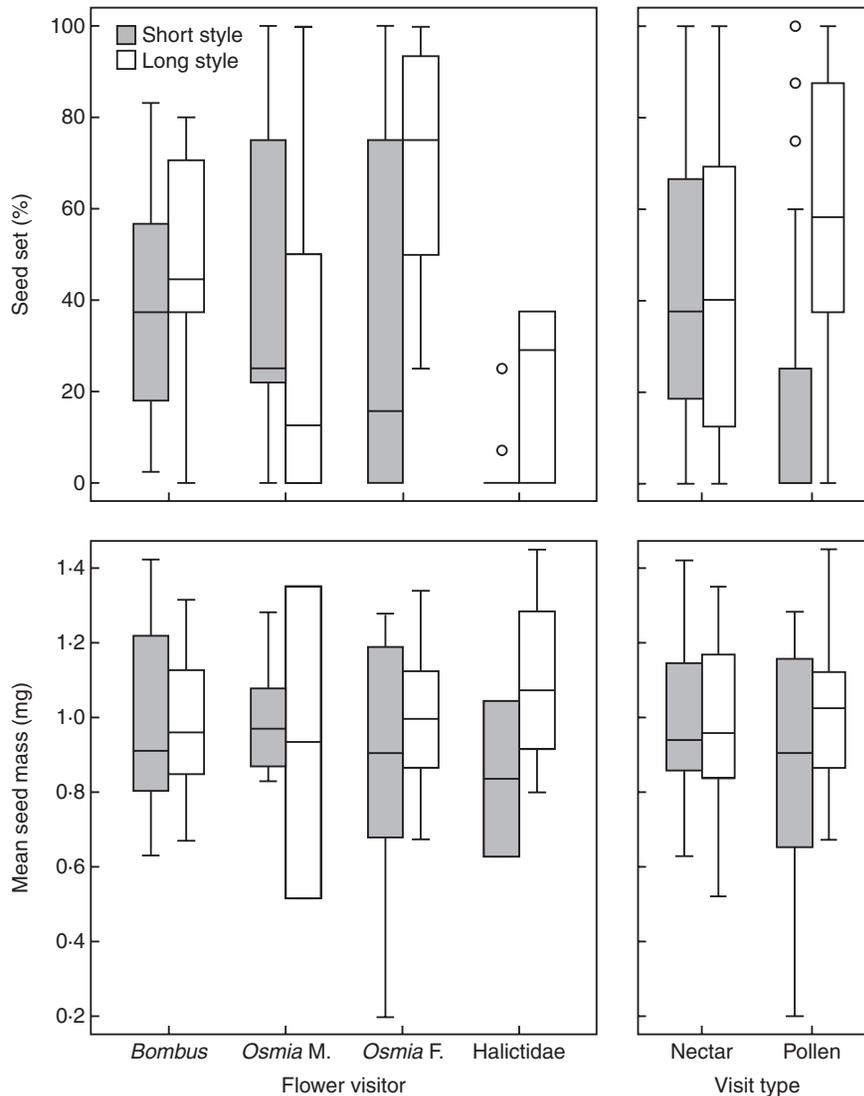


FIG. 6. Boxplots illustrating the effectiveness of different pollinator taxa, and different visit types, at fertilizing ovules of long- and short-styled plants. There is a significant style length  $\times$  flower visitor or visit type interaction for seed set, but not for mean seed mass.  $n = 6\text{--}25$  plants per treatment combination for seed set;  $n = 2\text{--}18$  for seed mass.

and inverse variation in anther height that is characteristic of truly heterostylous plants. While the latter type of stylar polymorphism is purely genetically controlled (by simple Mendelian inheritance; Lloyd and Webb, 1992), it is not yet known what causes *M. fusiformis* plants to differ in style length or stigma–anther separation. However, continuous variation in style length in other species is often strongly heritable (Kulbaba and Worley, 2008, and references therein). Moreover, true heterostyly and other discrete stylar polymorphisms are quite common within the Boraginaceae (Ganders, 1979; Ferrero et al., 2009, and references therein). The fact that herkogamy is consistent within individual *M. fusiformis* plants and does not scale with plant size leads us to expect that variation in this species, too, has a genetic basis, but this has yet to be confirmed with the necessary quantitative genetic experiments.

