Adaptation and diversification in bluebells (*Mertensia* spp., Boraginaceae)

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Abstract

Examining the ecological processes generating evolutionary patterns is crucial to understanding how biodiversity arises and evolves. One of the most striking examples of evolutionary diversification is provided by the flowering plants (angiosperms) and their flowers. Pollinators are traditionally considered to be the most important selective agents and drivers of floral diversity. However, many angiosperms have a generalized floral morphology and are visited by a diverse and overlapping suite of pollinators, making it unclear how pollinators could have driven diversification in these taxa. In addition, flowers and plant reproductive success are likely to be influenced by factors other than pollinators, such as herbivores, precipitation, and temperature. These factors need to be considered along with pollinators in order to improve our understanding of angiosperm evolution and diversification.

In my thesis, I focussed on the processes influencing adaptation and diversification in flowering plants in the genus *Mertensia* (Boraginaceae), which have relatively unspecialized flowers that attract a variety of nectar- and pollen-feeding insects. In Chapter One, I explored correlations among floral traits, vegetative traits, and flowering phenology across 12 *Mertensia* species. In Chapter Two, I assessed reproductive isolating barriers between related *Mertensia* species occurring in sympatry. In Chapter Three, I examined the ecological function of floral orientation in two *Mertensia* species with respect to pollinators and precipitation.

First, across *Mertensia* species, I found that early-flowering species were shorter, produced fewer flowers, and occurred at higher altitudes than late-flowering species—suggesting a life-history trade-off between plant size and flowering phenology, as well as an altitudinal effect on both traits. Interspecific variation in floral traits was not strongly associated with

variation in flowering phenology or plant size. Second, between sympatric *M. brevistyla* and *M. fusiformis* populations, I found weak reproductive isolating barriers and possible hybridization. Most pre-pollination barriers were weak, as the two *Mertensia* species shared similar habitats, flowering phenology, and pollinator assemblages. The two relatively strong barriers were floral (ethological and mechanical) isolation and post-pollination isolation: Pollinators transferred significantly more of a pollen analogue among conspecific than heterospecific plants in mixed-species arrays, and flowers yielded higher seed set when receiving conspecific rather than heterospecific pollen in hand-pollination experiments. Lastly, I found that floral orientation was more likely to be under selection by precipitation than by pollinators, in that paternal fitness (i.e., pollen germination) was reduced by contact with water and that pollinator-mediated selection via maternal fitness (i.e., seed set) was not detected. A more pendant floral orientation likely protects the relatively long and exposed anthers of *M. fusiformis* from rain, while the less pendant *M. brevistyla* does not require this protection because of its shorter, more concealed reproductive structures. Although I detected an effect of floral orientation on seed set, I was not able to identify the selective agents driving this effect.

In summary, my results suggest that pollinators play a minor role in influencing floral adaptation and diversification in *Mertensia*. Instead, the dominant influences on the traits I examined appear to be life-history trade-offs, environmental conditions that vary along altitudinal gradients, and abiotic variables (e.g., precipitation). It is important to consider these factors and their influences on paternal and maternal fitness in order to gain a broader perspective on floral evolution in plants with generalized pollination systems.

Résumé

Étudier les processus écologiques générant des patrons évolutifs est primordial afin de comprendre comment la biodiversité se forme et évolue. Un des exemples les plus frappant de diversification évolutive provient des plantes à fleurs (angiospermes) et leurs fleurs. Les pollinisateurs sont traditionnellement considérés comme étant les plus importants agents sélectifs et producteurs de diversité florale. Toutefois, plusieurs angiospermes ont une morphologie florale généralisée et sont visités par une grande diversité de pollinisateurs, rendant nébuleux comment les pollinisateurs pourraient avoir engendré la diversification de ces taxons. De plus, les fleurs et le succès reproducteur des plantes sont susceptibles d'être influencés par des facteurs autres que les pollinisateurs tels que les herbivores, les précipitations et la température. Ces facteurs doivent être considérés en plus des pollinisateurs afin d'améliorer notre compréhension de l'évolution et la diversification des angiospermes.

Lors de ma thèse, j'ai étudié les processus influençant l'adaptation et la diversification des plantes à fleurs du genre *Mertensia* (Boraginaceae), lesquelles possèdent des fleurs relativement peu spécialisées qui attirent une variété d'insectes se nourrissant de nectar et de pollen. Dans le premier chapitre, j'ai exploré les corrélations entre les traits floraux, les traits végétatifs et la phénologie de la floraison entre 12 espèces de *Mertensia.* Dans le second chapitre, j'ai étudié les barrières d'isolement reproductif entre des espèces de *Mertensia* apparentées se trouvant en sympatrie. Dans le troisième chapitre, j'ai examiné la fonction écologique de l'orientation florale pour deux espèces de *Mertensia* par rapport aux pollinisateurs et aux précipitations.

Premièrement, entre les espèces de *Mertensia*, j'ai observé que les espèces à floraison hâtive étaient de plus petite taille, produisaient moins de fleurs et se retrouvaient à plus haute

altitude comparativement aux espèces à floraison tardive—suggérant un compromis d'histoire de vie entre la taille des plantes et la phénologie de la floraison, ainsi qu'un effet de l'altitude sur chacun de ces traits. La variation interspécifique des traits floraux n'était pas fortement associée avec les variations de la phénologie de la floraison ou la taille de la plante. Deuxièmement, entre les populations sympatriques de *M. brevistyla* et *M. fusiformis*, j'ai trouvé un faible isolement reproductif et de l'hybridation potentielle. La plupart des barrières pré-pollinisation étaient faibles, puisque les deux espèces de *Mertensia* partageaient des habitats, une phénologie de la floraison et un assemblage de pollinisateurs similaires. Les deux barrières relativement fortes étaient l'isolation florale (éthologique et mécanique) et l'isolation post-pollinisation : les pollinisateurs ont transféré significativement plus d'un analogue au pollen entre les plantes conspécifiques que hétérospécifiques dans un dispositif avec espèces mélangées, et les fleurs ont produit une plus grande quantité de graines lorsque pollinisées avec du pollen conspécifique que du pollen hétérospécifique dans une expérimentation de pollinisation manuelle. Dernièrement, j'ai trouvé que l'orientation florale était plus probablement sous sélection par l'effet des précipitations que des pollinisateurs, dans le sens que le fitness paternel (c.-à-d., la germination du pollen) était réduit par le contact avec l'eau et que la sélection par les pollinisateurs par l'intermédiaire du fitness maternel n'a pas été observée. Une orientation florale plus pendante protège probablement les anthères relativement longues et exposées de *M. fusiformis* de la pluie, alors que les fleurs moins pendantes de *M. brevistyla* ne nécessitent pas cette protection à cause de ses structures reproductives plus courtes et abritées. Bien que j'ai trouvé un effet de l'orientation florale sur le nombre de graines produites, je ne suis pas parvenu à identifier l'agent sélectif produisant cet effet.

En résumé, mes résultats suggèrent que les pollinisateurs jouent un rôle mineur dans l'influence de l'adaptation florale et la diversification chez *Mertensia*. L'influence dominante sur les traits que j'ai examinés semble être les compromis d'histoire de vie, les conditions environnementales qui varient le long d'un gradient altitudinal et les variables abiotiques (ex. précipitations). Il est crucial de considérer ces facteurs et leurs influences sur le fitness paternel et maternel afin d'obtenir une perspective plus large sur l'évolution florale chez les plantes avec un système de pollinisation généralisé.

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Table of Contents

List of Tables

List of Figures

General Introduction

There are approximately 295,000 species of angiosperms in the world, and they are incredibly diverse in their life history, reproductive biology, and morphology (Christenhusz and Byng 2016). The biological processes generating angiosperm diversity, especially those involving the ecology and evolution of flowers, have intrigued biologists for centuries (Sprengel 1793, Darwin 1877, Grant 1949, Grant and Grant 1965, Stebbins 1970, Faegri and van der Pijl 1971, Lloyd and Barrett 1996, Harder and Barrett 2006, Crepet and Niklas 2009, van der Niet et al. 2014). Almost 88% of angiosperms rely on animal pollinators to visit flowers and effect sexual reproduction (Ollerton et al. 2011). Furthermore, clades associated with biotic pollination are more speciose than clades associated with abiotic pollination, suggesting that speciation in angiosperms is linked to evolutionary shifts in floral traits and pollinator identity (Kay et al. 2006). Indeed, some floral traits (e.g., nectar spurs and bilateral floral symmetry) have been found to promote speciation (Hodges and Arnold 1995, Sargent 2004, Kay et al. 2006), and several studies provide microevolutionary evidence of floral adaptation mediated by pollinators (reviewed by Harder and Johnson 2009). However, some studies have questioned the relative contributions of pollinators and other factors to angiosperm speciation, given that other biotic and abiotic selective agents may exert conflicting or stronger selection than pollinators, and given that a plant species' pollinators often vary over space and time—leading to inconsistent selection and consequently reduced opportunity for diversification (see Herrera 1996, Waser 1998, Waser 2001). To understand the complex interplay between pollinators and non-pollinator agents, and their roles in angiosperm reproduction and diversification, my thesis examines the following: (1) macroevolutionary patterns in trait correlations between flowering phenology and other plant traits across related species; (2) reproductive isolating barriers between related

species that share a generalized pollination system; and (3) ecological functions of a floral trait (i.e., floral orientation) in the context of pollination and the abiotic environment.

For my thesis, I focus on angiosperms in the genus *Mertensia* (Boraginaceae) occurring in western North America. This genus comprises 62 herbaceous perennials found in Asia and North America, with its geographic centre of diversity in the western United States (Nazaire and Hufford 2014, Nazaire et al. 2014). *Mertensia* species produce campanulate blue flowers, hence the common name "bluebells"; depending on the species, they can flower from early spring immediately after snowmelt to late summer, and their height can vary from 10 to 170 cm (Williams 1937). All species share a similar floral form (a narrow corolla tube that flares into a distinct corolla limb), with corolla length varying from 9 to 20 mm (Williams 1937). All species also share a relatively generalized pollination system, in that they have a fairly accessible floral morphology and those that have been studied are visited by a variety of pollinators, including small- to medium- sized bees (families Halictidae and Megachilidae), large bumblebees (*Bombus* spp.), hover flies (family Syrphidae), and butterflies (Lepidoptera). Overall, large interspecific variation in life-history and reproductive traits, as well as association with a variety of pollinators, make *Mertensia* a relevant and intriguing system for my research. At the same time, my research provides valuable natural history information on species that have previously been little studied.

Macroevolutionary patterns

Life-history theory provides a general framework for understanding how variation in traits such as body size, age at reproduction, number of offspring, and life span arise and evolve across different organisms (Stearns 1976, Lloyd 1989, Roff 1992). Organisms can employ different strategies to allocate limited resources (time, energy, and nutrients) to different life-history traits in order to maximize growth and reproduction (Stearns 1989). One axis of life-

history variation involves age and size at maturity: organisms that begin reproducing early in life should be smaller at the time of first reproduction than organisms reproducing late, and it is (theoretically) unlikely for organisms to reproduce early and be large at the same time (see Weiner 1988, Stearns 1992). In many angiosperms, similar reasoning can be applied to timing of flowering within a season, i.e., phenology, and several studies have indeed documented such a trade-off between plant size and flowering phenology (see Bolmgren and Cowan 2008, Du and Qi 2010). Across multiple herbaceous grassland species, Sun and Frelich (2011) found a positive correlation between maximum plant height and flowering phenology, indicating that early-flowering species are generally shorter than late-flowering species. Furthermore, phenological variation across angiosperm families has been shown to be fairly consistent across different habitats and years, suggesting that there are phylogenetic constraints acting on the reproductive schedules and/or life-history strategies across different species and families (see Kochmer and Handel 1986, Davis et al. 2010, Davies et al. 2013, Du et al. 2015).

While phylogenetic constraints and/or life-history strategies can influence flowering phenology, flowering time has also been shown to respond to selection by agents such as pollinators and herbivores (reviewed by Elzinga et al. 2007, Munguía-Rosas et al. 2011), and to reflect adaptation to the seasonal variation in pollinator availability (Kudo 2006, Kudo 2016, Mizunaga and Kudo 2017). Furthermore, flowering time has the potential to determine the selective agents and pressures acting on other plant traits, thereby generating correlations between flowering phenology and other traits. Within some plant species, traits such as flower colour and flower number exhibit a temporal pattern that matches the seasonal change in pollinator assemblages (Paige and Whitham 1985, Ehrlén and Münzbergová 2009, Sandring and Ågren 2009, Chen et al. 2017). At the community level, within temperate forests of eastern

North America, early-flowering species have lighter-coloured corollas than do late-flowering species, suggesting that the lighter colours may be an adaptive response to the preference of generalist pollinators available early in the flowering season (see Hensel and Sargent 2012). Identifying the relative importance of different processes generating macroevolutionary correlations between flowering phenology and other plant traits provides an important first step in understanding trait diversification and evolution. In my first chapter, I explore interspecific correlations between flowering phenology and other morphological traits (plant size and floral traits) in the genus *Mertensia* and discuss the potential mechanisms that may generate the observed macroevolutionary patterns.

Contributors to reproductive isolation

Speciation in angiosperms is often attributed to the accumulation of genetic differences that limit gene flow between populations and maintain species boundaries. These differences act as reproductive isolating barriers, and they can be categorized depending on when they take place: before fertilization (known as pre-zygotic barriers, and further classified as pre- and post-pollination barriers) or after fertilization (known as post-zygotic barriers; see Lowry et al. 2008, Widmer et al. 2009, Baack et al. 2015). The earliest-acting pre-zygotic barriers are generally geographical barriers or habitat differences that prevent species from sharing the same space (ecogeographical isolation and habitat isolation), and non-identical flowering times that limit interspecific pollination (phenological isolation). Next, specialized floral morphologies may prevent pollinators from transferring and depositing heterospecific pollen (mechanical isolation), and pollinators may additionally discriminate between genotypes or exhibit different behaviours when confronted with different plant genotypes (ethological isolation). Post-pollination barriers often involve precedence of conspecific pollen over heterospecific pollen in forming pollen tubes

or fertilizing ovules. Following fertilization, hybrid offspring may suffer reduced viability or fertility, leading to post-zygotic barriers.

