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The function of floral orientation in bluebells: interactions with pollinators and rain in two species of *Mertensia* (Boraginaceae)

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Abstract

Aims

Pollinators are traditionally considered to be the primary agent of selection on floral traits. However, floral traits may also be under selection from abiotic agents (e.g. rain), which makes considering the relative importance of pollinators and abiotic selective agents on floral traits essential. The functional significance of floral orientation is often ascribed to pollinator attraction, but orientation can also protect reproductive structures from rain. Therefore, a study that incorporates both factors will enhance our understanding of the ecological roles of floral orientation in plant fitness. *Mertensia brevistyla* and *M. fusiformis* are herbaceous species that differ in their floral orientations. A series of field and laboratory experiments was used to investigate the adaptive function of floral orientation in these species, particularly with respect to pollinators and rain.

Methods

We measured and compared floral orientation and visitor assemblages between *M. brevistyla* and *M. fusiformis* populations in western Colorado, USA. We manipulated floral stems and conducted a choice experiment with floral visitors, and also compared orientations of pollinator-visited stems with those of unvisited stems in a natural setting. We examined pollinator- and rain-mediated selection on floral orientation by manipulating orientation, conducting supplemental pollinations, applying watering treatments and measuring subsequent seed set. We also experimentally tested the likelihood of rain contact with anthers, and the effect of rainwater on pollen germinability.

Important Findings

Mertensia brevistyla had a significantly more upright floral orientation than *M. fusiformis*, and seed set was highest in upright *M. brevistyla* and in horizontal/pendant *M. fusiformis* stems, supporting an adaptive function (via female fitness) of the interspecific difference in orientation. However, floral visitor assemblages did not differ significantly between the two species; visitors did not exhibit significant preference for either orientation; and pollinator-mediated selection on orientation was undetectable. Similarly, there was little effect of water on seed set in either species, regardless of floral orientation. However, pollen germinability was reduced in both species by immersion in water; and water was more likely to contact anthers in *M. fusiformis* than in *M. brevistyla*, due to interspecific differences in floral morphology. We conclude that pollinators are likely not the primary selective agent driving differences in orientation in these *Mertensia* species. Instead, the negative effect of rain on pollen germinability helps explain the more pendant orientation of *M. fusiformis*, while short anthers in more upright *M. brevistyla* provide an alternative adaptation to rain. The selective agent driving effects of orientation on seed set remains unclear. This study illustrates the necessity of considering male fitness and abiotic agents in interpreting the functional significance of inflorescence traits.

Keywords: floral orientation, male and female fitness, *Mertensia*, pollinators, rain

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INTRODUCTION

Adaptation and diversification of floral traits in angiosperms are generally studied with the expectation that pollinator behaviour and morphology are the primary selective agents acting on these traits (reviewed by Fenster *et al.* 2004). Although animal pollinators are required for outcrossing in most angiosperms, flowers are also influenced by abiotic environmental

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conditions such as water availability and drought stress, which may also act as agents of selection on floral traits (Galen 2005; Strauss and Whittall 2006). Indeed, studies have found that abiotic factors can exert selection on stigma–anther separation (Elle and Hare 2002; Holtsford and Ellstrand 1992), flower colour (Warren and Mackenzie 2001) and flower size (Elle 2004). However, the relative importance of and interaction between pollinators and abiotic selective agents are rarely well characterized (but see Aronne *et al.* 2015; Chen *et al.* 2013), despite the fact that adaptive responses of floral traits to abiotic selection pressures may influence plant–pollinator interactions and overall plant reproductive success.

One floral trait that is likely to interact with both biotic and abiotic agents of selection is floral orientation—the angle at which open flowers are positioned relative to a vertical axis. Floral orientation is often overlooked in studies of pollinator-mediated selection, in favour of more conspicuous features, such as flower size and colour (Fenster *et al.* 2004). However, floral orientation can influence pollinator attraction (Fenster *et al.* 2009), and differences in floral orientation between related species can even facilitate reproductive isolation via differences in pollinator preference (e.g. *Aquilegia formosa* and *A. pubescens*; Hodges *et al.* 2002). Bumblebees, for example, have an innate preference for upright artificial flowers, although this preference might be overridden by bees experiencing other rewarding floral orientations (Makino and Thomson 2012). In addition to pollinator attraction, floral orientation has the ability to influence the effectiveness of flower visitors. Wang *et al.* (2014a) showed that when *Corydalis sheareri* flowers were rotated away from their natural horizontal orientation, pollinators became less effective at transferring pollen, which led to lower seed production in the manipulated inflorescences. These results demonstrate that pollinator attraction and effectiveness can vary with floral orientation, which may have important implications for plant reproductive success.

Flowers in different orientations also experience different abiotic conditions—such as rain exposure, which can be as important as pollinators in influencing plant reproductive success. For instance, pendant *Anisodus luridus* flowers protect pollen from being washed away and from reduction in pollen viability, thus benefitting the plant's male reproductive success (Wang *et al.* 2010). In contrast, water accumulation inside upright *A. luridus* flowers buffers flower temperature against fluctuating ambient temperature and creates a more stable microenvironment for the development of fertilized ovules, which improves female reproductive success in the plant (Wang *et al.* 2010). Given the contrasting ways in which pollinators (pollinator attraction vs. effectiveness) and rain (influencing male vs. female fitness) may exert selection on floral orientation, a study that incorporates both factors will enhance our understanding of the ecological roles of floral orientation in plant fitness.

Here, we focused on two herbaceous perennials, *Mertensia brevistyla* Watson (short-styled bluebells) and *M. fusiformis* Greene (dwarf bluebells; Boraginaceae). The two species appear to differ in floral orientation, with *M. brevistyla* flowers oriented horizontally or upright, and *M. fusiformis* flowers more pendant. The two species also differ in the length and exsertion of their reproductive structures, which may influence the costs and benefits of different floral orientations. As the species name suggests, the flowers of *M. brevistyla* have a short style and stamens concealed within the narrow corolla tube; therefore, due to surface tension, water may not come in contact with the reproductive parts even if the flowers face upward. In contrast, the flowers of *M. fusiformis* have a long style and stamens extending out of the floral tube; therefore, a more pendant floral orientation may be advantageous in this species in preventing rain damage to reproductive structures or in minimizing pollen loss to rain.