Similar variation in style length and herkogamy to that documented here has been reported in *Ipomopsis aggregata* (Waser and Price, 1984); in this species, the variation is correlated with functional gender (longer styled individuals having shorter corollas and a longer pistillate phase; Campbell, 1989). An association between ‘femaleness’ and long styles has also been noted in some heterostylous species (Beach and Bawa, 1980; Casper, 1992; Kohn and Barrett, 1992). We did not find any indication that longer styled plants are more functionally female in *M. fusiformis*; rather, style length, anther height and corolla length are all positively correlated in this species, and flowers are essentially adichogamous and invariant in ovule number. It is unlikely, therefore, that variation in stigma–anther separation reflects variation in functional gender in *M. fusiformis*, or that long- and short-styled plants are maintained by negative frequency-dependent selection.

We have instead hypothesized that variation in herkogamy is due to temporally or spatially varying selection via female function (i.e. seed set). This may seem incompatible with the finding that *M. fusiformis* in our study area were not strongly pollen limited in 2007–2008 (Forrest and Thomson, 2010), since an absence of pollen limitation suggests that selection to maximize pollen receipt should be weak at best. (Note that selection to protect stylar tissue from damage by frost should operate regardless of whether seed set is pollen limited.) However, our earlier study did not investigate whether pollen limitation was influenced by herkogamy and date of first flowering. Furthermore, multiyear studies frequently show that pollinator abundance and pollen limitation vary from year to year (e.g. Herrera, 1988; Baker *et al.*, 2000; Goodwillie, 2001; Buide, 2006); in our study area, occasional years with a dearth of pollinators could drive selection for increased pollen deposition. In fact, there is good reason to expect most plant populations to experience at least occasional pollen limitation and selection to maximize pollen receipt (Burd, 1994, 2008; Wilson *et al.*, 1994).

Based on our present results, we would predict pollen limitation to be most common in plants with short styles, perhaps especially in late-flowering populations: later flowering periods correspond to the flight season of pollen-collecting female solitary bees, which are comparatively unlikely to contact concealed stigmas. All else being equal, this should select for more exerted stigmas in late-flowering populations. In contrast, in early populations, which are visited mainly by bumble-bee queens and male solitary bees, most flower visitors will contact both short and long stigmas as they probe for nectar. Hence, pollinator-mediated selection on style length should be weak or absent in early-flowering populations.

However, despite a trend for longer styles in later populations, we were unable to explain most of the variation in style length among populations on the basis of flowering time, and, therefore, as an adaptive response to the seasonal transition in the pollinator community. There are several possible reasons for this. First, differences in selection between early and late populations may be insufficiently strong or consistent to produce measurable differences in stigma–anther separation. Inconsistent selection could result from interannual differences in spatial patterns of snow accumulation, such as those resulting from occasional avalanche runs. Even if spatial patterns of snowpack are consistent, however, among-year differences in the relative timing of plant flowering and insect emergence, or simply in the population sizes of different pollinator taxa, could produce interannual fluctuations in the pollinator community experienced by each *M. fusiformis* population. We are fairly confident that the qualitative pattern we have documented here (of proportionally more nectar visits early in the season) would be consistent, both because of the spatial replication in the present study and because most of the seasonal change in the pollinator community is the inevitable consequence of protandry (early male emergence) in solitary bees. However, quantitative changes in the relative abundances of bumble-bees and solitary bees, for example, could change the strength of selection on floral traits. Secondly, seed- or pollen-mediated gene flow between patches could override differences in selection gradients and

prevent morphological differentiation between populations. Seed dispersal from patches with different flowering times might explain the reversal of the expected pattern in style length at our ‘SP’ sites, both of which – unlike most other sites – had moderately bimodal frequency distributions of stigma exertion. [A bimodal pattern was not observed at the other ‘reversed’ sites (‘SG’), however.] Both gene flow and weak selection would tend to prevent or obscure local adaptation.