Understanding the relative importance of these isolating barriers can help identify the primary processes driving speciation in angiosperms. Pre-zygotic isolation is approximately twice as strong as post-zygotic isolation across the 19 pairs of plant taxa reviewed by Lowry et al. (2008); and pollinator-mediated isolation is considered an important driver of speciation in certain taxa (Fulton and Hodges 1999, Kay 2006, Schiestl and Schlüter 2009). However, there remain some gaps in our knowledge of reproductive isolation in angiosperms. For example, 14 out of the 31 reproductive isolation studies reviewed by Lowry et al. (2008) and Baack et al. (2015) focussed on the genera *Costus*, *Helianthus*, *Iris*, *Mimulus*, and *Orchis*. In addition, much emphasis has been placed on taxa with specialized floral traits (e.g., nectar spurs in *Aquilegia* spp. and Orchidaceae; Fulton and Hodges 1999, Schiestl and Schlüter 2009) or having relatively specialized pollination syndromes (e.g., pollination by hawkmoths or hummingbirds, Fulton and Hodges 1999, Kay 2006, Schiestl and Schlüter 2009). For taxa with generalized pollination systems (i.e., with generalized floral morphologies and visited by multiple pollinator taxa), pollinator-mediated isolation may not be as important in maintaining species integrity as other barriers (Waser 2001). Barriers that can be equal in strength to or stronger than pollinator-mediated isolation include habitat differences (Goldblatt and Manning 1996, Burge et al. 2013), flowering phenology (Pascarella 2007), pollen competition (Brys et al. 2013, Ishizaki et al. 2013), and inability to form hybrid seeds (Costa et al. 2007). In my second chapter, I focus on previously unstudied sympatric populations of *M. brevistyla* and *M. fusiformis*, both of which have relatively generalized floral morphology and are visited by a variety of pollinators, and I evaluate the importance of pollinator-mediated isolation in relation to other barriers.

Selective agents on floral traits

Pollinators are traditionally considered to play a major role in the adaptation of flowers because their behaviours and morphologies can select for floral traits that are attractive and mechanically fit the morphologies of the pollinators, which significantly influence plant reproductive success (Grant and Grant 1965, Stebbins 1970). However, selection on floral traits can be complicated by visitation from multiple pollinator taxa (especially in plants with generalized pollination systems), as different pollinators can exert conflicting selection on floral traits within the same plant (e.g., in *Raphanus raphanistrum*; Sahli and Conner 2011). Furthermore, interactions between non-pollinator selective agents (both biotic and abiotic) and flowers can also influence plant fitness (Strauss and Whittall 2006). For instance, Gómez (2003) found that pollinator-mediated selection on floral traits can be disrupted by conflicting selection from herbivores. Meanwhile, abiotic factors can exert selection on floral traits by directly influencing floral traits or indirectly affecting female fitness (Caruso et al. 2019). One abiotic selective agent is soil water availability, which is a limiting resource that can significantly alter resource allocation to flower production, influence floral attractiveness, and impact pollinator-mediated selection (Galen 2005, Carroll et al. 2001). For example, in *Polemonium viscosum*, bumblebees prefer larger flowers, but the production of large flowers is unfavourable under drought conditions at high altitudes—leading to potential conflicting selection between pollinators and drought stress (Galen 1989, Galen 2000). These different studies demonstrate that floral adaptation is unlikely to be mediated solely by pollinators. Detailed examinations of the relative importance of pollinators and non-pollinator agents of selection on floral traits are necessary to understand the adaptive response of floral traits (reviewed by Strauss and Whittall 2006, Caruso et al. 2019). In my final chapter, I focus on floral orientation as a trait that is likely

to be under selection by both pollinators and non-pollinator agents (i.e., rain) and examine paternal (pollen germination) and maternal (seed set) fitness in relation to pollinators and rain.

Chapter 1

Examining interspecific correlations among flowering phenology, size, and floral traits in *Mertensia* (Boraginaceae)

Abstract

Flowering phenology marks the transition from vegetative growth to reproduction and exposes plants to seasonally varying selective environments during flowering. Life-history trade-offs between time and size at reproduction suggest that early-flowering species should generally be smaller than late-flowering species. Furthermore, consistent seasonal variation in environmental conditions may generate seasonally varying selection on morphological traits (such as vegetative size and floral traits), potentially causing correlations between morphological traits and flowering phenology. Similarly, trait correlations may be driven by multiple traits responding in parallel to environmental gradients; for example, low air temperature and high wind velocity in high-altitude habitats might favour smaller species that also flower rapidly after snowmelt. To assess interspecific correlations between flowering phenology and other traits, I focused on 12 species of *Mertensia* (Boraginaceae) that have similar vegetative and floral morphology but differ greatly in size, flowering phenology, and altitudinal distribution. I developed two novel techniques to estimate flowering phenology of each species based on a single day's survey, measured size and reproductive traits of each species, and used phylogenetic comparative methods to test for interspecific correlations among flowering phenology, size, floral traits, and altitude. Early-flowering *Mertensia* species were shorter in stature, produced fewer flowers, and occurred at higher altitudes than late-flowering species. However, traits of individual flowers (stigma–anther separation and floral volume) were not strongly associated with variation in flowering phenology or plant size. The evolution of flowering phenology within *Mertensia* has been associated with changes in plant size but not floral traits. Early-flowering species may be smaller because they have less time for growth before reproduction, and because short stature and early flowering are both adaptations to high-altitude environments.

Introduction

Timing of flowering is a complex life-history trait that dictates the set of environmental conditions experienced by a plant during reproduction (Rathcke and Lacey 1985, Kudo 2006, Ehrlén 2015). Plant species vary enormously in flowering phenology traits, such as timing of first and peak flowering, and this interspecific variation—particularly variation among closely-related species—can help us understand how species adapt to changes in biotic (e.g., pollinators) and abiotic (e.g., temperature and precipitation) conditions. Flowering phenology can represent an adaptive response to conditions favourable for reproduction, and previous studies have found that plant reproductive success can be influenced by flowering phenology and the temporal availability of reliable and effective pollinators. For example, within-season variation in reproductive success of bumblebee-pollinated plants appears to be linked to the annual life cycle of the bumblebee colony (Kudo and Suzuki 2002, Kudo et al. 2011, Mizunaga and Kudo 2017). Because bumblebee queens overwinter and emerge in spring (Plowright and Laverty 1984), queens are important pollinators early in the flowering season, while workers are produced and become more abundant than queens later in the season. Flowering phenology can also define the selective environment for floral traits due to temporal variation in the composition of pollinator assemblages and other agents of selection. Within-species studies have found that the magnitude and direction of selection on floral traits (e.g., corolla length or flower size) can change based on pollinator availability and abundance, which vary within and across seasons (Schemske and Horvitz 1989, Eckhart 1992). Among spring-flowering plants in temperate forests, there is a significant correlation between flowering phenology and corolla colour, with many early-flowering species having light-coloured corollas that may be preferred by generalist pollinators (see Hensel and Sargent 2012). Thus, there is potential for floral traits (by affecting which

pollinators are most effective) to influence selection on flowering phenology, as well as for phenology to influence selection on floral traits.

At the same time, reproduction in flowering plants requires allocation of resources to flower and seed production—resources that might otherwise have been allocated to vegetative growth (Obeso 2002). As many plant species need to reach a minimum size threshold before initiating reproduction (Lacey 1986, Weiner 1988), I expect plant size at reproduction and timing of flowering onset to be linked. Given that it is impossible to both flower early and have a long period of resource acquisition before flowering, Bolmgren and Cowan (2008) predicted a trade-off between plant size and flowering phenology within and among species. Although perennial species can accumulate resources from previous seasons in preformed buds or storage organs (thereby potentially decoupling the seasonal timing of flowering from time for resource acquisition), many studies have nevertheless found significant positive correlations between plant size and flowering time within perennial herbaceous species as well as across species (i.e., shorter species flowering earlier than taller species; see Vile et al. 2006, Bolmgren and Cowan 2008, Du and Qi 2010). Sun and Frelich (2011) also documented a negative correlation between maximum plant height and growth rate and a positive correlation between maximum plant height and flowering phenology, indicating that early-flowering herbaceous species generally reach their maximum height faster but nevertheless remain shorter than late-flowering species. Reproducing early in life (or, for herbaceous perennials, early in a flowering season) therefore typically entails reproducing at a small size, because early reproduction allows little time for vegetative growth between the onset of the growing season and the start of reproduction (see Austen et al. 2017 and references therein). The potential benefits of early flowering include reduced risk of mortality or herbivory before flowering, reduced competition for pollinators or

abiotic resources, and abundant time for seed maturation and dispersal. Conversely, late-flowering plants can attain a large vegetative size before reproducing (and therefore high fecundity), but risk being damaged by herbivores or outcompeted before seed production. Furthermore, because floral display (number of flowers produced) is another component of plant size, I also expect an interspecific association between flowering time and floral display. Viewed another way, small species that produce few flowers may be adapted to flowering early in the season, when they face fewer competitors for pollinator services, whereas large species that produce many flowers may be better competitors in a more crowded reproductive environment later in the season. However, the relationship between flowering phenology and floral display has so far been examined only in intraspecific studies (e.g., Ehrlén and Münzbergová 2009, Sandring and Ågren 2009, Sletvold et al. 2010).

Plant size might also constrain the evolution of floral traits independent of its association with flowering phenology. Flower size and floral display may exhibit a size–number trade-off wherein species either produce many small flowers or few large flowers (Schoen and Dubuc 1990, Morgan 1993). Indeed, previous studies have found a negative correlation between flower size and number across plant taxa (Worley et al. 2000, Sargent et al. 2007, but see Worley and Barrett 2000, Ashman and Majetic 2006). Alternatively, species with many flowers may be overall larger and produce large flowers as a completely different life-history strategy (see Ågren and Schemske 1995) or as an allometric relationship between vegetative and reproductive biomass (Samson and Werk 1986, Weiner 2004). While some studies have examined seasonal patterns in morphological traits within plant individuals and populations (Rathcke and Lacey 1985, Ishii and Harder 2012), our current knowledge about joint evolution of flowering phenology and morphological traits across species is limited.

Correlations between flowering phenology and morphological traits could also arise from selection by a common environmental factor. For example, the short growing seasons at high altitude require rapid flowering early in the season (which may still be quite late in terms of calendar date, due to a general delay in phenology with altitude) to allow sufficient time for seed development (Bliss 1971, Körner 2003, Inouye and Wielgolaski 2013). At the same time, high altitudes, owing to low air temperatures, high solar radiation, and high wind velocity, favor short-statured plants because they are closer to the ground and less affected by ambient temperature and atmospheric circulation (see Billing and Mooney 1968, Körner et al. 1989, Körner 2007). Therefore, variation in altitudinal distributions could lead to interspecific correlations between flowering phenology and certain morphological traits.

In this study, I investigate interspecific associations among flowering phenology, plant size, and floral traits in western North American members of the genus *Mertensia* Roth (Boraginaceae). *Mertensia* species are herbaceous perennials that produce mostly blue campanulate flowers, hence the common name bluebells (Williams 1937). Despite sharing similar habitat and floral morphology, *Mertensia* species exhibit substantial interspecific variation in timing of peak flowering, size, floral traits, and altitudinal range (from sea level to > 4000 m), making them an intriguing study system. For instance, timing of flowering of different species ranges from early spring to summer; plant height varies from 10 to 170 cm; and corolla length varies from 9 to 20 mm (Williams 1937).

By sampling 12 *Mertensia* species, developing methods to quantify flowering phenology based on a single day of surveying, and measuring plant size (i.e., plant height, leaf area, and total number of flowers per plant) and floral traits (i.e., floral volume, stigma height, anther height, and stigma–anther separation) for each species, I aim to examine interspecific

correlations between flowering phenology and morphological (size and floral) traits and suggest mechanisms that may contribute to phenological and morphological trait evolution in flowering plants. If early- and late-flowering *Mertensia* species have different life-history strategies, consistently interact with different types of pollinators, and/or occur at different altitudes, then I expect correlations between flowering phenology and morphological traits among *Mertensia* species. Studies of individual species have shown that early-season pollinators (e.g., bumblebee queens and male solitary bees) are more effective at pollinating wide *M. ciliata* flowers and short-styled *M. fusiformis* flowers, while late-season pollinators (e.g., bumblebee workers and female solitary bees) are better at pollinating narrow *M. ciliata* flowers and long-styled *M. fusiformis* flowers (Suzuki 1994, Forrest et al. 2011). If similar patterns hold true across *Mertensia* species, then I expect a negative interspecific correlation between flowering phenology and flower width or volume, and a positive interspecific correlation between flowering phenology and stigma height or stigma–anther separation. Furthermore, if environmental conditions at higher altitude select for species with small stature and early flowering phenology (relative to the onset of the growing season), then I expect negative correlations between altitude and flowering phenology and between altitude and size traits across species.

Material and Methods

Study species

Mertensia Roth is a genus of 62 herbaceous perennial species distributed in Asia, Beringia, and North America, with 79% of the species found in the Rocky Mountains of western North America (Nazaire and Hufford 2014, Nazaire et al. 2014). Plants produce cymose inflorescences with campanulate blue flowers (~ 7.5 to 17 mm in length, N = 12 species) that

have a slender floral tube and a widened bell-shaped floral limb (Fig. 1-1; Williams 1937, Morris 1996). I used the most comprehensive molecular phylogeny for this genus and selected 12 species from five (out of 12) North American subclades distributed broadly in the phylogenetic tree (Nazaire and Hufford 2014). These species are found along an altitudinal gradient from 2145 m to 4165 m. I selected a single locality per species based on voucher locations in Nazaire and Hufford (2014); i.e., I make the assumption that interspecific variation is larger than intraspecific variation. Between 2014 and 2016, I visited the study species in Colorado and Arizona (Figs. 1-2 and 1-3), estimated flowering phenology, and measured morphological (size and floral) traits of each species (explained further below).

Estimating flowering phenology

Estimating flowering phenology of each *Mertensia* species from a single day's survey is challenging. I could not collect phenological data from herbarium specimens, as other studies have done for other taxa (Primack et al. 2004, Davis et al. 2015), because taxonomic uncertainties in *Mertensia* made it impossible to confidently match herbarium specimens from a given locality to the species identified in the recently constructed phylogeny of Nazaire and Hufford (2014). Therefore, I developed two methods—the first based on observations of the focal *Mertensia* species, and the second based on observations of the co-flowering community.