While we do not know the genetic basis of floral orientation in *Mertensia*, work with *Aquilegia* has detected a quantitative trait locus for floral orientation (Hodges *et al.* 2002), suggesting that interspecific differences in this trait could result from different responses to selection. We hypothesize that the observed difference in floral orientation between the two *Mertensia* species reflects an adaptive response to the ways in which pollinators and rain interact with floral orientation to influence plant fitness. More specifically, based on prior studies with artificial flowers and other species (Fulton and Hodges 1999; Makino and Thomson 2012), we expect pollinators to select for more upright floral orientation in both species. However, varying degrees of countervailing selection from rain exposure may explain the observed differences in floral orientation between the two species. In particular, we expect stronger selection from rain for pendant floral orientation in *M. fusiformis* than *M. brevistyla*, given the exserted floral organs in *M. fusiformis*.

We tested these expectations using several field observations and experiments. First, we measured and compared floral orientation and visitor assemblages between *M. brevistyla* and *M. fusiformis*. Second, we manipulated cut stems of both species into three floral orientations (pendant, horizontal and upright), presented them to approaching floral visitors, and recorded the visitors' choices. Third, we manipulated stems of intact plants into different floral orientations and examined pollinator-mediated selection by comparing seed set (female reproductive success) between open-pollinated and pollen-supplemented plants of each orientation. Fourth, we manipulated floral orientation in another set of plants and applied two different watering treatments to examine the combined effects of floral orientation and rain on seed set. Fifth, we examined whether water can enter the corolla tube of both species and experimentally tested whether water exposure affects pollen germination rate (a proxy for male reproductive success). Together, our results help to explain the functional significance of floral orientation in *M. brevistyla* and *M. fusiformis*, while still leaving some questions unanswered.

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MATERIAL AND METHODS

Study species and study sites

Mertensia brevistyla Watson and *M. fusiformis* Greene are herbaceous perennials found on the western slope of the Rocky Mountains in Colorado, USA, including our study area near Crested Butte, CO. Both species occur at our Kebler Pass site (KP; 38°51′9.26″N, 107°6′4.72″W); only *M. fusiformis* occurs at our Rocky Mountain Biological Laboratory site (RMBL; 38°57′.66″N, 106°59′8.30″W). Both species produce blue, campanulate flowers with the ~1 cm-long corolla consisting of a distinct tube and limb (Fig. 1). The flowers are clustered on a determinate cymose inflorescence. At KP, measured individuals were similar in plant height above ground (mean ± standard error [SE], *M. brevistyla*: 10.6 ± 0.8 cm; *M. fusiformis*: 9.6 ± 0.3 cm). *Mertensia brevistyla* produced 1.6 ± 0.1 stems/plant and 31.0 ± 2.8 flowers/plant, and *M. fusiformis* produced 1.5 ± 0.1 stems/plant and 44.5 ± 4.0 flowers/plant (n = 71 and 89 plants, respectively). In *M. brevistyla* flowers, the anthers are found on short filaments, inserted in the corolla tube, and the style is short and concealed in the corolla tube (Nazaire and Hufford 2014). In *M. fusiformis*, anther filaments are long, and style length varies continuously among plants from shorter to longer than the length of the corolla tube (Forrest *et al.* 2011).

Floral orientation

To quantify mean floral orientation for a given flowering stem of either species (relative to a vertical axis), we attached a suspended weight to the centre of a protractor, held the straight edge of the protractor tangential to the flowering stem, and recorded the angle between the suspended weight and the