#### *How are short styles maintained?*

Our study addresses not only the issue of adaptation of local populations to seasonally changing conditions, but also a broader question about how genetic diversity is maintained within populations (Mitchell-Olds *et al.*, 2007): are seemingly maladapted phenotypes present because they are being constantly generated by mutation? Or do they result from some form of balancing selection that favours different phenotypes in different conditions? If the latter, is it a case of temporally varying selection within individual populations, or of local adaptation and migration of genotypes between populations (i.e. migration–selection balance, or balancing selection at a larger scale; Mitchell-Olds *et al.*, 2007)?

A significant interaction between pollinator type and floral morphology is a necessary condition for pollinator-mediated adaptive diversification in floral form, but fewer studies have succeeded in detecting such an interaction than would be expected from the level of interest in angiosperm diversity (Wilson and Thomson, 1996). Most examples of strong pollinator  $\times$  flower type interactions (e.g. Schemske and Bradshaw, 1999; Aigner, 2004; Muchhala, 2007; Streisfeld and Kohn, 2007) have come from contrasts between very dissimilar pollinator types (e.g. bats vs. hummingbirds, birds vs. bees). Our study is unusual in documenting a difference in effectiveness between relatively closely related pollinators visiting naturally co-occurring floral variants (though see also Harder and Barrett, 1993). Clearly, morphological or behavioural differences among bee genera, and between sexes of the same bee species, can have important implications for selection on floral traits. However, a significant pollinator  $\times$  flower type interaction is not a sufficient condition for diversification, which additionally requires the existence of a trade-off between adaptations to different pollinator types (Aigner, 2001). Our data do not point to a strong trade-off, since no situation in which long styles were inferior could be found. A slight tendency for greater pollination effectiveness of male *Osmia* on short- than on long-styled plants was observed, perhaps indicating that longer styles, which can be pushed outside the ring of anthers during a nectar visit, are less reliably positioned to contact pollen-bearing parts of these insects’ bodies. However, seed set from male *Osmia* visits was extremely variable, and the trend was not significant. This leaves us with the challenge of explaining the maintenance of short styles in *M. fusiformis* populations.

It is possible that the observed variation in herkogamy is selectively neutral, as we have not yet measured selection *per se*, but our results suggest that selection for long styles is likely, if only under certain conditions. In fact, we can imagine additional reasons, beyond those investigated here,

why stigma exertion might be advantageous. Long styles can potentially facilitate mate choice by females (Mulcahy, 1979; Skogsmyr and Lankinen, 2002). They also seem better suited to avoiding deposition of autogamous pollen (Webb and Lloyd, 1986), which could, in principle, clog stigmatic surfaces or prevent tube growth from compatible pollen (e.g. Ockendon and Currah, 1977; Parra-Tabla and Bullock, 2005). It was previously found that prior or simultaneous deposition of self-pollen had no detectable negative effect on seed set in *M. fusiformis*, provided cross pollen was available (A. Gorischek, unpubl. res.) – suggesting that there may in fact be little or no cost to having a stigma positioned where self-pollen deposition is likely. Nevertheless, receipt of self-pollen could have a negative indirect effect by triggering corolla abscission before the flower has been effectively pollinated (Forrest and Thomson, 2010), thereby reducing chances of further pollinator visits. Clearly, studies of maternal fitness as a function of style length and flowering time, in both open-pollinated and pollen-supplemented plants, will be needed to evaluate the importance of pollinator-mediated selection via female function in this system. We plan to conduct such studies in a future season.

Our hypothesis that short styles might be more protected from radiative heat loss was not supported by either our observational data or the results of the frost experiment. Small differences in temperature or cooling rate between exposed and concealed stigmas may simply have been undetectable using our methods: *M. fusiformis* flowers are too small to permit us to insert thermocouples into the stylar or stigmatic tissue itself, so our measurements may not have precisely captured the relevant temperatures. On the other hand, the typically pendent flower orientation, tightly clustered inflorescences and pubescent leaves of *M. fusiformis* may shield even long styles from heat loss to the night sky (or allow them to benefit from heat radiating from the soil), and our inability to detect cooler temperatures in exerted stigmas may reflect a real lack of thermal cost of longer styles. The fact that a previous night's frost did not reduce seed set of long-styled plants any more than that of short-styled plants supports the latter interpretation. In contrast, it seems inevitable that pollen tubes would require longer to reach and fertilize ovules when starting from a more distant stigma, and that pollen tube growth in both long and short styles would be slower at low temperatures. Nevertheless, we found no evidence (in the post-pollination temperature experiment) that the combination of long styles and cool night-time temperatures reduced seed set. Even during cool nights, pollen of *M. fusiformis* may be quite capable of reaching ovules, regardless of style length. It is worth noting that our experiments were aimed at testing effects of temperature on style tissue and pollen tube growth, but not pollen germination, which can also be affected by temperature (Galen and Stanton, 2003); this remains a possible area for future research in our system. Experiments using artificial flowers or the flowers of larger species as models could also help to test how floral orientation and herkogamy affect stigmatic temperature; however, they would be of limited value in determining whether temperature is an important agent of selection in field populations of *M. fusiformis*.