Method 1: "Mertensia phenology"

First, I used a high-resolution, long-term phenological dataset (1973–1977 and 1979–2017; deposited in the Open Science Framework: doi.org/10.17605/osf.io/jt4n5) on two *Mertensia* species (*M. fusiformis* and *M. ciliata*) at Rocky Mountain Biological Laboratory, Colorado (at an average altitude of 2898 m, $N = 30$ plots), to characterize, to the best of my

ability, the shape of the flowering curve for the genus. This was achieved by calculating, for each of the two species, the mean proportion of open flowers for each day of the flowering season (averaged across all years). Phenology data were recorded almost every other day over the 44-year dataset, so I estimated the proportion of open flowers for days without data as the average of the previous and following days' proportions. I examined the fit of different distributions (Weibull, gamma, and lognormal) to each flowering curve using the function fitdist in the R package fitdistplus (Delignette-Muller and Dutang 2015; R Core Team 2018) and comparing the likelihood of each distribution. For both *Mertensia* species, Weibull distributions had higher log-likelihood values than other distributions. Next, I fitted a two-parameter Weibull distribution using the function nls2 in the R package nls2 (Grothendieck 2013) to the data for each species separately. I then calculated the mean parameters from the two fitted distributions: shape parameter = 1.66 (*M. ciliata*: 2.00; *M. fusiformis*: 1.32) and scale parameter = 22.7 (*M. ciliata*: 23.2; *M. fusiformis*: 22.3; Fig. 1-4). These parameters described a flowering curve with a duration (i.e., period in which > 99.5% of flowers opened; L_{2898}) of 62.1 days (*M. ciliata*: 53.4 d; *M. fusiformis*: 78.9 d) and a mode or peak flowering day (M_{2898}) of 13.0 days after the start of flowering (*M. ciliata*: 16.4 d; *M. fusiformis*: 7.6 d) at 2898 m. I assumed that the flowering curve of all *Mertensia* species corresponded, approximately, to this mean Weibull distribution.

Next, when I surveyed each *Mertensia* species, I began by qualitatively (for three species in 2014) or quantitatively (for nine species in 2015–2016) estimating the proportion of opened (i.e., currently open or already senesced) flowers in the population. To qualitatively assess the proportion of opened flowers, I inspected at least 25 randomly selected individuals and estimated whether 0, 25, 50, 75, or 100% of the flowers had already opened in these individuals. To quantify the proportion of opened flowers, I randomly selected a subset of individuals in the

population (N = 12–25 individuals per population) and counted the number of flower buds, open flowers, and wilted flowers on each. I calculated the proportion of opened flowers by adding the number of open and senesced flowers in each population and dividing the sum by the total number of buds plus open and senesced flowers. I applied the proportion of opened flowers of each species to the quantile function qweibull in the R package stats to determine the day (W_{2898}) at which that proportion of opened flowers would be observed within a flowering period having the same parameters as that of the combined *M. fusiformis* + *M. ciliata* flowering curve.

Given that phenologies are both compressed (shorter) and delayed at high altitudes, and because my sampling locations occurred over a 2019 m altitudinal range, I took the following steps, detailed below, to estimate the peak flowering day for a given sampled species, were that species to occur at a common reference altitude of 2898 m: (1) I estimated an altitude-specific flowering-period duration for each sampling locality; (2) I estimated an altitude-specific peak flowering day, based on the observed proportion of opened flowers, the day of year of observation, and the appropriately stretched or compressed flowering-period distribution; and (3) I corrected the estimated peak flowering day for altitude.

(1) I estimated the flowering-period duration (l_a) at a given altitude (a) by shortening or lengthening the *M. fusiformis* + *M. ciliata* flowering curve by 3.3 days for every 100 m upward or downward from 2898 m, respectively (adopted from the correction factor developed by Hopkins (1919) for plants and animals; see also Fitzjarrald et al. 2001, Elmore et al. 2012):

$$l_a = L_{2898} - 0.033\ (a - 2898)$$

(2) I determined the day within the flowering period on which the flowering peak was expected to occur at the altitude of the sampling site (m_a) by calculating the fraction of the flowering

period at which peak flowering occurred at 2898 m and multiplying this fraction by the altitude-adjusted length of the flowering period:

$$m_a = (M_{2898} / L_{2898}) \times l_a$$

I also adjusted the Weibull score (W_{2898}) in a shortened (or lengthened) flowering period by multiplying it by the ratio between l_a and L_{2928}. I denote the adjusted score by W_a:

$$W_a = W_{2898} \times (l_a / L_{2898})$$

Then, to estimate the peak flowering date at the study site, given the estimated (altitude-appropriate) flowering duration, I calculated the difference between W_a and m_a, and added this difference to the day of year on which the observation took place (d_{obs}).

(3) Finally, I corrected for the altitudinal effect on phenology by shifting 3.3 days for every 100 m change in altitude from 2898 m (Hopkins 1919). I refer to this peak flowering date estimate as "*Mertensia* phenology", D:

$$D = (d_{obs} + m_a - W_a) - 0.033\ (a - 2898)$$

For example, *M. alpina* was sampled on day 192 (July 10) at an altitude of 3770 m, by which time 78.0% of the flowers in the population had opened; this corresponded to $W = 31.0$ days. The local flowering duration (l_{3770}) was estimated as 35.7 days, the local peak flowering day (m_{3770}) as day 8.1 (of the total 35.7 d duration), and the adjusted Weibull score (W_{3770}) as 16.2 days. Therefore, I estimate that peak *M. alpina* flowering would have occurred on day 184 (July 2) at 3770, or on day 155 (June 3) if the species occurred at 2898 m (Tables 1-1 and 1-2).

Method 2: "Co-flowering phenology"

For the second method, I determined the flowering phenology of the plant community associated with each *Mertensia* species by surveying and identifying all the plant species that were flowering concurrently with the focal *Mertensia* species. I determined the first month of flowering (which was then converted to day of year) of each co-flowering species based on an online guide on regional (Colorado, New Mexico, Arizona, and Utah) flora (Schneider 2018) and, from these values, calculated the mean co-flowering date associated with each *Mertensia* species. Again, to correct for the effect of altitude on phenology, I shifted the mean co-flowering date by adding or subtracting 3.3 days for every 100 m upward or downward from 2898 m, respectively (Hopkins 1919). I refer to this estimate of the first flowering date as "co-flowering phenology". Note that I could not simply obtain an estimate of the phenology of each *Mertensia* species from the same regional flora because of uncertainties in *Mertensia* taxonomy; also, some *Mertensia* species are not listed in the guide. *Mertensia* phenology and co-flowering phenology are positively but non-significantly correlated with each other (Pearson correlation, $r = 0.39$, $P = 0.21$, $N = 12$).

Measuring size and floral traits

I haphazardly selected at least ten individuals per species. When in doubt, I ensured that plants were in fact distinct individuals by brushing away the top layer of the substrate and examining the subterranean portion of the shoot system. I measured the following size traits on each individual: plant height (maximum vertical height above the substrate), leaf area (mean elliptical surface area of the three largest leaves, obtained by measuring the major and minor leaf axes of each leaf), and total number of flowers (combined number of flower buds, open flowers, and senesced flowers). Next, I selected approximately three flowers per individual (from the

same individuals as above) and measured the following floral traits on each individual (Fig. 1-1): floral volume (combined volumes of a truncated cone [by measuring the corolla limb width and length] and a cylinder [by measuring the corolla tube width and length]), stigma height (distance from top of the stigma to the base of the ovary), anther height (distance from top of longest anther to the base of the ovary), and stigma–anther separation (difference between stigma height and anther height; positive values indicate taller stigmas than anthers).

Data analyses

Phylogenetic principal components analyses

To incorporate phylogenetic relationships among species into my interspecific trait correlation analyses, I pruned the most recent *Mertensia* phylogeny to the 12 species sampled in this study using the function drop.tip in the R package phytools (Revell 2012; Nazaire and Hufford 2014). Furthermore, to account for morphological measurements of multiple individuals per species, I split each terminal branch into a polytomy with a short branch (1/100 the length of the species' branch length) for each individual (see Berardi et al. 2016). Each trait type (phenology, size, and floral traits) was characterized as a linear combination of each of its constituent traits by conducting a phylogenetic principal component analysis (pPCA) using the function phyl.pca in the R package phytools. For size and floral pPCAs, I selected as many principal components (PCs) as necessary to account for at least 80% of the variance in each dataset. For the phenology pPCA (with only two variables), I selected the first PC and used the dichotomous species-level tree because phenology traits were characterized at the population (rather than individual) level.

Phylogenetic signal

To assess whether interspecific trait variation is influenced by the phylogenetic relatedness of *Mertensia* species, I calculated two measures of phylogenetic signal (Pagel's λ and Blomberg's K) for all traits and PCs using the function phylosig in the R package phytools. For peak *Mertensia* phenology, co-flowering phenology, and the associated phenology PC, I had no measure of within-species error; for size and floral traits and the associated PCs, I included standard errors in the analyses to account for within-species variation. Pagel's λ ranges from 0 to 1 and measures the similarity of covariances among species relative to the covariances expected under the Brownian-motion model (Pagel 1999); when $\lambda = 1$, trait evolution is well-explained by phylogeny under a Brownian-motion model. Pagel's λ values for all variables and PCs in this study were zero, indicating that a star phylogeny (i.e., multifurcation from a single node) explains trait variation under a Brownian-motion model and/or that sample size is too small (discussed below). Blomberg's K measures the partitioning of trait variance within and among clades (Blomberg et al. 2003). When $K < 1$, closely related species are less similar than expected under the Brownian-motion model (i.e., there is more variance within clades); conversely, when $K > 1$, closely related species resemble each other more than expected under Brownian motion (i.e., more variance among clades).

Phylogenetic generalized least-squares

To test for correlations between PC values for phenology, size, and floral morphology, as well as between altitude and all PCs, I performed phylogenetic generalized least-squares (PGLS) analyses using the functions pgls.SEy and pgls.Ives in the R package phytools, which account for within-species variation (i.e., standard errors) in one or both variables, respectively. Because model convergence in pgls.Ives can be sensitive to starting conditions, I repeated each analysis

500 times, selected the top 25 models with the highest likelihood, and calculated the mean intercept, slope, and likelihood for these models to determine an average model (see Garamszegi 2014). To examine the importance of incorporating phylogeny in these analyses, I repeated the same analyses as above but with a star phylogeny (i.e., assuming no phylogenetic signal; $\lambda = 0$). I then conducted likelihood-ratio tests between models with and without λ using the function pchisq in the R package stats.

Results

Trait variation

The 12 *Mertensia* species sampled in this study occurred from northern Colorado to central Arizona (Fig. 1-3), at altitudes varying from 2146 to 4165 m (Table 1-1). Based on my calculation of peak flowering date (standardized to an altitude of 2898 m), the earliest and latest *Mertensia* phenologies were June 3 (day 155) and July 26 (day 207) for *M. alpina* and *M. longipedunculata*, respectively (Table 1-1). Size traits in *Mertensia* varied by several orders of magnitude (Table 1-3): *M. longipedunculata* reached approximately 73 cm in height and produced > 3500 flowers per plant, while *M. perplexa* was only about 4 cm tall and produced on average seven flowers per plant. Floral traits also varied greatly across species—for example, floral volume in *M. lateriflora* was 83% less than *M. ciliata* (52.4 mm^3 versus 311.6 mm^3, respectively); stigma height in *M. brevistyla* was 83% less than *M. ciliata* (2.2 mm versus 12.9 mm, respectively); and anther height in *M. brevistyla* was 70% less than *M. ovata* (3.2 mm versus 10.9 mm, respectively). Nine and three species exhibited approach and reverse herkogamy, respectively (Table 1-3). Five of my 12 study species were described in detail by Nazaire and Hufford (2014), and my stigma–anther separation measurements qualitatively

matched these descriptions, except for *M. parvifolia*, which showed approach herkogamy in my study but not in the specimen illustrated by Nazaire and Hufford (2014).

Principal components and phylogenetic signals

The first principal component (PC) from phenology pPCAs explained 72% of the variance in *Mertensia* phenology (Fig. 1-5A). The first and second size PCs were primarily associated with number of flowers (floral display) and plant height, respectively (Fig. 1-5B). Stigma–anther separation and stigma height loaded strongly on the first floral PC, while floral volume and anther height contributed primarily to the second floral PC (Fig. 1-5C). Phylogenetic signals in all variables and PCs were not statistically significant based on Blomberg's K (Table 1-4). Weak phylogenetic signals indicate that closely related species do not have more similar traits than distantly related species; however, phylogenetic signals are known to be unreliable at small sample size (e.g., < 20 species) and can have high type II errors (Münkemüller et al. 2012).

Phylogenetic generalized least-squares

Correlations between phenology PC1 and size PC1 were positive and statistically significant ($P < 0.01$ with both resolved and star phylogenies; Table 1-5 and Fig. 1-6A), indicating that early-flowering species produced fewer flowers per plant than late-flowering species. There was a significant positive relationship between phenology PC1 and size PC2 ($P = 0.037$ with star phylogeny only; Table 1-5 and Fig. 1-6B), suggesting that early-flowering species were shorter than late-flowering species. There were no significant correlations between phenology PC1 and floral PCs (PC1: stigma–anther separation; PC2: floral volume; Figs. 1-6C and 1-6D). Floral PC1 was weakly positively correlated with size PC1 (≈ floral display; $P = 0.064$ with star phylogeny only; Table 1-5 and Fig. 1-7A) and with size PC2 (≈ plant height; $P <$

0.01 with resolved phylogeny and $P = 0.055$ with star phylogeny; Table 1-5 and Fig. 1-7C), indicating that taller plants tended to have flowers with greater stigma–anther separation. Meanwhile, correlations between floral PC2 (≈ floral volume) and plant size PCs were negative but not statistically significant (Table 1-5 and Figs. 1-7B and 1-7D).

There was a significant negative correlation between altitude and phenology PC1 (i.e., early flowering at high altitude; $P < 0.01$ with both resolved and star phylogenies; Table 1-5 and Fig. 1-8A). The correlations between altitude and size PC1 (≈ floral display) and between altitude and size PC2 (≈ plant height) were also significantly negative, indicating smaller display and shorter stature at high altitude (PC1: $P < 0.01$ with both resolved and star phylogenies; PC2: $P < 0.05$ with both resolved and star phylogenies; Table 1-5 and Figs. 1-8B and 1-8C). There were no significant correlations between altitude and floral PC1 (≈ stigma–anther separation) or between altitude and floral PC2 (≈ floral volume; Table 1-5 and Figs. 1-8D and 1-8E). For all correlation analyses, the model with resolved phylogeny ($\lambda = 1$) was either inferior to or not statistically different from the model with a star phylogeny ($\lambda = 0$), indicating a weak phylogenetic effect on phenological and morphological trait correlations among the study species.