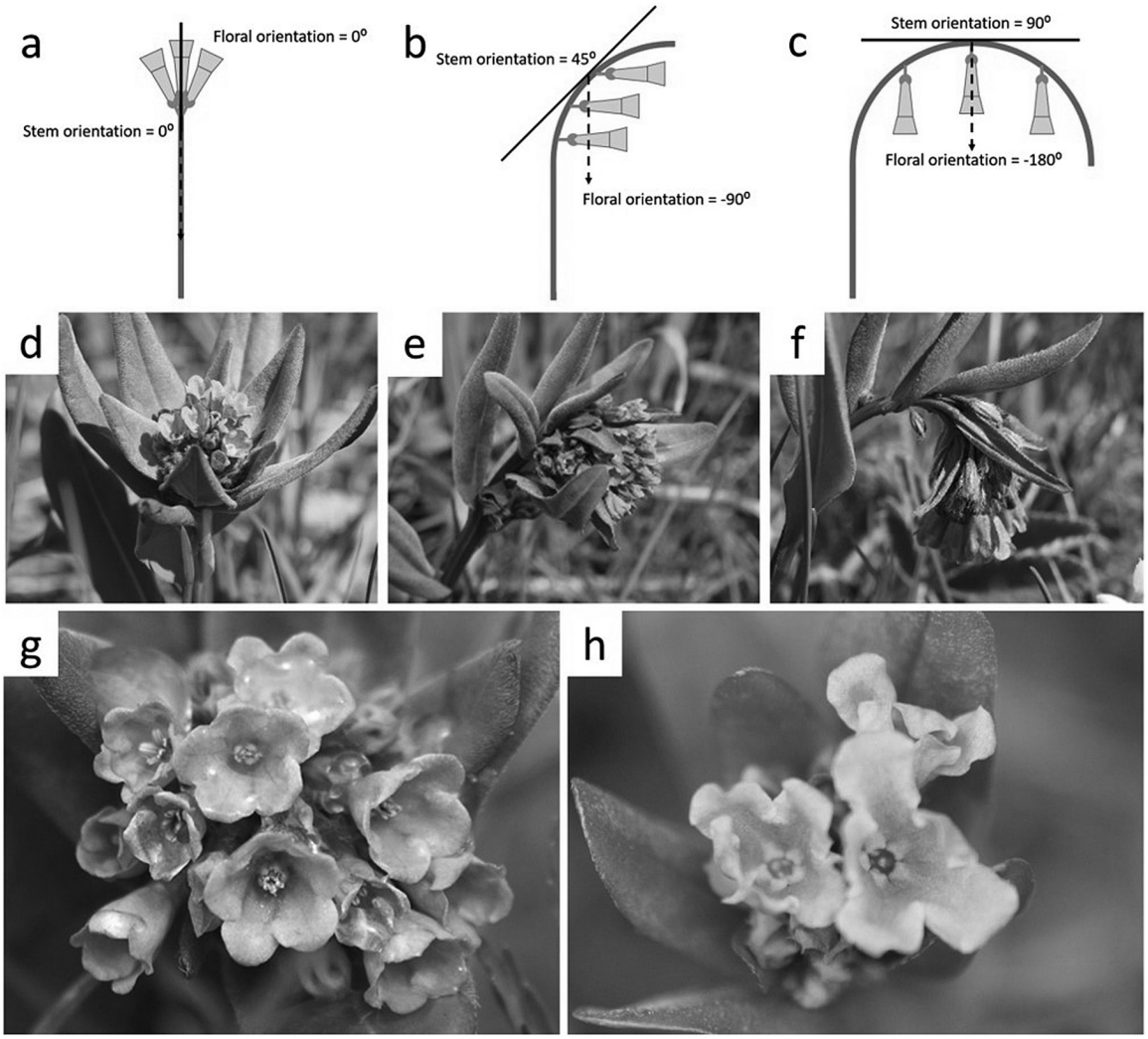

Figure 1: diagrams illustrating the technique used to measure (**a**) upright, (**b**) horizontal and (**c**) pendant floral orientations (the solid lines represent the edge of a protractor and the dashed arrows represent a suspended weight; see Materials and Methods). Examples of unmanipulated *M. fusiformis* in (**d**) upright, (**e**) horizontal and (**f**) pendant floral orientation. Photographs showing the (**g**) exserted stamens and style in *M. fusiformis* flowers with water droplets in some of the flowers and (**h**) inserted stamens and style in *M. brevistyla* flowers.

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straight edge of the protractor (Fig. 1). We measured each stem three times to calculate a mean angle, and then multiplied this value by −2 to represent mean floral orientation in a 180° context (i.e. 0° is completely upright and −180° is completely pendant; Fig. 1). We measured floral orientation of both species at KP in 2014, and compared orientation between species with a Mann–Whitney U test using the 'wilcox.test' function in the 'stats' package in R v. 3.2.3 (R Core Team 2015).

Floral visitor assemblages

To determine if visitor assemblages differed between *M. brevistyla* and *M. fusiformis*, we observed any insects visiting *Mertensia* flowers on warm, calm, dry days during the flowering season between 2014 and 2017 at KP and RMBL. We identified these floral visitors as: bumblebees (*Bombus* spp., Apidae), megachilid bees (Megachilidae), halictid bees (Halictidae), syrphid flies (Syrphidae) or other flies (Diptera). We pooled our observations with data from Forrest *et al.* (2011) on *M. fusiformis* visitors at KP and RMBL sites (Table 1). We performed a nonmetric multidimensional scaling (NMDS) on proportion of observed individual visitors per site and year using the 'metaMDS' function in the 'vegan' package (Oksanen *et al.* 2017). We then conducted a permutational multivariate analysis of variance (perMANOVA with 10 000 permutations) using the 'adonis' function, accompanied by a permutational multivariate homogeneity of group dispersions (PERMISP with 10 000 permutations) using the 'betadisper' function, to determine differences in NMDS centroid and dispersion between the two species.

Floral visitor preferences

To determine if floral visitors preferred a certain floral orientation, we conducted a choice assay in 2015 by presenting an 'interview stick' (Thomson 1981) to individual insects visiting *Mertensia* flowers (at KP for *M. brevistyla* and at RMBL for *M. fusiformis*). The interview stick was about 1 m long with three floral aquapics attached at one end. Each aquapic contained a cut stem of *M. brevistyla* or *M. fusiformis* that was manipulated into pendant, horizontal or upright orientation using a bent and looped metal wire inserted into the aquapic. We ensured each stem was accessible to the floral visitors and presented the three orientations simultaneously, and recorded the identity of the visitor and the orientation(s) selected by the visitor. Prior to each interview, we replaced any stem that was visited by the previous visitor or was losing its rigidity, and randomly arranged the aquapics (with different orientations) at the end of the interview stick. For the 32 visitors (pooled across the two plant species; $n = 1$ *M. brevistyla* and 31 *M. fusiformis* visitors), we performed a χ^2 test using the 'chisq.test' function in the 'stats' package to determine if the observed choices differed significantly from expected.

Second, we spent 7 hours in 2016 observing pollinator visits to *M. brevistyla* and *M. fusiformis* plants. For each flower visitor, we recorded orientation of the visited flowering stem and of the nearest unvisited flowering stem of similar height and number of flowers (on a different plant). We examined floral orientation of the visited and unvisited flowering stems for each species separately using linear mixed-effects models, in which we considered stem status (visited or unvisited) as the fixed factor, and individual pollinator as the random factor. We fitted the model using the 'lmer' function in the 'lme4' package (Bates *et al.* 2015) and tested significance of the factors using the 'lmerTest' package (Kuznetsova *et al.* 2016).

Pollinator-mediated selection on floral orientation

In 2014, we haphazardly selected flowering plants within a natural population of each species (*M. brevistyla* at KP and *M. fusiformis* at RMBL) and assigned each plant to a floral orientation treatment (pendant, horizontal or upright). We manipulated floral orientation by fixing one flowering stem per plant around a bent and looped metal wire inserted into the ground. In 2014, we manipulated 30 plants per orientation per species and supplemented pollen to half of the plants in each treatment to test for pollinator-mediated selection on floral orientation. Pollen from two different plants (at least 3 m from the focal plant) was collected and applied to the

Table 1: proportions of floral visitors observed visiting *M. brevistyla* and *M. fusiformis* flowers in 2010 and 2014–2017 at KP and RMBL (see Methods)

	M. brevistyla				*M. fusiformis*						
	2014	2015	2016	2017	2010		2014	2015	2016	2017	
Visitors	KP (9/12)	KP (39/11)	KP (8/7)	KP (26/1.75)	KP (132/28)	RMBL (359/54)	KP (13/12)	KP (50/12)	KP (5/4)	KP (5/0.25)	RMBL (17/1)
Bombus	0	0.23	0	0.04	0.19	0.20	0	0.26	0.40	0.20	0.59
Megachilidae	0.33	0.18	0.13	0.31	0.46	0.63	0.08	0.22	0.20	0	0.29
Halictidae	0.22	0.21	0.75	0.62	0.26	0.15	0.85	0.32	0.20	0.80	0.12
Syrphidae	0.33	0.10	0.13	0.04	0.08	0.01	0.08	0.16	0.20	0	0
Diptera	0.11	0.28	0	0	0.01	0	0	0.04	0	0	0

The numbers beside each site represent the sample sizes for total number of observed floral visitors and the number of observation-hours spent at each site, respectively.