If exposure to a hostile abiotic environment is not a threat to long-styled plants, we must look elsewhere for factors that have maintained short styles in the population. Another possible advantage of shorter styles is that concealment within the corolla tube may provide protection from herbivory or mechanical damage by pollinators (cf. Parra-Tabla and Bullock, 2005). We did not test this possibility here because we have rarely observed herbivore damage to flowers in our study area (notwithstanding the frequent destruction of developing seeds after flowering), and the damage we have observed appears to affect the entire gynoecium and not merely the tip of an exposed style. Nevertheless, the possibility of differential damage to long and short styles merits further investigation.

In some plants, aspects of stigma morphology may in fact have evolved in response to selection via male function (i.e. pollen export), rather than selection to maximize pollen receipt, a possibility we did not consider here. For example, in *Mimulus aurantiacus*, the large, bilobed stigma closes shortly after receiving pollen – apparently a behaviour that has evolved to minimize interference between male and female functions (Fetscher, 2001). One could imagine that short styles might similarly facilitate pollen export in *M. fusiformis* (cf. Kohn and Barrett, 1992; Stone and Thomson, 1994). It is also possible that flowers with protruding styles might deter some prospective flower visitors; if so, the greater likelihood of pollen receipt by long styles (given a pollinator visit) could be offset by a lower probability of receiving a visit in the first place. It seems unlikely to us that the small, unobtrusive stigma of *M. fusiformis* would interfere with pollinator attraction or pollen export, regardless of its placement relative to the anthers. However, tests of pollinator preference, and analyses of pollen removal following single visits to long- and short-styled plants, would be necessary to confirm this.

### Conclusions

Early- and late-flowering populations of *M. fusiformis* differ consistently in the set of insects that frequent their flowers. Although our study took place over only a single season, we expect that this qualitative difference would be maintained over multiple years. This seasonal transition in pollinators should favour long-styled plants in later-flowering areas, due to the greater effectiveness of pollen-collecting bees in fertilizing long-styled plants. However, this expectation requires further validation, since we do not yet have measurements of selection on style length in open-pollinated plants. Also, the observed relationship between flowering time and floral morphology across sites, although consistent in direction with our prediction, was not significant – indicating either that local adaptation is not present, or that it is too weak to be detected with our limited sample size. Inconsistency among years in population flowering time and immigration from nearby areas with differing floral morphology may be obscuring any local adaptation that is occurring. The former possibility is easily tested in principle, although it would require several years of observation. The prevalence of non-locally adapted immigrant genotypes is more difficult to assess using direct observations, given the difficulties inherent in tracking seed dispersal. However, these immigrants should

be relatively more abundant in smaller populations. Given sufficient study sites, a correlation between adaptation and population size could be tested. Also, because we observed consistent differences in abiotic conditions (temperature) between early- and late-flowering populations, we might predict corresponding adaptations in physiological or morphological traits (other than the floral traits measured here), provided local adaptation is possible. Thus, comparisons of the spatial scale of adaptation in floral and vegetative characters could be revealing. Future investigations along these lines should help us explain the maintenance of within- and among-population variation in floral morphology.

#### SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxfordjournals.org](http://www.aob.oxfordjournals.org) and consist of the following. Table S1: co-ordinates and peak flowering dates of *Mertensia fusiformis* for all study sites. Table S2: taxa recorded visiting flowers of *M. fusiformis* in Gunnison County, Colorado, USA (2006–2010).

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