Discussion

Examining the macroevolutionary relationship between flowering phenology and morphological traits in a single genus provides a first step in determining the potential drivers of variation in flowering phenology and morphological traits. Across 12 *Mertensia* species, I found size traits (floral display and plant height) to be significantly and positively correlated with flowering phenology: that is, early-flowering species produce fewer flowers and are shorter than late-flowering species. Furthermore, species from higher altitudes flowered earlier (relative to the start of the growing season, which is itself delayed at high altitude) and had fewer flowers per

plant than species from lower altitudes. However, I did not find strong correlations between floral traits and flowering phenology, nor between floral and size traits—with the possible exception of an association between plant height and stigma–anther separation. I also did not detect phylogenetic signal in the traits and trait correlations examined. Below, I propose several explanations for my findings.

Flowering phenology and size traits

Flowering phenology and plant size are both fitness-related traits constrained by limiting resources. Interspecific correlations between these two traits may reflect species evolving different life-history strategies (e.g., small but early versus large but late) in the face of inescapable trade-offs (reviewed in Roff and Fairbairn 2007, Agrawal et al. 2010). Indeed, my finding of a positive correlation between size traits and flowering phenology among *Mertensia* species supports previous studies that have found positive interspecific correlations between plant height and flowering time (Vile et al. 2006, Bolmgren and Cowan 2008, Du and Qi 2010, Sun and Frelich 2011). Alternatively, the correlation between flowering phenology and size may be driven by environmental conditions—such as those associated with altitude—selecting on both traits concurrently (see Tranquillini 1964, Billings and Mooney 1968).

Comparing phenologies of different species that occur across a large altitudinal gradient is challenging, and this challenge was compounded in my study by the need to quantify phenology from a single survey for each species. I therefore developed two independent metrics to estimate flowering phenology of my 12 *Mertensia* species. The two metrics are weakly positively correlated (see Methods), suggesting that they measure similar components of *Mertensia* phenology. I also incorporated altitude in my metrics to correct for altitudinal variation in the length of the flowering season. I found a strong negative correlation between

altitude and phenology PC1—even though the uncorrected estimated peak flowering date tends to be later for higher-altitude *Mertensia* species (Pearson's correlation, $r = 0.60$, $P < 0.05$, $N = 12$). I also found negative correlations between altitude and size PCs. Overall, then, higher-altitude *Mertensia* species flower early (relative to the short available growing season), have smaller floral displays, and are shorter in stature than lower-altitude species.

I cannot be certain whether the observed association between plant size and flowering phenology in my study is due to size–time trade-offs (as described above) or independent responses of size and phenology to environmental conditions along an altitudinal gradient—or some combination of the two processes. Other environmental stressors, such as drought, could also impose selection on flowering phenology and morphological traits. For example, species growing in xeric conditions may flower early and produce small flowers to avoid drought stress (e.g., *Clarkia* spp.; Schneider and Mazer 2016). Incorporating environmental conditions during the flowering period (e.g., mean temperature, precipitation, wind speed) in future phylogenetic comparative analyses may help us understand the relative importance of phenology and environment in explaining size variation among *Mertensia* species.

Flowering phenology and floral traits

Flowering phenology and floral traits could be under correlational selection influenced by temporal variation in availability of selective agents, such as pollinators emerging in predictable seasonal patterns (e.g., bumblebee queens emerging before workers, and male solitary bees emerging earlier than females; Plowright and Laverty 1984, Forrest et al. 2011). If different pollinators exert differing selection on floral traits, flowering phenology might plausibly be correlated with floral traits. In my study, I focused only on floral traits that I expected to exhibit a seasonal pattern. However, I observed insignificant correlations between flowering phenology

and floral traits (predominantly stigma–anther separation and floral volume) across *Mertensia* species, suggesting that any temporal pattern in selective forces on floral traits is weak or inconsistent. Interannual differences in pollinator composition can alter the direction and magnitude of selective pressures on floral traits (Schemske and Horvitz 1989); this could lead to insignificant correlations between flowering phenology and floral traits. Furthermore, flowering phenology and floral traits may be under conflicting selection by non-pollinator selective agents, such as herbivores or environmental conditions (Strauss and Whittall 2006). Unfortunately, it was logistically impossible for me to observe pollinators and other potential selective agents throughout the season for every species; thus, I cannot be certain why there was no association between flowering phenology and floral traits.

Size traits and floral traits

In addition to being correlated with flowering phenology, vegetative size could be positively associated with floral size if there is an allometric relationship, such that species with large vegetative biomass (e.g., taller stature or large leaves) also have more reproductive biomass (e.g., large flowers; reviewed by Cheplick 2005, Weiner et al. 2009). Alternatively, floral display size (i.e., flower number) could be negatively associated with flower size (e.g., floral volume) if they directly compete for the same limiting resources (e.g., Worley et al. 2000, Sargent et al. 2007). In my study, I found that floral volume was unrelated to measures of plant size within *Mertensia* (Figs. 1-7B and 1-7D); i.e., there was no indication of either an allometric relationship or a resource-allocation trade-off involving flower size in this set of species. Interestingly, I detected a weak positive correlation between altitude and floral volume, supporting the notion that plants at high altitudes may compensate for pollinator scarcity via greater investment in floral structures (Fabbro and Körner 2004). I also documented a weak association between plant

size and stigma–anther separation (Fig. 1-4C), which might indicate an allometric relationship between these two traits or that small-statured plants associated with harsh climates tend to bear flowers with more protected (less exserted) stigmas. However, I am cautious about over-interpreting the relationship between plant size and stigma–anther separation, given the marginal significance levels, the lack of an a priori prediction for this relationship, and the number of separate comparisons I conducted. Nevertheless, my results suggest that future work might further test for links between altitude and floral morphology in *Mertensia*. Indeed, studies on other species have found morphological clines in floral traits along altitudinal gradients, suggesting that floral traits may be influenced by pollinator-mediated selection and climatic conditions varying across these gradients (Maad et al. 2013, Zhao and Wang 2015).

Conclusion

Comparative phylogenetic analyses like those I report here are necessary in examining trait evolution across species. However, I found low phylogenetic signal in all traits and trait correlations, which may be due to my relatively small sample size or may truly reflect little phylogenetic pattern in these traits. Nevertheless, a larger sample size would improve my assessment of phylogenetic relatedness in this genus. Furthermore, explaining the patterns observed in this study may require reconstructing the evolutionary history of other potential drivers (e.g., Molnár et al. 2012). Quantifying pollinator assemblages and environmental conditions associated with each *Mertensia* species would provide additional evidence of how these factors influence selection on flowering phenology and morphological traits. For example, short anthers in *M. brevistyla* may be an adaptive response to avoid negative effects of rain on pollen germination (Lin and Forrest 2019). Similarly, studies on *Datura wrightii* and *Collinsia parviflora* showed that water availability can influence stigma–anther separation and flower size

across individuals and populations (Elle and Hare 2002, Elle 2004). However, gathering these data requires, at a minimum, observing pollinators throughout the flowering season and collecting seasonal and annual weather data for each *Mertensia* species—a major logistical challenge for species distributed throughout the Rocky Mountains of western North America. Nonetheless, my results point to altitude as a major driver of species' life-history strategies and morphologies. Further, focused studies of individual species will be essential to better understand the significance of floral trait variation in *Mertensia*.

Table 1-1: Altitude and phenological estimates of each *Mertensia* species. Refer to Methods for details on phenological estimation.

Species (*n* plants)	Altitude (m)	Sampling day of the year (d_{obs})	Proportion of opened flowers (%)	Estimated flowering-period duration at sampling site (l_a, days)	Estimated flowering peak within duration at sampling site (m_a, days)	Adjusted Weibull score (W_a)	Uncorrected peak flowering at sampling site (day of year)	Altitude-corrected peak flowering at 2898 m (D, day of year)	Altitude-corrected co-flowering phenology at 2898 m (day of year)
M. alpina (15)	3770	192	78.0	35.7	8.1	16.2	184	155	154
M. bakeri (15)	3519	184	28.0	44.0	9.9	7.9	186	166	147
M. brachyloba (15)	2145	175	92.2	89.3	20.2	55.3	140	165	203
M. brevistyla (51)	2994	163	25	61.3	13.9	10.2	167	164	132
M. ciliata (25)	3132	182	38.8	56.7	12.8	13.0	182	174	147
M. clokeyi (15)	2744	174	69.0	69.6	15.7	27.0	163	168	154
M. franciscana (20)	2829	194	42.5	66.7	15.1	16.5	193	195	163
M. lateriflora (15)	2584	187	57.6	74.8	16.9	24.1	180	190	156
M. longipedunculata (10)	2378	206	75	81.6	18.5	35.0	189	207	184
M. ovata (12)	3635	189	66.4	40.1	9.1	14.9	183	159	154
M. parvifolia (20)	3440	189	84.8	46.6	10.5	24.1	175	158	153
M. perplexa (24)	4165	205	75	22.7	5.1	9.72	200	159	141

Table 1-2: Description of parameters to estimate phenology of each *Mertensia* species. Refer to Methods for details on phenological estimation.

Parameters	Description
L_{2898}	Estimated flowering duration (62.1 days) at altitude 2898 m from long-term *M. ciliata* and *M. fusiformis* dataset
M_{2898}	Estimated peak flowering day (13.0 days) at altitude 2898 m from long-term *M. ciliata* and *M. fusiformis* dataset
W_{2898}	Estimated day during the flowering period (L_{2898}) when the survey took place, at altitude of 2898 m from long-term *M. ciliata* and *M. fusiformis* dataset
d_{obs}	Day of the year when the survey took place
l_a	Adjusted flowering duration of a species at altitude *a*
m_a	Adjusted peak flowering day of a species at altitude *a*
W_a	Adjusted day during the flowering period when the survey took place at altitude *a*
D	Calculated peak flowering day of a species at altitude 2898 m

Table 1-3: Size and floral traits (mean ± SE) of each *Mertensia* species. Refer to Methods for details on morphological measurements.

	Size traits			Floral traits			
Species (*n* plants)	Plant height (mm)	Mean surface area of largest leaves per plant (mm^2)	Number of flowers per plant	Floral volume (mm^3)	Stigma height (mm)	Anther height (mm)	Stigma–anther separation (mm)
M. alpina (15)	52.2 ± 5.7	89.5 ± 7.6	17.3 ± 2.8	87.0 ± 4.5	2.7 ± 0.1	3.4 ± 0.1	−0.72 ± 0.087
M. bakeri (15)	226.7 ± 5.8	524.7 ± 34.9	99.2 ± 23.0	155.7 ± 7.4	10.2 ± 0.3	8.9 ± 0.3	1.32 ± 0.077
M. brachyloba (15)	171.5 ± 16.6	599.9 ± 57.9	158.2 ± 48.9	55.9 ± 5.0	7.1 ± 0.2	6.3 ± 0.2	0.77 ± 0.11
M. brevistyla (51)	118.2 ± 11.3	381.8 ± 25.8	30.1 ± 3.3	175.5 ± 5.6	2.2 ± 0.1	3.2 ± 0.1	−0.97 ± 0.056
M. ciliata (25)	315.1 ± 10.6	1283.5 ± 83.5	32.0 ± 4.8	311.6 ± 8.3	12.9 ± 0.1	10.2 ± 0.1	2.68 ± 0.096
M. clokeyi (15)	233.9 ± 12.3	489.4 ± 55.5	85.1 ± 28.3	130.2 ± 10.7	8.6 ± 0.3	8.0 ± 0.2	0.62 ± 0.20
M. franciscana (20)	572.8 ± 21.2	1714.3 ± 116.4	1420.5 ± 412.2	104.8 ± 5.8	11.1 ± 0.2	10.2 ± 0.2	0.91 ± 0.086
M. lateriflora (15)	269.5 ± 30.0	263.6 ± 32.5	1915.0 ± 842.9	52.4 ± 2.8	8.3 ± 0.2	6.9 ± 0.1	1.37 ± 0.10
M. longipedunculata (10)	733.0 ± 82.2	6203.8 ± 481.1	3581.1 ± 1097.8	136.1 ± 15.9	11.8 ± 0.1	9.1 ± 0.2	2.70 ± 0.18
M. ovata (12)	73.5 ± 6.3	448.0 ± 42.5	17.6 ± 3.1	200.4 ± 7.4	11.2 ± 0.3	10.9 ± 0.2	0.41 ± 0.20
M. parvifolia (20)	147.8 ± 5.9	334.0 ± 16.1	47.1 ± 12.3	156.9 ± 7.5	9.8 ± 0.3	8.2 ± 0.1	1.63 ± 0.27
M. perplexa (24)	43.0 ± 2.8	256.7 ± 27.2	7.6 ± 0.71	178.4 ± 14.7	5.6 ± 0.1	6.7 ± 0.1	−1.07 ± 0.14

Table 1-4: Phylogenetic signal in flowering phenology, size traits, and floral traits using Blomberg's *K*.

Variables	Blomberg's *K*	*P*
Mertensia phenology	0.48	0.61
Co-flowering phenology	0.75	0.16
Plant height	0.50	0.58
Leaf area	0.43	0.78
Number of flowers	0.55	0.43
Floral volume	0.72	0.14
Stigma height	0.54	0.40
Anther height	0.62	0.25
Stigma–anther separation	0.44	0.79
Phenology PC1	0.53	0.46
Size PC1	0.49	0.60
Size PC2	0.49	0.66
Floral PC1	0.46	0.72
Floral PC2	0.69	0.13

Table 1-5: Comparison of phylogenetic generalized least squares (PGLS) between variables with star phylogeny (λ = 0) and resolved phylogeny (λ = 1) included in the analyses. Significant *P* values from likelihood-ratio (LR) tests between λ = 0 and λ = 1 indicate that the model with λ = 0 is significantly better.