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stigmas of all open flowers on the manipulated flowering stem. This was done several times throughout the flowering season to ensure that pollen was supplemented to all flowers on the focal stem. Seed set from the manipulated stems of all surviving plants was measured at the end of the growing season as the proportion of mature seeds produced relative to number of ovules, where ovule number = 4 × number of flowers. For each species separately, we compared seed set among the three floral orientation treatments and between the two pollination treatments (open-pollinated vs. pollen-supplemented) by fitting a generalized linear model (GLM) with a binomial error distribution using the 'stats' package. In each model, we considered floral orientation, pollination treatment, and interaction between orientation and pollination treatments as the predictor variables. We tested the significance of the predictor variables using likelihood-ratio χ^2 statistics (Morrell 1998). We also conducted Tukey's post hoc pairwise contrasts between orientations using the 'glht' function in the 'multcomp' package (Hothorn *et al.* 2008).

Effect of floral orientation on seed set

In 2015, we conducted the same experiment as above but we used only 15 plants per orientation per species and we did not conduct pollen supplementation because pollinator-mediated selection was not detected in 2014. For each species, we compared seed set from 2014 (both pollination treatments combined) and 2015 among the three orientation treatments by fitting a generalized linear model with binomial error. In each model, we considered study year, orientation and interaction between year and floral orientation as predictor variables. We tested significance of the predictor variables using likelihood-ratio χ^2 statistics.

Effects of water and floral orientation on seed set

To test whether water on flowers differentially affected maternal fitness in *M. brevistyla* and *M. fusiformis* of varying floral orientation, we set up four 1.0 × 1.0 m plots in natural populations of each species in 2016. We placed a 1.2 × 1.2 m corrugated polycarbonate sheet (Palram Americas, Sacramento, USA) on an angle over each plot (raised above the ground using plastic pipes) so rainwater would run off. Eight haphazardly selected plants in each plot were allocated to one of four treatments in a 2 × 2 factorial design: Each plant was manipulated into either a pendant or upright floral orientation and was subjected to one of two watering treatments (either water was sprayed over the entire plant, including the flowers, both from above and from the sides; or water was sprayed on the soil while avoiding the flowers). Five flowers on each manipulated plant were haphazardly selected for hand pollination (receiving pollen from pollen donors at least 3 m away on two separate occasions per flower to overcome possible pollen limitation) and marked with a permanent marker on the sepal. Each focal plant received two applications of 65 ml of water, resulting in a total of 130 ml of water per plant (June precipitation in Crested Butte averages ~3.3 cm, and we assumed each plant covers a surface area of ~39 cm^2). One of the two applications occurred ~2 hours before a hand-pollination event, and the other occurred after another hand-pollination event to simulate natural precipitation events occurring before and after pollination, respectively. Seeds were collected from the marked flowers at the end of the flowering season. For each species, we compared seed set among the four treatments by fitting a generalized linear mixed-effects model (GLMM) with a binomial error distribution using the 'glmer' function in the 'lme4' package. In the model, plot, floral orientation treatment, watering treatment and interaction between floral orientation and watering treatments were the fixed factors; plant was the random factor. We tested significance of the predictor variables using likelihood-ratio χ^2 statistics.

Water accumulation in corolla tubes

To assess if floral structure prevents water entry into the corolla tube of *M. brevistyla* or *M. fusiformis* flowers, we selected 15 plants of each species and removed two flowers from each. We placed each flower in an upright orientation, made a small incision on the corolla tube, and filled the corolla from above with tap water using a micropipette. Next, we inserted a 10 µl microcapillary tube into the incision to withdraw any water entering the corolla tube. Due to the high number of flowers without any water accumulation in the corolla tube, we treated water presence as a binary variable and tested for a difference between the two species by fitting a generalized linear mixed-effects model with binomial distribution. Species was the fixed factor, and flower nested within plant was the random factor. We tested significance of the species term using likelihood-ratio χ^2 statistics.

Effect of water on pollen germination

To test the effect of water on pollen germination rate, we randomly selected 15 plants of each species and removed three flowers with freshly dehisced anthers from each. Three anthers from each flower were removed, placed on microscope slides at room temperature, and randomly assigned to one of three treatments (one anther per treatment): 3 hours in pollen germination medium (5% sucrose solution with additional nutrients in tap water; Brys *et al.* 2008); 1 hour in tap water followed by 3 hours in germination medium; or 1 hour untreated followed by 3 hours in germination medium. At the end of each treatment, the proportion of pollen grains with growing pollen tubes relative to the total number of pollen grains was scored under three haphazardly chosen fields of view (mean ± SE number of pollen grains scored per slide: 10.0 ± 0.6; n = 270 flowers) with a Leitz Laborlux D light microscope at 200× magnification. We compared proportion of germinating pollen grains among treatments using a generalized linear mixed-effects model with a binomial error distribution. We considered treatment, species, flower, field of view and interaction between treatment and species as fixed

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factors; plant was treated as a random factor. We tested significance of the predictors using likelihood-ratio χ^2 statistics followed by Tukey's post hoc tests.

RESULTS

Floral orientation and visitor assemblages

Mertensia brevistyla and *M. fusiformis* differed significantly in their natural floral orientations (Wilcoxon, $W = 4857$, $P < 0.01$): *M. brevistyla* (mean ± SE, −120 ± 5°; range: −7° to −179°, $n = 71$ stems) had a less pendant floral orientation than *M. fusiformis* (mean ± SE, −152 ± 4°; range: −27° to −180°, $n = 89$ stems). Furthermore, upright floral orientation (i.e. between 0 and −90°) was more common in *M. brevistyla* (15 out of 71 stems; 21%) than *M. fusiformis* (5 out of 89 stems; 6%).

Halictid and megachilid bees accounted for most of the visitors to *M. brevistyla* (mean ± SE, halictid: 44.8 ± 13.8%; megachilid: 23.6 ± 5.0%, $n = 4$ year × site) and *M. fusiformis* (halictid: 38.5 ± 11.6%; megachilid: 26.9 ± 8.2%, $n = 7$ year × site; Table 1). Syrphid flies and bumblebees were the third most common visitors to *M. brevistyla* (15.0 ± 6.4%) and *M. fusiformis* flowers (26.3 ± 7. 0%), respectively. Visitor assemblages were similar between *M. brevistyla* and *M. fusiformis* (perMANOVA, $R^2 = 0.11$, $F = 1.08$, $P = 0.33$; PERMDISP, $F = 0.09$, $P = 0.76$, Fig. 2).

Floral visitor preferences

Based on our floral visitor choice experiment, there were more visits (all by *Bombus* spp.) to pendant stems than to horizontal or upright stems (pendant: 20 visits; horizontal: 14 visits; upright: 11 visits), but the observed frequencies did not significantly differ from expected (χ^2 test, $\chi^2 = 2.8$, df = 2, $P = 0.25$, $n = 32$ pollinators).

We also did not detect a significant difference in floral orientation between visited ($n = 7$ halictid bees, 2 megachilid bees, 2 *Bombus* spp., and 2 syrphid flies) and nearest unvisited stems in *M. brevistyla* or *M. fusiformis* (linear mixed-effects models, *M. brevistyla*: stem status [visited or unvisited]: $t_{14} = 0.79$, $P = 0.44$; *M. fusiformis*: stem status [visited or unvisited]: $t_4 = 1.37$, $P = 0.24$), although the visited stems were generally more upright than the unvisited stems in both species (see online supplementary Fig. S1).

Pollinator-mediated selection on floral orientation (2014 experiment)

Upright floral orientation produced the highest seed set in *M. brevistyla* (GLM, main effect of orientation: $F_{2,79} = 12.5$, $P < 0.001$; Fig. 3a), while horizontal orientation yielded the highest seed set in *M. fusiformis* (GLM, main effect of orientation: $F_{2,83} = 22.7$, $P < 0.001$; Fig. 3b). There was a weak but significant negative effect of pollen supplementation on seed set in *M. fusiformis*, with pollen-supplemented plants having lower seed set than open-pollinated plants on average (i.e. 11% vs. 14% mean seed set in the pendant orientation and 18% vs. 24% mean seed set in the horizontal orientation; supplementation: $F_{1,82} = 8.4$, $P < 0.01$). Among upright *M. fusiformis* stems, mean seed set was slightly higher in the pollen-supplementation treatment (i.e. 14% vs. 13%), but there was no evidence of pollinator-mediated selection on floral orientation (supplementation × orientation: $F_{2,80} = 1.6$, $P = 0.21$). There was also no evidence of pollen limitation in *M. brevistyla*, nor any indication that pollen limitation depended on floral orientation in this species (supplementation: $F_{1,78} = 0.15$, $P = 0.70$; supplementation × orientation: $F_{2,76} = 2.1$, $P = 0.12$).

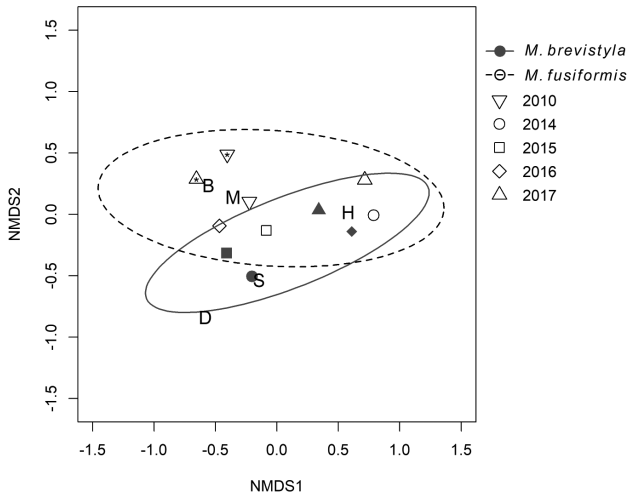

Figure 2: non-metric multidimensional scaling (NMDS; stress = 0.08) of pollinator assemblages (represented by letters in the figure: B = *Bombus*; H = halictid bees; M = megachilid bees; S = syrphid flies and D = other flies) of *M. brevistyla* and *M. fusiformis* in 2010 and 2014–2017. Asterisks indicate observations from RMBL; all other observations were conducted at KP. NMDS is based on Bray–Curtis similarities, and the ellipses indicate a 95% confidence interval.

Effect of floral orientation on seed set (2014 and 2015 experiments)

Mean seed set was highest in the upright orientation for *M. brevistyla* in both years, and seed set was higher in this species in 2014 than in 2015 (GLMM, orientation: $F_{2,83} = 6.4$, $P < 0.01$; year: $F_{1,82} = 14.0$, $P < 0.001$; Fig. 4a). In 2014, mean seed set in the upright orientation was 5% and 30% higher than horizontal and pendant orientations, respectively; and in 2015, it was 20% and 64% higher than horizontal and pendant orientations, respectively—leading to a marginally significant interaction between floral orientation and study year in *M. brevistyla* (orientation × year: $F_{2,80}$=2.9, $P = 0.055$).

In *M. fusiformis*, seed set was highest in the horizontal and pendant orientations in 2014 and 2015, respectively. *Mertensia fusiformis* seed set depended significantly on floral orientation, study year and the interaction between floral orientation and study year (GLMM, orientation: $F_{2,118} = 18.8$, $P < 0.001$; year: $F_{1,117} = 14.0$, $P < 0.001$, orientation × year: $F_{2,115} = 22.2$, $P < 0.001$; Fig. 4b). On average, mean *M. fusiformis* seed set

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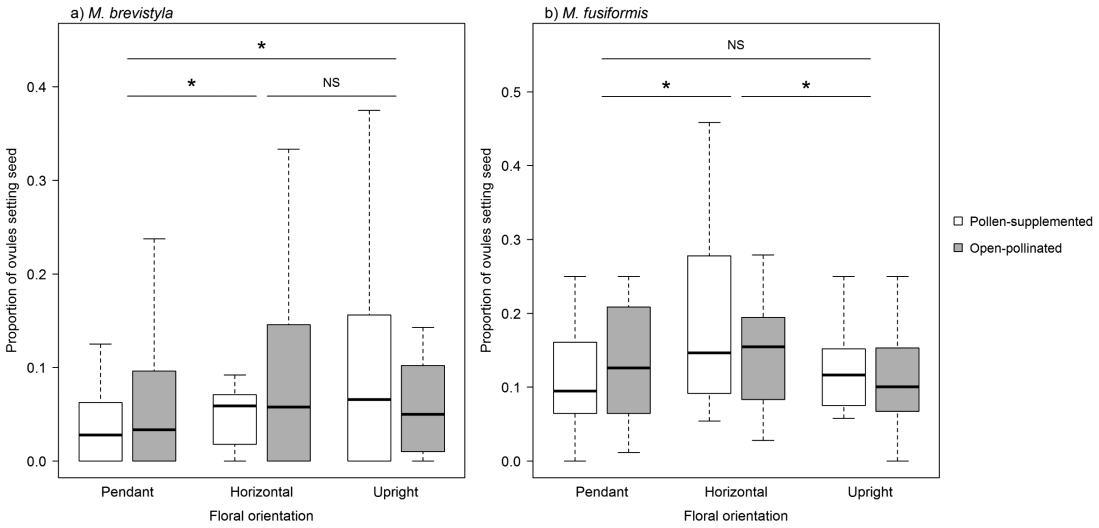

Figure 3: boxplots showing the proportion of ovules setting seed for each supplementation and floral orientation treatment for (**a**) *Mertensia brevistyla* and (**b**) *M. fusiformis* (n = 12 to 15 plants per treatment combination; total n = 118 *M. brevistyla* and 125 *M. fusiformis* plants). Boxes show the median and interquartile range. Lines above boxplots indicate Tukey's pairwise comparisons between orientations (*P < 0.05). Note difference in y-axis range between panels.