		λ = 0			λ = 1			LR test	
Variable 1	Variable 2	Slope	*P*	LL	Slope	*P*	LL	χ^2	*P*
Phenology PC1	Size PC1	1.22	< 0.001	−11.9	1.25	< 0.001	−13.0	−2.07	0.15
	Size PC2	0.41	0.037	−11.7	0.44	0.052	−13.4	−3.53	0.060
	Floral PC1	0.44	0.19	−19.3	0.52	0.16	−21.7	−4.73	0.030
	Floral PC2	−0.32	0.14	−13.6	−0.21	0.31	−15.0	−2.70	0.10
Size PC1	Floral PC1	0.12	0.065	−28.7	0.35	0.99	−29.8	−2.37	0.12
	Floral PC2	−7.24	0.52	−28.1	−0.46	0.99	−28.6	−0.99	0.32
Size PC2	Floral PC1	0.088	0.054	−19.4	0.088	< 0.01	−21.2	−3.71	0.054
	Floral PC2	−1.91	0.066	−19.9	−0.99	0.97	−21.8	−3.69	0.055
Altitude	Phenology PC1	−0.0016	< 0.01	−12.6	−0.0017	< 0.01	−16.0	−6.76	< 0.01
	Size PC1	−0.0017	< 0.01	−15.6	−0.0017	< 0.01	−16.0	−0.88	0.35
	Size PC2	−0.00078	< 0.05	−11.4	−0.0010	< 0.05	−12.3	−1.80	0.18
	Floral PC1	−0.00068	0.30	−19.7	−0.0012	0.15	−21.6	−3.83	0.050
	Floral PC2	0.00068	0.087	−13.2	0.00053	0.25	−14.8	−3.28	0.070

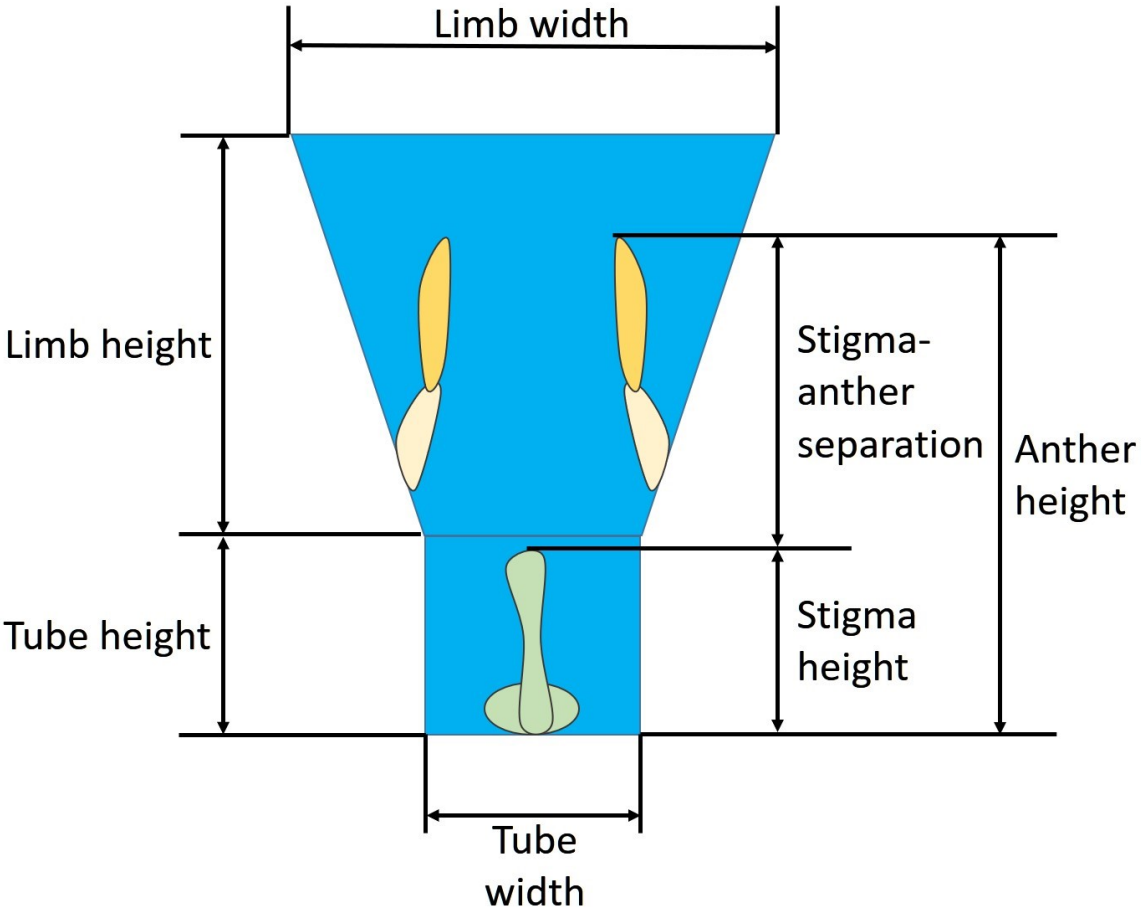

Figure 1-1: Diagram showing floral traits measured in this study.

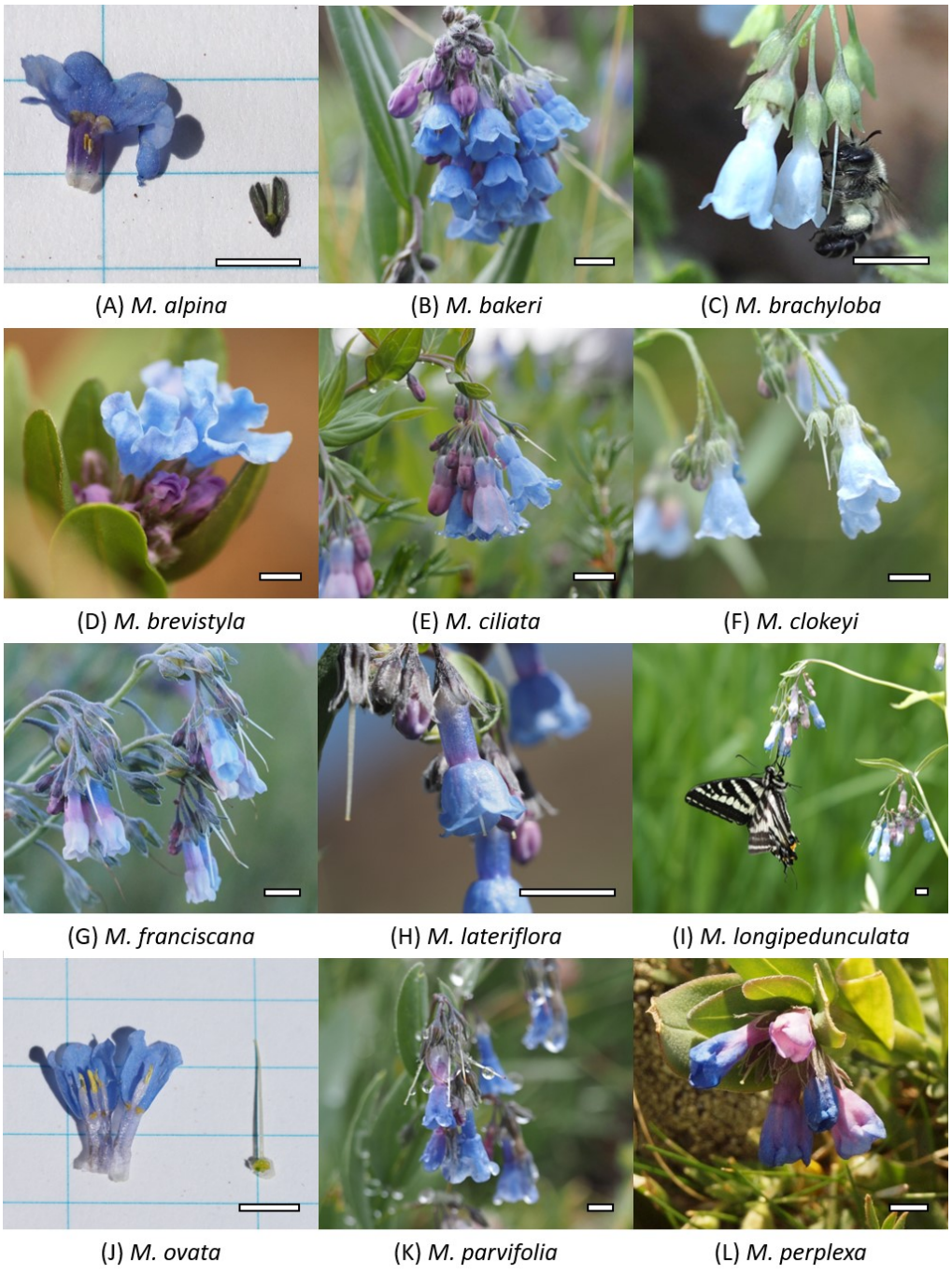

Figure 1-2: Photographs of *Mertensia* study species found in Arizona (*M. franciscana*) and Colorado (all others) between 2014 and 2016. In *M. alpina* and *M. ovata*, the style and ovary have been separated from the corolla (with attached stamens). The white bars represent ~ 0.5 cm in each photograph.

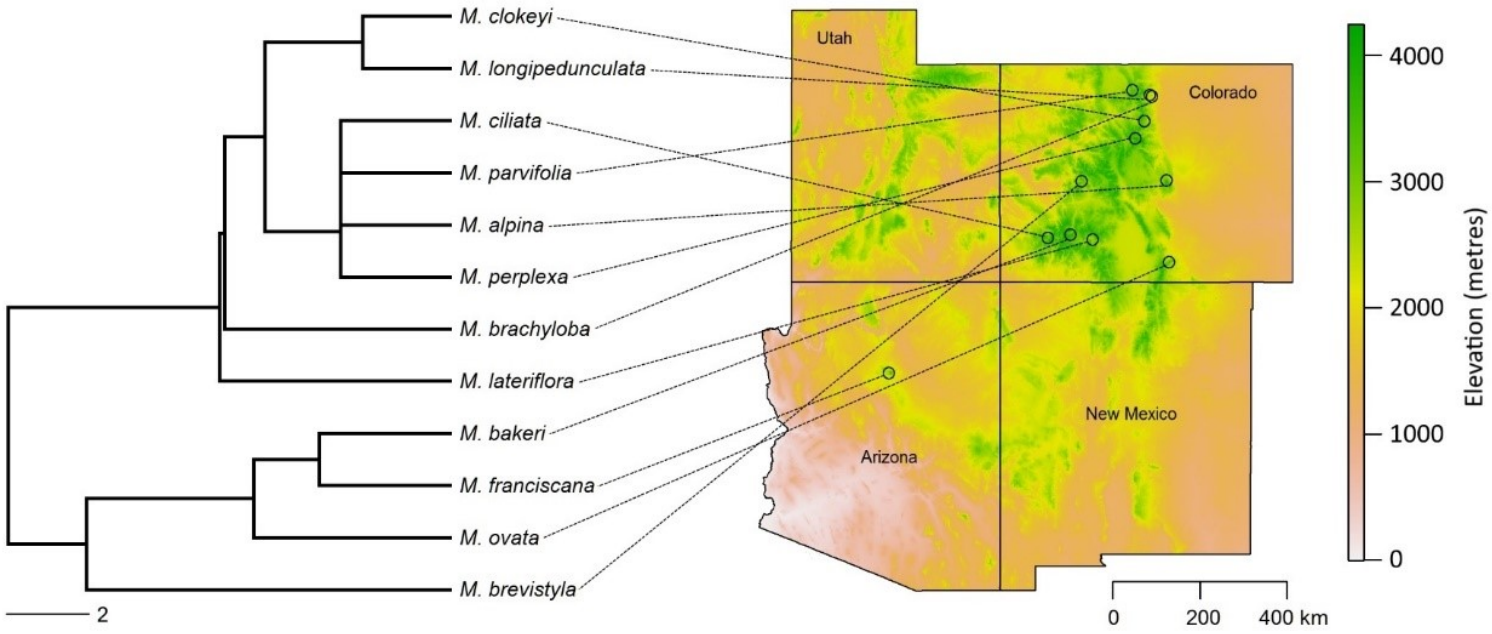

Figure 1-3: Modified phylogeny (pruned from Nazaire and Hufford 2014) along with sampling locations and altitudes of the 12 *Mertensia* species examined in this study. The scale bar below the phylogeny denotes two substitutions per locus for the concatenated plastid genes used to construct the phylogeny.

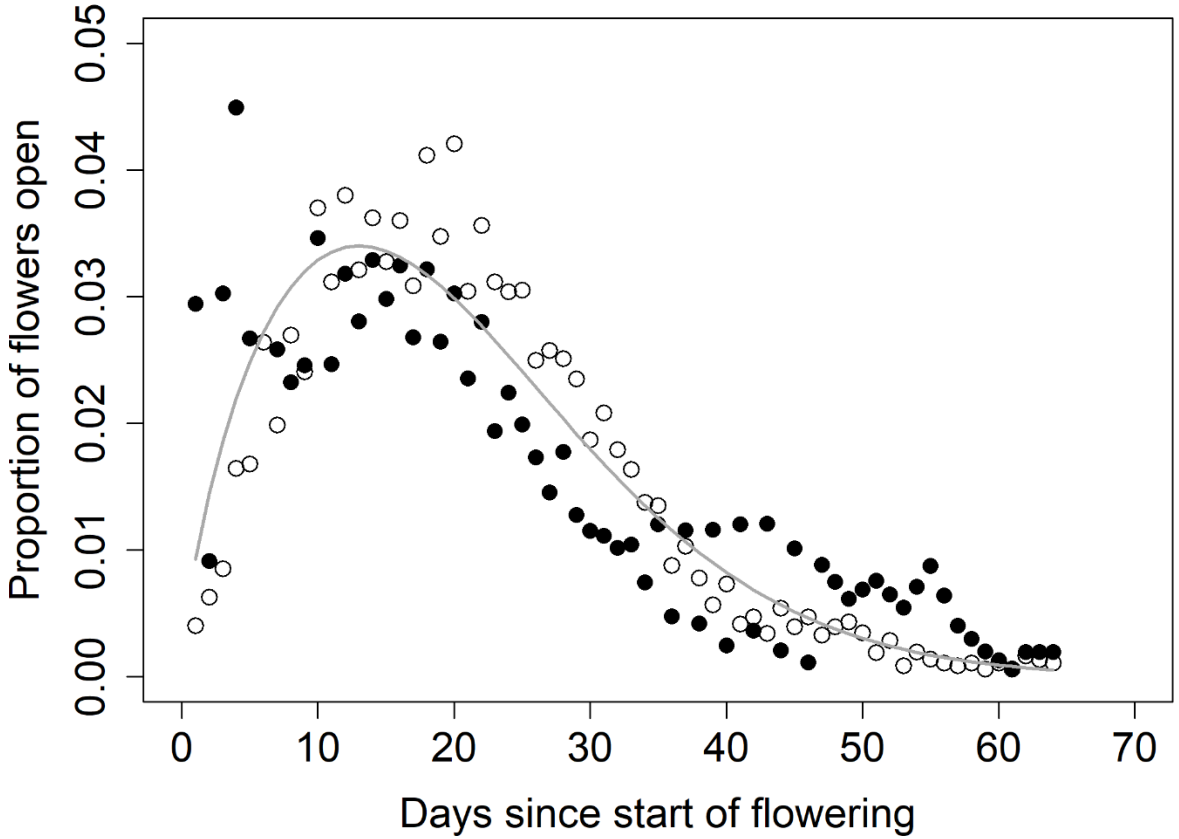

Figure 1-4: Scatterplot representing the mean proportion of open flowers for each day during the flowering season for *M. ciliata* (filled circles) and *M. fusiformis* (open circles) across all plots and year between 1973 and 2017 (except 1978). The mean Weibull distribution (grey line) has a shape parameter = 1.66 and a scale parameter = 22.7 (averaged between *M. ciliata* and *M. fusiformis*).