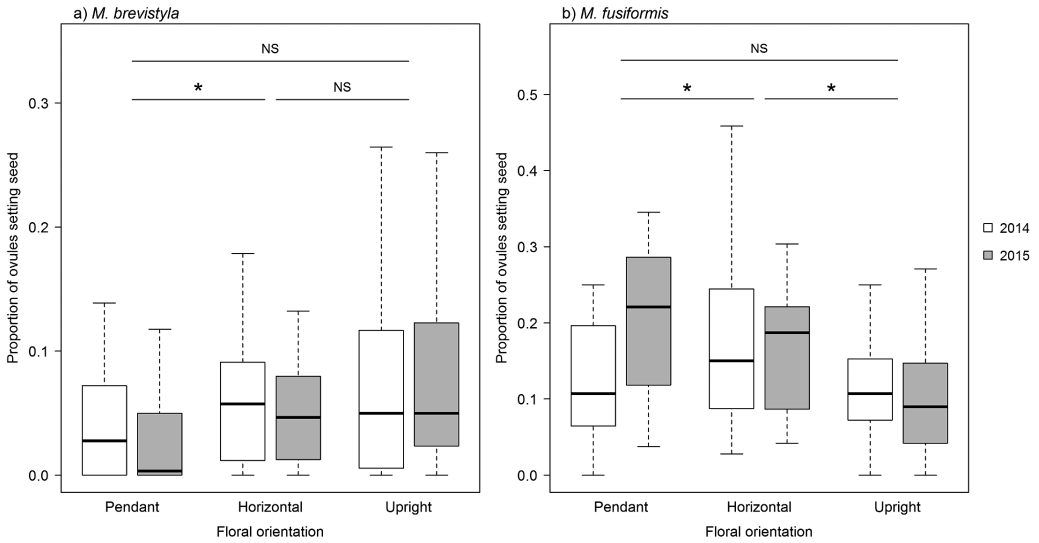

Figure 4: boxplots showing the proportion of ovules setting seed for each orientation treatment in 2014 and 2015 for (**a**) *Mertensia brevistyla* and (**b**) *M. fusiformis* (n = 10 to 28 plants per treatment combination; total n = 60 *M. brevistyla* and 80 *M. fusiformis* plants). Boxes show the median and interquartile range. Lines above boxplots indicate Tukey's pairwise comparisons between orientations (*P < 0.05). Note difference in y-axis range between panels.

was 36% and 21% higher in the horizontal orientation than in the upright and pendant orientations, respectively.

Effect of water and floral orientation on seed set

Seed set in *M. brevistyla* was marginally significantly different among the four plots and was marginally higher when water addition occurred over the ground instead of over the flowers, regardless of floral orientation (GLMM, plot: $\chi^2 = 4.2$, $P = 0.040$; watering treatment: $\chi^2 = 4.4$, $P = 0.037$; orientation: $\chi^2 = 1.5$, $P = 0.23$; watering treatment × orientation: $\chi^2 = 0.027$, $P = 0.87$; Fig. 5a). Contrary to our expectations based on floral morphology, watering treatment and floral orientation had no significant influence on *M. fusiformis* seed set (GLMM, plot: $\chi^2 = 0.33$, $P = 0.57$; watering treatment: $\chi^2 = 0.060$, $P = 0.81$; orientation: $\chi^2 = 0.14$, $P = 0.70$; watering treatment × orientation: $\chi^2 = 0.32$, $P = 0.57$; Fig. 5b).

Water accumulation in corolla tube

Water accumulation inside corolla tubes occurred in 3% and 10% of *M. brevistyla* and *M. fusiformis* flowers examined,

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respectively (mean ± SE water volume, *M. brevistyla*: 0.094 µl; *M. fusiformis*: 0.49 ± 0.19 µl, $N = 1$ *M. brevistyla* and 3 *M. fusiformis* flowers with water out of 30 flowers per species), but water presence did not differ significantly between species (GLMM, species: $z = 1.57$, $P = 0.12$). Thus, in nature, the stigma and anthers of *M. brevistyla* (located within the corolla tube) would rarely be submerged, despite the more upright orientation of these flowers. The anthers of *M. fusiformis* could be submerged, given their exsertion beyond the corolla tube (Fig. 1g), except that the typically pendant orientation of these flowers tends to prevent this.

Effect of water on pollen germination

To explicitly test the effect of water exposure on pollen germination without confounding influences of floral orientation and floral morphology, we removed anthers from flowers and subjected them to different exposure treatments. Pollen germination rate was significantly influenced by treatment, species and interaction between treatment and species (GLMM, treatment: $\chi^2 = 1362.9$, $P < 0.01$, species: $\chi^2 = 13.7$, $P < 0.01$, treatment × species: $\chi^2 = 123.1$, $P < 0.01$; Fig. 6). Pollen germination rates were generally higher in *M. brevistyla* than in *M. fusiformis*, except in the treatment where anthers were

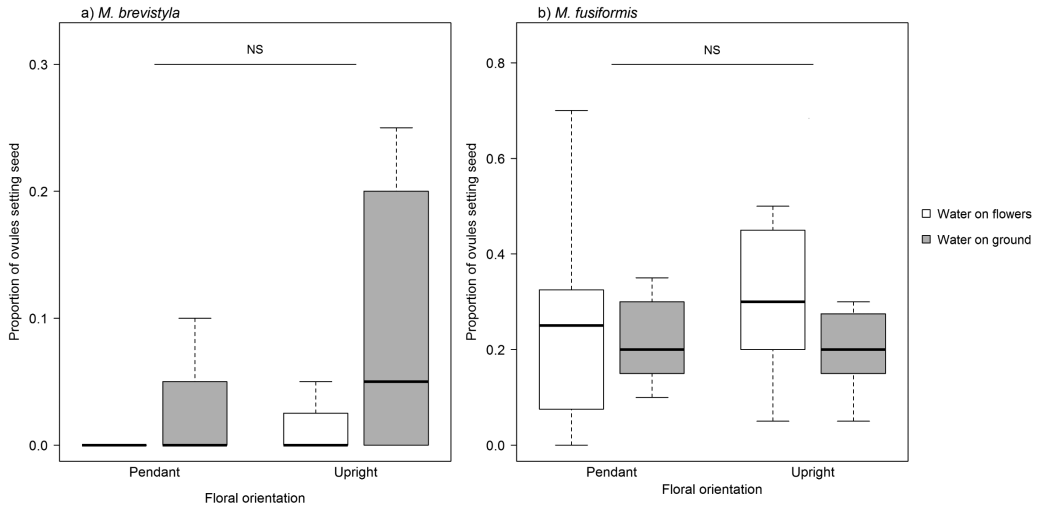

Figure 5: boxplots showing proportion of ovules setting seed for each watering and floral orientation treatment for (**a**) *Mertensia brevistyla* and (**b**) *M. fusiformis* ($n = 5$ to 8 plants per treatment combination; total $n = 26$ *M. brevistyla* and 25 *M. fusiformis* plants). Boxes show the median and interquartile range. Lines above boxplots indicate Tukey's pairwise comparisons between orientations. Note difference in *y*-axis range between panels.

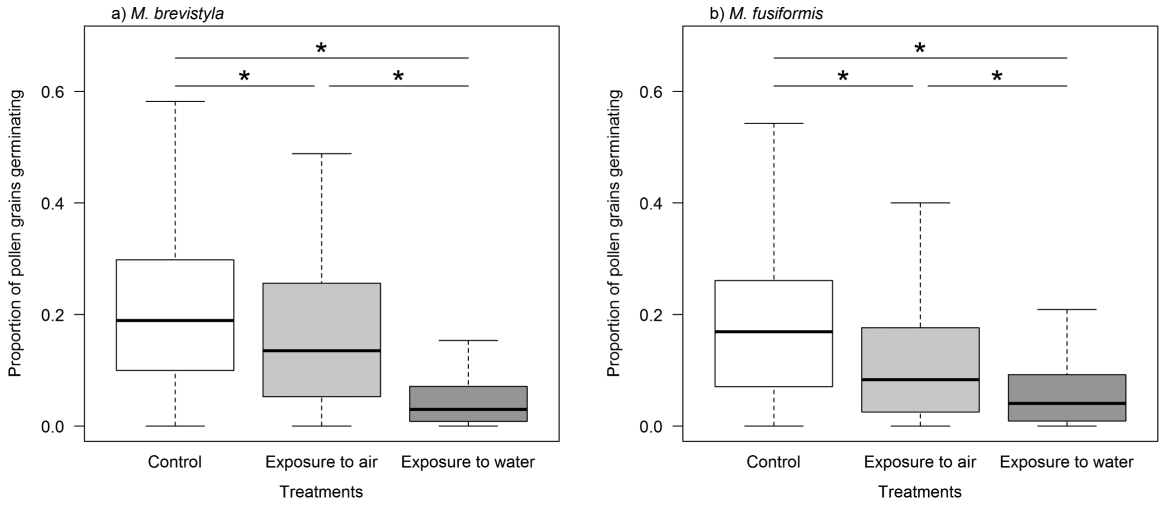

Figure 6: boxplots showing proportion of pollen grains germinating in each treatment for (**a**) *Mertensia brevistyla* and (**b**) *M. fusiformis* ($n = 15$ plants per treatment per species). Boxes show the median and interquartile range. Lines above boxplots indicate Tukey's pairwise comparisons between germination treatments (*$P < 0.05$).

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exposed to water for 1 hour first. Pollen from both species had a significantly reduced germination rate when exposed to air or water for 1 hour before being placed in the germination medium, compared to pollen grains placed immediately in the germination medium after removal from anthers (i.e. control; Tukey's post hoc tests, control vs. exposure to air: $z = 7.5$, $P < 0.01$; control vs. exposure to water: $z = 28.1$, $P < 0.01$). In addition, exposure to water further reduced pollen germination by >45% in *M. brevistyla* and *M. fusiformis* compared to exposure to air (i.e. from 17% to 5% in *M. brevistyla* and from 13% to 7% in *M. fusiformis*; $z = 21.3$, $P < 0.01$ for the two species combined).

DISCUSSION

Floral traits that influence visibility and accessibility to pollinators, and that simultaneously protect reproductive organs from unfavourable environmental conditions, may be under selection from both biotic and abiotic agents. We examined the functional significance of floral orientation with respect to pollinators and rain in *Mertensia brevistyla* and *M. fusiformis*, related species that differ in floral orientation and placement of reproductive structures. Our manipulative experiments showed that seed set was highest in upright *M. brevistyla* and in horizontal or pendant *M. fusiformis*, which corresponds fairly well with the natural floral orientations of the two species. However, the floral visitor assemblage was similar between the two species; visitors showed weak preferences for floral orientation; and pollinators exerted no detectable selective pressure on floral orientation in either species—all of which suggests that pollinators have not been the primary selective force driving the interspecific difference in floral orientation. Instead, our results suggest that an interaction between rainwater and floral morphology contributes to the adaptive significance of floral orientation in the two species.

Pollinators and floral orientation

Pollinator preference for floral orientation is highly variable across studies and it can depend on pollinator type and foraging behaviour. Some studies show that upright flowers are more attractive to floral visitors (Fenster *et al.* 2009; Fulton and Hodges 1999; Wang *et al.* 2014a), while other studies find no difference in visitor attraction between floral orientations (Chen *et al.* 2013; Huang *et al.* 2002; Tadey and Aizen 2001; Ushimaru and Hyodo 2005; Wang *et al.* 2010). In our field observations of *Mertensia* spp., we found no consistent floral visitor preference for a particular orientation; nor was there a difference in preference between the two plant species (although our sample size for this comparison is small). Given the similarity in floral visitor assemblages between *M. brevistyla* and *M. fusiformis*, it seems unlikely that the direction of pollinator-mediated selection would differ between them (but see seed set and floral orientation).