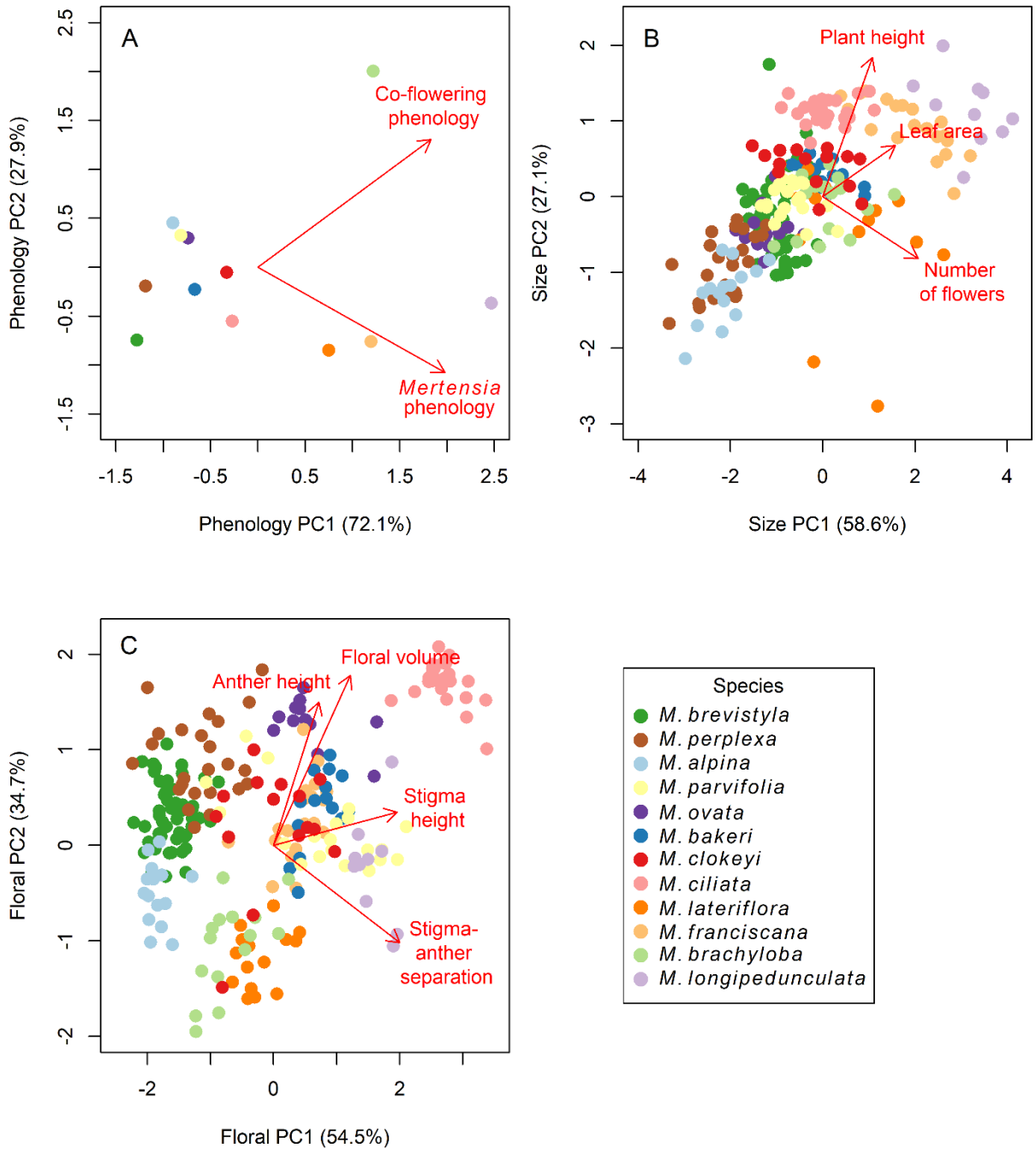

Figure 1-5: pPCA biplots of a) phenology, b) size, and c) floral traits across 12 *Mertensia* species.

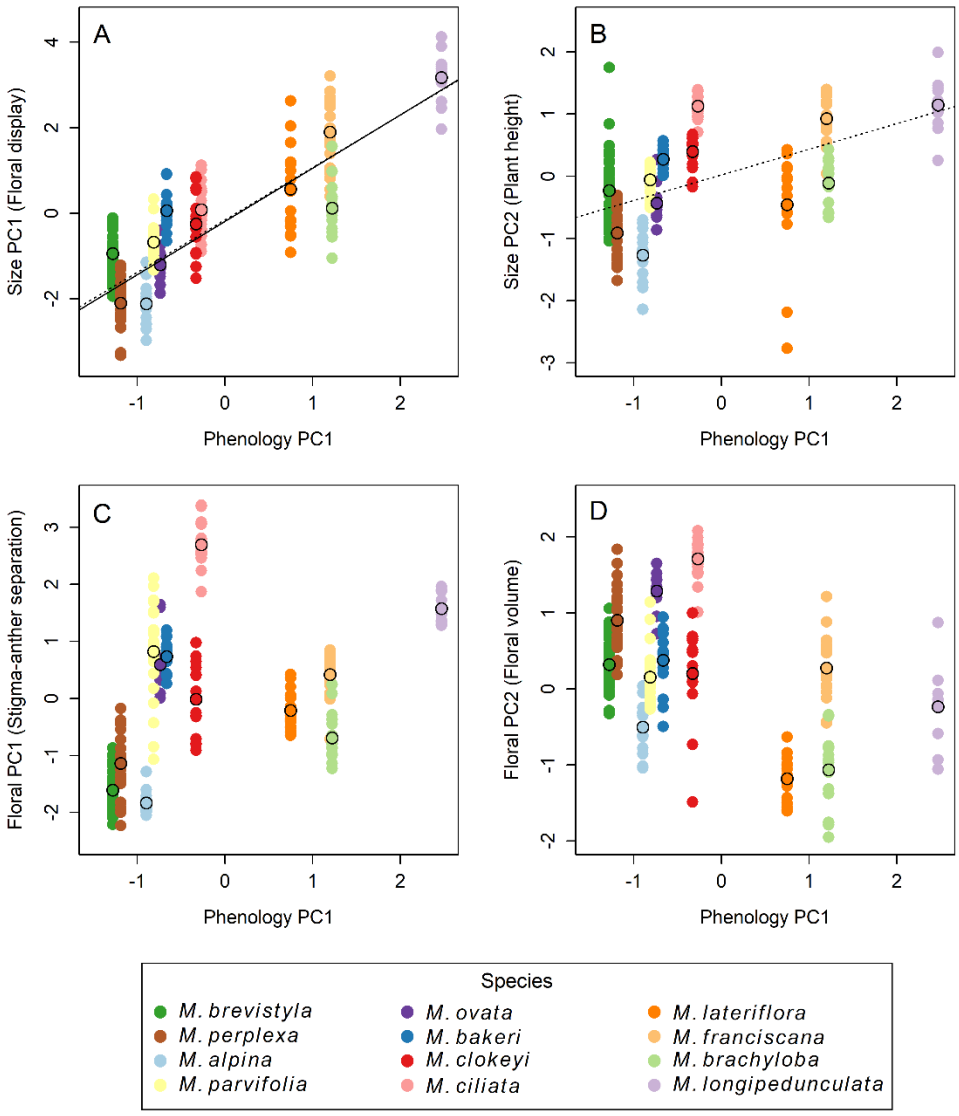

Figure 1-6: Scatterplots showing the relationships between phenology PC1 and (A) size PC1, (B) size PC2, (C) floral PC1, and (D) floral PC2 across 12 *Mertensia* species. The y-variable with the largest loading in each PC is indicated in the axis label. The outlined circles represent the species' mean values for each PC. Only statistically significant slopes ($P < 0.05$; solid and dotted lines are PGLS regressions with resolved phylogeny ($\lambda = 1$) and star phylogeny ($\lambda = 0$), respectively) are shown (see Results).

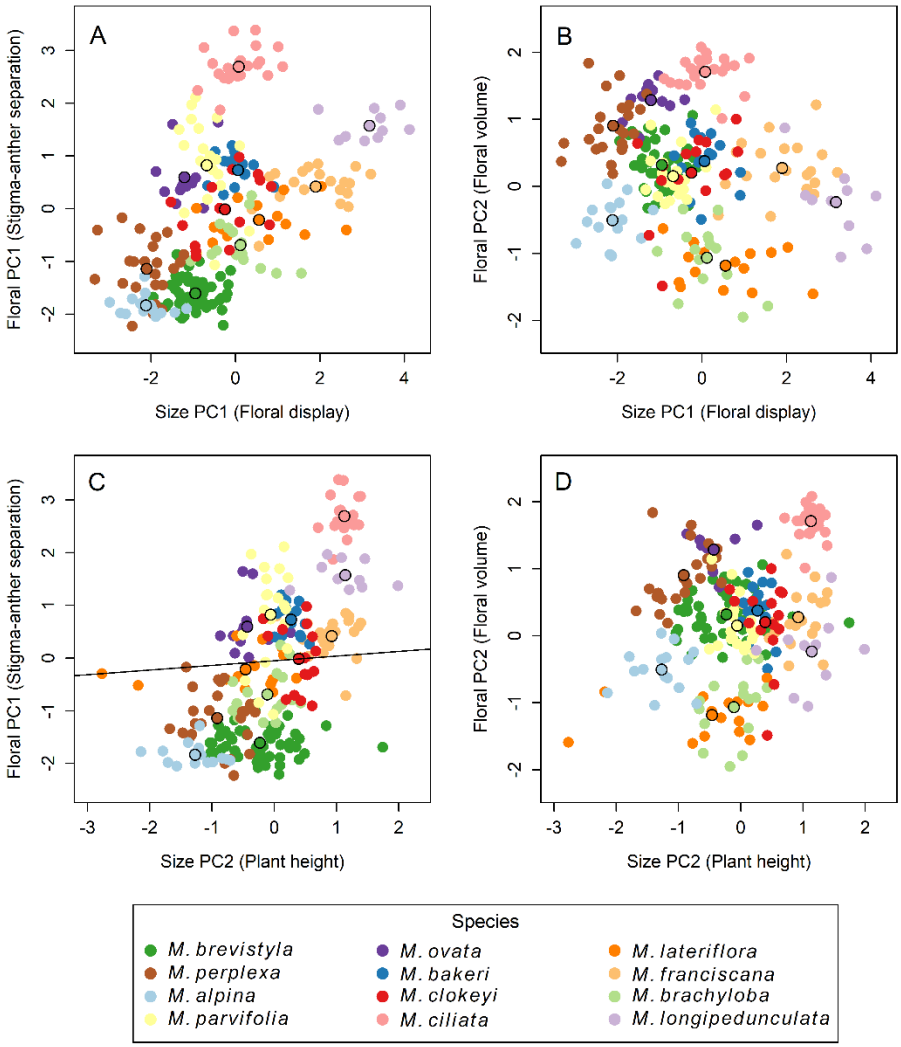

Figure 1-7: Scatterplots showing the relationships between (A) size PC1 and floral PC1, (B) size PC1 and floral PC2, (C) size PC2 and floral PC1, and (D) size PC2 and floral PC2 across 12 *Mertensia* species. The variable with the largest loading in each PC is indicated in the axis label. The outlined circles represent the species' mean values for each PC. In panel C, the statistically significant slope shown is PGLS regression with resolved phylogeny ($\lambda = 1$, $P < 0.01$); however, the PGLS regression with star phylogeny is marginally significant ($\lambda = 0$, $P = 0.054$), and the latter model is marginally statistically superior ($P = 0.054$; see Results).

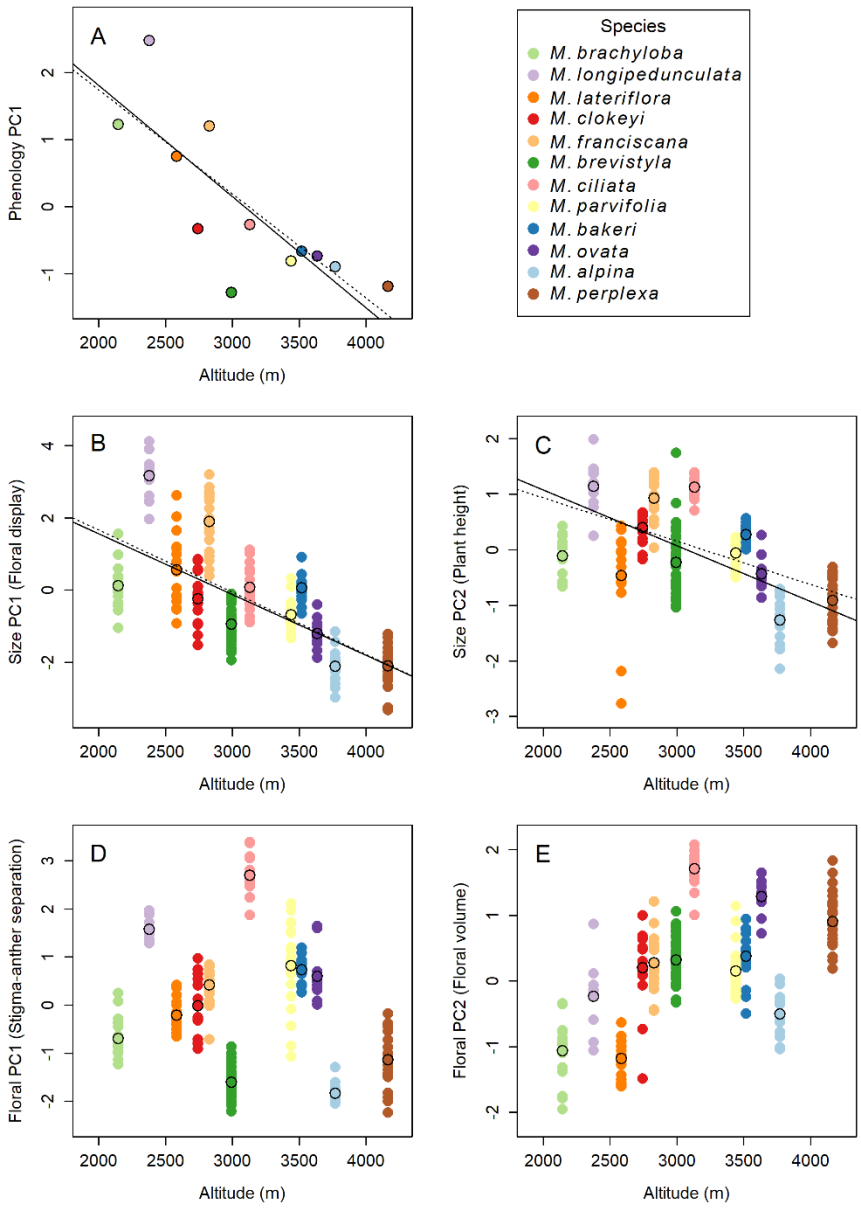

Figure 1-8: Scatterplots showing the relationships between altitude and (A) phenology PC1, (B) size PC1, (C) size PC2, (D) floral PC1, and (E) floral PC2 across 12 *Mertensia* species. The y-variable with the largest loading in each PC is indicated in the axis label. The outlined circles represent the species' mean values for each PC. Only statistically significant slopes ($P < 0.05$; solid and dotted lines are PGLS regressions with resolved phylogeny ($\lambda = 1$) and star phylogeny ($\lambda = 0$), respectively) are shown (see Results).

Chapter 2

Weak reproductive isolation between sympatric *Mertensia* populations associated with generalized pollination ecology

Abstract

Examination of reproductive isolation between lineages provides critical insight into the ecological processes that lead to adaptive divergence and maintain species boundaries. In angiosperms, pre-pollination barriers have been documented to be strong between taxa with distinctly different pollination syndromes (e.g., insect versus bird pollinators). Other important pre-pollination barriers can include differences in habitat, flowering phenology, and floral traits. However, in taxa with generalized pollination systems, these pre-pollination barriers may not be sufficient to maintain reproductive isolation, since visitation from overlapping pollinator assemblages is more likely to lead to heterospecific pollen transfer. Thus, post-pollination barriers influencing pollen viability and hybrid seed formation may play a greater role in maintaining reproductive isolation in these taxa. I evaluated the contributions of pre- and post-pollination barriers between sympatric *Mertensia brevistyla* and *M. fusiformis* (Boraginaceae) populations at Kebler Pass, Colorado. Both species produce blue, campanulate flowers and are pollinated by generalist bees and flies. For pre-pollination barriers, I examined differences in habitat (by surveying co-occurring plants), flowering phenology, pollinator assemblages, and conspecific and heterospecific pollinator movements in mixed-species arrays. For post-pollination barriers, I compared seed set following conspecific and heterospecific hand-pollination, and compared pollen germination rates of both species and putative hybrids. Within the small area of sympatry I studied, I found habitat isolation to be weak due to the close proximity of heterospecifics. Both species shared similar flowering phenology and pollinator assemblages, indicating weak phenological and pollinator isolation. Pollinators transferred more fluorescent dye (a pollen analog) between conspecifics than between heterospecifics, leading to a relatively strong pre-pollination barrier involving floral isolation. Flowers hand-pollinated with

conspecific pollen yielded higher seed set than those pollinated with heterospecific pollen, and this was the strongest barrier examined (i.e., 0.55 and 0.40 in *M. brevistyla* and *M. fusiformis*, respectively). However, I detected similar pollen germination rates among the parental species and putative hybrids, suggesting potential hybrid fertility. Overall, reproductive isolating barriers between sympatric *M. brevistyla* and *M. fusiformis* populations were incomplete and likely led to interspecific gene flow at Kebler Pass. This study expands our knowledge of the importance of pre- and post-pollination isolating barriers beyond the well-known specialized systems.

Introduction

Angiosperms are one of the most speciose groups of organisms, and they provide an excellent system with which to examine the forces that contribute to species formation and that maintain species boundaries. Speciation, the formation of species, can occur when divergent selection acts on genetically based differences within a taxon, followed by the formation of reproductive isolating barriers separating sister lineages (Mayr 1942, Coyne and Orr 2004). For example, *Mimulus cardinalis* and *M. lewisii* are sister species that differ in flower colour. This trait differentially—and dramatically—influences visitation by bumblebee and hummingbird pollinators, suggesting that speciation could have been driven by divergence in pollinator identity following a single genetic change in flower colour (see Schemske and Bradshaw 1999, Bradshaw and Schemske 2003). Following speciation, when related species come into secondary contact, reproductive isolating barriers may be favoured by selection (a process known as reinforcement) because they reduce the wastage of resources on inviable reproductive efforts (Dobzhansky 1937, Levin 1978, reviewed by Hopkins 2013). Overall, reproductive isolation is essential to the maintenance of species boundaries, and the strengths of various barriers to reproduction have been documented for a number of angiosperm taxa (reviewed by Lowry et al. 2008, Widmer et al. 2009, Baack et al. 2015). However, most studies have focused on pairs of taxa with relatively specialized and distinctly different pollination syndromes (e.g., bees versus hummingbirds; reviewed by Grant 1994, Campbell and Aldridge 2006). For plants with more generalized pollination ecology and sharing similar pollinator assemblages, the relative strength of reproductive isolating barriers has been less studied (but see Costa et al. 2007, Pascarella 2007, Ostevik et al. 2016).

Reproductive isolating barriers occur sequentially during the various life-history stages of angiosperms, and barriers that take place before pollination are known as pre-zygotic, pre-pollination barriers. First, ecological processes and/or physical barriers that prevent large-scale spatial overlap between related species are known as ecogeographic isolation (see Sobel 2014, Baack et al. 2015, Duffy and Jacquemyn 2019), while ecological processes that produce local-scale habitat differences are considered habitat isolation (see Kay 2006, Rieseberg and Willis 2007). If species occur in sympatry, then divergence in flowering phenology (phenological isolation), morphological/mechanical misfit between flowers and pollinators (mechanical isolation), and pollinators exhibiting differential preference for floral traits (ethological isolation) can all be important barriers preventing heterospecific pollen transfer (see Martin and Willis 2007, Lowry et al. 2008, Widmer et al. 2009). Once interspecific pollination has occurred, conspecific pollen may have precedence over heterospecific pollen due to pollen–pistil interactions or interspecific pollen competition, which are considered pre-zygotic, post-pollination barriers (see Diaz and Macnair 1999, Howard 1999, Campbell et al. 2003). If heterospecific pollen does successfully fertilize the ovules and produce hybrid seeds, then hybrids may experience inviability and infertility. The latter factors are referred to as post-zygotic barriers and may be intrinsic (genetic) or extrinsic (environmental) in nature (see Coyne and Orr 2004, Rieseberg and Willis 2007). As a result of selection to avoid gamete wastage and production of unfit hybrid offspring, early-acting pre-zygotic isolation is estimated to be twice as strong as the late-acting post-zygotic isolation in angiosperms (Lowry et al. 2008).

However, given that species with generalized pollination systems are visited by a wide range of pollinators and may be more likely to experience interspecific pollination (Fang and Huang 2013), post-zygotic barriers may be stronger than pre-zygotic barriers in these species

(Scopece et al. 2007). To understand how reproductive isolation operates in species with generalized pollination, I focused on the barriers between two sympatric *Mertensia* species (*M. brevistyla* and *M. fusiformis*, Boraginaceae) at Kebler Pass, Colorado, USA. I quantified six pre-pollination and post-pollination barriers between *M. brevistyla* and *M. fusiformis*, including spatial proximity between parental species, differences in flowering phenology and pollinator assemblages, visitation rate in mixed-species arrays, seed set in conspecific and heterospecific pollen crosses, and pollen viability. All *Mertensia* produce campanulate flowers that are pollinated by generalist bees and flies (Pelton 1961, Suzuki 1994, Forrest et al. 2011, Lin and Forrest 2019). Therefore, to assess the potential for pollinators to discriminate between my study species, I also compared vegetative and floral traits between *M. brevistyla*, *M. fusiformis*, and putative hybrids found in an overlapping region at Kebler Pass. My main objective was to evaluate the relative strength of pre- and post-pollination isolating barriers between related species with generalized pollination systems in an area of sympatry.

Materials and Methods

Study species and study site

Mertensia brevistyla Watson and *M. fusiformis* Greene are herbaceous perennials found in the Rocky Mountains of western North America. They generally occupy dry, open habitats, and begin flowering in early spring (Weber and Wittmann 2012). The two species were historically considered to be closely related based on similarities in leaf and root morphologies (Williams 1937) and are thought to have diverged from each other approximately 2 million years ago (Nazaire and Hufford 2014, Nazaire et al. 2014). Accessions of *M. fusiformis* from Colorado and Utah form a monophyletic clade with accessions of *M. brevistyla* from Utah and Nevada, while an accession of *M. brevistyla* from Colorado forms a polyphyletic clade with *M. arizonica*

and *M. toyabensis* (Nazaire and Hufford 2014). The accessions of *M. fusiformis* and *M. brevistyla* collected by Nazaire and Hufford (2014) from Colorado are not sister to each other, and their Colorado accession of *M. brevistyla* came from the same locality as the *M. brevistyla* population examined in this study (voucher specimen to be deposited at the Museum of Nature, Ottawa, Canada). *Mertensia fusiformis* individuals examined in this study appear to be the same species that occurs throughout the East River valley and Coal Creek drainage (and identified as such in previous studies: Miller-Rushing and Inouye 2009, Forrest and Thomson 2010, Forrest et al. 2011, Nazaire and Hufford 2014).

Both *Mertensia* species are short (maximum height less than 30 cm above ground) and produce blue campanulate flowers consisting of a cylindrical floral tube and a broader floral limb with lobes (Weber and Wittmann 2012). The flowers of *M. brevistyla* have a more broadly flared floral limb and five short stamens and a style concealed inside the floral tube, while those of *M. fusiformis* have exserted stamens and a style that can be shorter or longer than the floral tube (Forrest et al. 2011; Nazaire and Hufford 2014; see Fig. 2-1). The two species also differ significantly in floral orientation, with *M. fusiformis* inflorescences more pendant than those of *M. brevistyla* (Lin and Forrest 2019). The floral visitors of both species are primarily bumblebees (*Bombus* spp., Apidae), solitary bees (Halictidae and Megachilidae), and syrphid and other flies (Diptera; Forrest et al. 2011, Lin and Forrest 2019).

I examined six barriers that may contribute to reproductive isolation between sympatric populations of *M. brevistyla* and *M. fusiformis* at Kebler Pass, Colorado, USA (38°51’27.41”N, 107°5’42.25”W). The two species occur in adjacent meadows at the pass, and both species co-occur (i.e., less than 20 m between heterospecific individuals) in a region of overlap where putative hybrids (with flared floral limbs similar to *M. brevistyla* and a long style similar to *M.*

fusiformis) are present (Fig. 2-1). I quantified each barrier following the equations of Kay (2006). Each equation measures the proportion of shared (i.e., heterospecific or interspecific) relative to non-shared (i.e., conspecific or intraspecific) interactions between *M. brevistyla* and *M. fusiformis* and subtracts each proportion from one (see Table 2-1 for calculations). Reproductive isolating barriers generally range between zero and one, where zero indicates no barrier preventing interspecific reproduction and one indicates complete isolation. Negative barriers would indicate a greater interspecific than intraspecific interaction, such as greater hybrid viability or fertility. Because my results suggested that pollinators can discriminate between the two species (see Results), I also measured several traits of the two plant species that may allow pollinators to distinguish between them.

Vegetation community and habitat isolation

To assess whether habitat use differed between *M. brevistyla* and *M. fusiformis*, I examined the vegetation community in three locations (*M. brevistyla* habitat, *M. fusiformis* habitat, and overlapping region), as a proxy for habitat-specific environmental differences. I set up six transects (1 × 300 m) that crossed all three habitat types. Along each transect, I placed a 1 × 1 m sampling quadrat at every 100 m (i.e., 4 quadrats per transect) and identified all plant taxa (to the family, genus or species level) inside the quadrat. Using the R Statistical Software version 3.4.4 (R Core Team 2018), I performed nonmetric multidimensional scaling (NMDS) using the function metaMDS in the vegan package (Oksanen et al. 2018) on the presence or absence of plant taxa within the quadrats. I then conducted a permutational multivariate analysis of variance (perMANOVA with 10000 permutations) using the function adonis to test for differences in the plant community (NMDS centroid) among the three habitats. Before those analyses, I examined homogeneity of group covariances, an assumption of perMANOVA, with a permutational

multivariate homogeneity of group dispersions test (PERMDISP with 10000 permutations) using the function betadisper. Lastly, to determine if certain plant species were indicative of the different habitats, I calculated indicator value indices (indval) for each plant species (based on the number of quadrats where each species is present) using the function indval in the labdsv package (Roberts 2016).