In fact, we did not detect pollinator-mediated selection on floral orientation in either species, which may be a result of *Mertensia* being visited by a variety of floral visitors of different body sizes and foraging strategies. Small-bodied pollinators such as flies have difficulty landing on and entering pendant flowers (Ushimaru and Hyodo 2005; Ushimaru *et al.* 2009); therefore, these pollinators may prefer more upright *Mertensia* flowers. Meanwhile, for large-bodied (and potentially more effective) pollinators such as bumblebees, access is less constrained by floral orientation because they can grab onto and probe flowers while hanging upside down (Ushimaru and Hyodo 2005). For instance, in manipulated *Geranium refractum* flowers, bumblebees and solitary bees removed more pollen grains from pendant than upright flowers, and the opposite effect was observed for muscoid flies (Wang *et al.* 2014b). Alternatively, we may have failed to detect pollinator-mediated selection on floral orientation because we tested for it in a single season, during which seed set may have been limited by other factors. For example, a late snowfall that occurred on 8 June 2014 and covered some of the manipulated plants may have limited ovule development (although it did not cause observable frost damage). Regardless, in a study system where pollinators do not exhibit strong preference or exert consistent selection on a floral trait, it becomes important to identify other selective agents, such as abiotic factors, that can help explain the ecological significance of the trait in question.

Rain and floral orientation

Rain can influence both maternal and paternal fitness in flowering plants. Previous studies have shown that rain exposure can reduce seed set (Bynum and Smith 2001), wash pollen grains from anthers (Wang *et al.* 2010) and lower pollen viability (Corbet 1990). Conversely, rainwater accumulation inside flowers might regulate intrafloral microclimatic conditions (Galen 2005), resulting in higher seed set (e.g. in *Anisodus luridus*; Wang *et al.* 2010). The results of our watering experiment suggest that rain exerted minimal selective pressure on floral orientation via maternal fitness (seed set). Instead, directly wetting the anthers significantly reduced pollen germinability—a component of male fitness—in both *Mertensia* species. Similarly, other studies show a consistent pattern of rain selecting for pendant flowers via paternal fitness (i.e. pollen export, viability and/or germinability; Aizen 2003; Eisikowitch and Woodell 1974; Huang *et al.* 2002; Tadey and Aizen 2001; Wang *et al.* 2014b). Species with more upright flowers may exhibit other traits that protect their pollen from rain, such as closing the corolla during storms (Bynum and Smith 2001), producing water-resistant pollen (Eisikowitch and Woodell 1974; Mao and Huang 2009), or forming a waxy rim around the corolla tube (Aronne *et al.* 2015). In *M. brevistyla*, short reproductive structures inserted in the narrow corolla tube may be an alternative adaptive response to avoid rain exposure.

Seed set and floral orientation

Given that pollinator- and rain-mediated selection on floral orientation are weak with respect to female fitness in our

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study species, other factors must explain the observed differences in seed set between species and orientations. First, it is important to note that we only examined pollinator-mediated selection in one season, even though interannual variability in the strength and direction of selection is common (Siepielski *et al.* 2009). In fact, we found selection on floral orientation (via female fitness) to be relatively consistent between 2014 and 2015—seed set was highest in upright *M. brevistyla* flowers and in horizontal or pendant *M. fusiformis* flowers. Nevertheless, as noted above, pollinator-mediated selection may be only occasionally detectable. Solitary bees are the most common floral visitors for both species, making it unlikely they would drive opposing selection on orientation. However, selection on orientation could still differ between plant species if the same pollinators interact differently with each, owing to differences in floral morphology. Measuring pollinator-mediated selection across multiple years (ideally via male as well as female fitness), and determining the effectiveness and orientation preferences of all floral visitors to each plant species, would help determine the role of pollinators in the evolution of floral orientation.

Second, could the result be an artefact of the experimental design? Stems that have been manipulated furthest from their natural orientation (i.e. upright *M. fusiformis* and pendant or upright *M. brevistyla*) may endure more physical strain and therefore exhibit higher mortality or lower seed set. We did observe higher mortality of pendant and upright *M. brevistyla* stems (33% and 20%, respectively), but not significantly more than the 16% mortality in the more natural horizontal orientation ($\chi^2 = 0.075$, df = 2, $P = 0.96$, $n = 45$ stems per orientation). Furthermore, seed set in *M. brevistyla* was highest in the upright (least natural) orientation. Stem mortality in *M. fusiformis* was consistent across all three orientations (22–24%; $\chi^2 = 0.0014$, df = 2, $P = 0.99$, $n = 45$ stems per orientation). Overall, these data do not support the idea that treatment effects on seed set are artefacts of manipulation—although we cannot rule out the possibility that experimental artefacts may have contributed to the higher seed set in pendant and horizontal *M. fusiformis*.

Third, there may be reinforcing selection favouring more upright *M. brevistyla* and more pendant *M. fusiformis* plants. Previous studies have shown that orientation and other floral traits in *Aquilegia* and *Zaluzianskya* species promote discriminatory pollinator choices (ethological isolation; Campbell *et al.* 2016; Hodges *et al.* 2002). In areas where both *Mertensia* species occur (e.g. at KP), pollinators may visit plants of similar floral orientation regardless of species. If interspecific crosses reduce seed production, we would observe lower seed set in more pendant *M. brevistyla* and in relatively upright *M. fusiformis* plants (i.e. those phenotypes most resembling the other species). However, this mechanism (reinforcement) does not explain low seed set in upright *M. fusiformis* plants in areas of allopatry (e.g. at RMBL). The latter result could instead reflect selection imposed by a selective agent we did not consider (e.g. herbivores, although these are rarely observed on our study species).

Finally, the existence of substantial intraspecific variation in both species may indicate that selection on floral orientation is inconsistent, and/or that environmental influences on floral orientation are strong. Furthermore, our measurements and manipulations of floral orientation were conducted at the inflorescence level, while the orientations of individual flowers varied around the mean set by the angle of the inflorescence. This among-flower variation also limits the effectiveness of selection on floral orientation. While these factors (variable selection and variable phenotype) do not explain the patterns of selection we did observe, they add to the challenge of understanding the adaptive significance of floral orientation and may have limited our ability to detect pollinator- or rain-mediated selection on this trait.

CONCLUSION

We have shown that the pendant floral orientation of *M. fusiformis* provides a likely ecological advantage by minimizing pollen exposure to rain. A relatively pendant orientation also increases seed set in this species, for reasons that remain unclear. Meanwhile, the more upright orientation of *M. brevistyla* is favoured via female fitness, and the short reproductive structures of this species' flowers prevent rain from entering the corolla tube and reducing male fitness, in spite of their orientation. Although pollinators are undoubtedly necessary for most plant reproduction, our results support the idea that other selective agents (including abiotic ones) can be equally important. Broader thinking about the multiple selective pressures that affect flowers, via both male and female fitness, will improve the study of the adaptation and diversification of floral traits.

SUPPLEMENTARY DATA

Supplementary material is available at *Journal of Plant Ecology* online.

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