I also investigated the potential for local-scale habitat isolation between *M. brevistyla* and *M. fusiformis* inside the overlapping region. To do this, I calculated the extent of co-occurrence of the two species in randomly generated virtual "plots". First, I mapped the coordinates of all visibly distinguishable *M. brevistyla* and *M. fusiformis* individuals using a high-accuracy GPS unit (GeoXT, Trimble, California, USA; see Fig. 2-1). I imported the coordinates of all mapped individuals (N = 82 *M. brevistyla* and 24 *M. fusiformis* individuals) using the function SpatialPoints in the sp package (Pebesma and Bivand 2005, Bivand et al. 2013). Next, inside a rectangular polygon (48.3 m × 129.5 m) encompassing all individuals, I randomly generated 10000 points that served as centres for circular virtual plots (varying in radius from 10 to 50 m, in 10 m increments) using the function circles in the dismo package (Hijmans et al. 2017). For each radius size, I divided the number of heterospecific plots (i.e., those in which both species co-occurred) by the sum of heterospecific and conspecific (i.e., focal species only) plots, and subtracted this value from one (see Table 2-1). This was done separately for *M. brevistyla* and *M. fusiformis*.

Phenological isolation

I compared phenology between *M. brevistyla* and *M. fusiformis* in 2014. Between 6 June and 5 July, I counted the number of open flowers in 42 *M. brevistyla* and 50 *M. fusiformis* individuals (a subset of the individuals located outside the overlapping region and the same ones

sampled for morphological comparisons; see below) every two to five days. I determined the first flowering date and peak flowering date (the date with the most open flowers) of all individuals. The phenological differences (in first flowering and peak flowering date) between species were analyzed using non-parametric Wilcoxon tests in the stats package.

To quantify phenological isolation, I first determined that the flowering phenology of both species overlapped between 11 June (beginning of *M. fusiformis* phenology) and 24 June (end of *M. brevistyla* phenology). I then divided the number of open flowers produced by all sampled individuals of each species during this overlapping period by the total number of open flowers produced throughout the flowering season, and subtracted this value from one (see Table 2-1; Fig. 2-2).

Visitor assemblages

I contrasted the composition of insect visitors to *M. brevistyla* and *M. fusiformis* plants at Kebler Pass between 2014 and 2017 (total sampling effort: *M. brevistyla*: 30.25 observer-hours; *M. fusiformis*: 29.75 observer-hours; Table 2-2). During favourable weather conditions (warm and dry days), I recorded insect visitors to *Mertensia* flowers and identified them as: bumblebees (*Bombus* spp., Apidae), megachilid bees (Megachilidae; mainly *Osmia* spp.), halictid bees (Halictidae), syrphid flies (Syrphidae) or other flies (Diptera). Due to variation in sampling effort, I calculated the proportion of each visitor type per site and year, and performed NMDS on the proportions. I then conducted a perMANOVA (as described for vegetation community) to evaluate differences in NMDS centroid between visitors to *M. brevistyla* and *M. fusiformis*. Data included in this analysis are a subset of the pollinator data reported by Lin and Forrest (2019).

Floral (ethological and mechanical) isolation

To determine ethological and mechanical isolation (i.e., floral isolation; Schiestl and Schlüter 2009) between *M. brevistyla* and *M. fusiformis*, I quantified the ability of pollinators to transfer pollen between species by setting up two to six experimental arrays each day between 18 June and 25 June 2015 ($N = 24$ *M. brevistyla* and 20 *M. fusiformis* arrays in total) inside the natural population of each species and examining the movement of fluorescent dyes inside the arrays (as a proxy for pollen transfer; Kearns and Inouye 1993). Each array consisted of a central ("donor") potted plant that was the same species as the surrounding natural population, encircled by three potted ("recipient") plants of each species (six plants total) placed approximately 30 cm away from the central plant and arranged alternately in a circle. Fluorescent dye was applied to all flowers of the central plant each day, and dye transfer inside the array was quantified the following day by examining dye inside flowers of the surrounding recipient plants using a black light source and a hand lens. Any potted plants that had received dye in their flowers and/or were becoming wilted at the end of the day were replaced. I quantified the proportion of conspecific and heterospecific plants (relative to the identity of the central plant) that had fluorescent dye in at least one flower. To test the effect of donor and recipient species identities on dye transfer, I performed a generalized linear mixed model (GLMM) with a zero-inflated binomial distribution on proportion of recipient plants with dye. Type of dye deposition (interspecific versus intraspecific) and species were the predictor variables, and array was included as a random effect. This analysis was conducted using the function glmmadmb in the glmmADMB package (Fournier et al. 2012, Skaug et al. 2016).

Morphological and colour differences

I haphazardly selected and measured vegetative and reproductive traits on 66 *M. brevistyla* individuals (51 plants sampled outside the overlapping region in 2014, and 15 plants inside the overlapping region in 2016), 72 *M. fusiformis* individuals (57 plants outside the overlapping region in 2014, and 15 plants inside the overlapping region in 2016), and 15 putative hybrids inside the overlapping region in 2016 (Fig. 2-1). For all individuals, I measured the following traits: plant height (maximum vertical height above substrate), average maximum leaf area (the elliptical leaf area from major and minor leaf axes of the three largest leaves), and number of flowers (the sum of the number of flower buds and open and senesced flowers). From each individual, I randomly selected up to three flowers and measured floral limb width and length, floral tube width and height, anther height, and stigma height. I performed two multivariate analyses of variance (MANOVA) to examine differences in the three size traits (plant height, leaf area, and number of flowers) and six floral traits (floral limb width and length, floral tube width and length, stamen height [distance from base of ovary to top of tallest anther], and style length [distance from base of ovary to top of stigma]) among the five "ecotypes": *M. brevistyla* (inside and outside the overlapping region), *M. fusiformis* (inside and outside the overlapping region), and putative hybrids. All variables were log-transformed to achieve normality and homoscedasticity. These tests were analyzed using the function manova in the stats package (R Core Team 2018).

To determine if there were colour differences between *M. brevistyla* and *M. fusiformis* corollas during the peak flowering period in 2014, I randomly removed at least one flower per individual from 45 *M. brevistyla* and 57 *M. fusiformis* individuals in the field (a subset of the same individuals as above) and placed the flowers in a cooler before measuring corolla

reflectance in the laboratory within the same day. I recorded the reflectance spectra of the corollas using a JAZ spectrometer coupled with a LS-1 tungsten halogen light source (Ocean Optics, Dunedin, Florida, USA). The spectra measurements were calibrated relative to a white standard (WS-1 diffuse reflectance standard). I removed the corolla from the reproductive structures and kept the UV-VIS optical fiber at a constant 45-degree angle on the corolla. The spectrometer was controlled by the SpectraSuite software and adjusted to scan each corolla ten times and to provide the average dominant reflective wavelength of the corolla based on the chromaticity diagram (see Backhaus 1991, Heuschen et al. 2005). For individuals with more than one corolla sample, I calculated the mean dominant wavelength for each individual. The interspecific colour difference in dominant reflective wavelength was analyzed using a non-parametric Wilcoxon test.

Post-pollination experiment

I measured reproductive success resulting from intraspecific versus interspecific crosses in both *Mertensia* species. I haphazardly selected 30 individuals per species (removing any wilted or open flowers prior to the experiment) outside the overlapping region and covered them with pollinator-exclusion bags. As new flowers opened over the following days, two stems per individual were randomly chosen to receive either conspecific or heterospecific pollen, and ten flowers on each stem were hand-pollinated twice from pollen donor plants located at least five metres from the recipient plant (to avoid transferring pollen from closely related individuals). Once the seeds were mature, I collected the seeds and calculated seed set as the proportion of mature seeds produced relative to number of ovules, given that each *Mertensia* flower contains four ovules. I also determined the autonomous selfing ability of *M. brevistyla* and *M. fusiformis* by examining seed set in non-pollinated stems (i.e., where flowers received no pollen) in ten of

the bagged plants. To test the effects of donor and recipient species identities on seed set, I performed a GLMM with a binomial distribution with proportion of seeds produced as the response variable, type of pollen cross (interspecific versus intraspecific) and species as the predictor variables, and plant as the random effect. This was done using the function glmer function in the lme4 package (Bates et al. 2015).

Pollen viability

I evaluated pollen viability in *M. brevistyla*, *M. fusiformis*, and putative hybrids, as a proxy for male fertility. I removed one flower with freshly dehisced anthers from 15 *M. brevistyla* and 15 *M. fusiformis* individuals, and 13 putative hybrids. I removed three anthers from each flower and placed them in a pollen germination medium (5% sucrose solution with additional nutrients in tap water; Brys et al. 2008) for three hours. Afterwards, I used a Leitz Laborlux D light microscope and haphazardly selected one field of view per anther. I scored the proportion of pollen grains with growing pollen tubes (i.e., germinated pollen grains) relative to the total number of pollen grains in each field of view. To test for differences in pollen viability among taxa, I conducted a GLMM with a binomial distribution with proportion of germinated pollen grains as the response variable, species as the predictor variable, and plant as the random effect. This was done using the function glmer in the lme4 package.

Results

Vegetation community and habitat isolation

The vegetation community differed significantly among the overlapping region and the *M. brevistyla* and *M. fusiformis* habitats despite considerable overlap in plant community composition among the three habitats (perMANOVA, $R^2 = 0.25$, $F = 3.48$, $P < 0.01$; PERMDISP,

$F = 1.01$, $P = 0.28$; Fig. 2-3A). *Salix* sp. (indval = 0.50, $P < 0.05$) and *Bistorta* sp. (indval = 0.42, $P < 0.05$) were indicative species of the overlapping region, while *Helianthella* sp. (indval = 0.67, $P < 0.01$), *Thalictrum fendleri* (indval = 0.67, $P < 0.01$), *Lathyrus lanszwertii* (indval = 0.53, $P < 0.05$), and *Carex egglestonii* (indval = 0.50, $P < 0.05$) were representative of the *M. fusiformis* habitat. No plant species was indicative of the *M. brevistyla* habitat (Table 2-3).

For *M. brevistyla* and *M. fusiformis* individuals in the overlapping region, the mean distance to the nearest conspecific neighbour was 35.9 m (range: 0.6 to 129.7 m) and 8.2 m (range: 0.7 to 24.2 m), respectively; and the mean distance between the nearest heterospecific neighbours was 54.1 m (range: 18.4 to 109.1 m). Based on my analyses of habitat isolation, as the radius of the virtual plots increases, the fraction of heterospecific plots also increases, and $RI_{habitat}$ decreases (from 1.0 at 10 m to 0.25 at 50 m for *M. brevistyla*, and from 0.98 at 10 m to 0.0 at 50 m for *M. fusiformis*; Fig. 2-4).

Phenological isolation

In 2014, there were significant differences between *M. brevistyla* and *M. fusiformis* in timing of flowering onset (mean day of year ± SE, *M. brevistyla* = 158.2 ± 0.3; *M. fusiformis* = 163.5 ± 0.3, $N = 42$ *M. brevistyla* and 50 *M. fusiformis* individuals) and peak flowering phenology (*M. brevistyla* = 162.5 ± 0.5 day of year; *M. fusiformis* = 165.9 ± 0.6 day of year; Wilcoxon, onset: $W = 149$, $P < 0.001$; peak: $W = 620$, $P < 0.001$). However, based on the flowering durations of both species (Fig. 2-2), there was a phenological overlap of 13 days (from 11 June and 24 June), and during this period, approximately 59.9% and 97.6%, respectively, of *M. brevistyla* and *M. fusiformis* flowers were open, leading to $RI_{phenology} = 0.40$ for *M. brevistyla* and 0.024 for *M. fusiformis*.

Visitor assemblages

Insect visitor assemblages, at least at the level of taxonomic resolution I considered, were not statistically different between *M. brevistyla* and *M. fusiformis* at Kebler Pass (perMANOVA, $R^2 = 0.076$, $F = 0.50$, $P = 0.80$; PERMDISP, $F = 0.91$, $P = 0.35$; Fig. 2-3B)—leading to $RI_{pollinators} = 0.20$ and 0.10 for *M. brevistyla* and *M. fusiformis*, respectively.

Floral isolation

In the mixed-array experiment, a higher proportion of conspecific than heterospecific recipient plants received dye from the central plant (Fig. 2-5A). The proportion of plants receiving dye was influenced by type of dye deposition (heterospecific versus conspecific) but not by recipient species (zero-inflated binomial regression, deposition: $z = -2.02$, $P = 0.043$; species: $z = -0.92$, $P = 0.36$). Overall, RI_{floral} was 0.47 for *M. brevistyla* and 0.23 for *M. fusiformis*.

Morphological and colour differences

Most traits were significantly different among the parental species and the putative hybrids (Fig. 2-6, Table 2-4). *M. brevistyla* had significantly shorter floral tubes and stigmas (Fig. 2-6G and H, Table 2-4), and *M. fusiformis* had significantly longer stigmas and anthers (Fig. 2-6H and I, Table 2-4). The putative hybrids exhibited intermediate floral traits for stigma and anther height, and they also had significantly smaller leaf area than either parental species (Fig. 2-6B, Table 2-4). Furthermore, morphologies of the parental species measured in two separate years outside (2014) and inside (2016) the overlapping region were significantly different, suggesting an effect of year or site on the morphologies. Specifically, between inside and outside the overlapping region, *M. brevistyla* differed in plant height, floral limb width and length, and

floral tube width (Figs. 2-6A, D, E, and F), while *M. fusiformis* differed in plant height, floral tube length, and anther height (Figs. 2-6A, G, and I). Corollas of both *Mertensia* species were predominantly blue (dominant wavelengths: *M. brevistyla*: 471.9 ± 2.3 nm; *M. fusiformis*: 479.6 ± 3.3; $N = 45$ *M. brevistyla* and 57 *M. fusiformis* individuals), and the dominant wavelength did not significantly differ between species (Wilcoxon: $W = 1554.5$, $P = 0.067$; Fig. 2-7).

Post-pollination experiment

In both *Mertensia* species, flowering stems that received conspecific pollen yielded a higher seed set than stems that received heterospecific pollen (Fig. 2-5B). Overall, there was a significant effect of cross type on seed set in both species (GLMM, species: $\chi^2 = 3.5$, $P = 0.060$, type: $\chi^2 = 65.4$, $P < 0.001$, species × type: $\chi^2 = 2.3$, $P = 0.13$), leading to $RI_{post\text{-}pollination} = 0.55$ for *M. brevistyla* and 0.40 for *M. fusiformis*. Stems not receiving any pollen did not yield any seeds, indicating that neither species is capable of autogamous selfing.

Pollen viability

Pollen viability of *M. brevistyla* was slightly higher (mean ± SE, 16.7 ± 4.7%, $N = 15$ plants) than that of putative hybrids (10.7 ± 1.7%, $N = 13$ plants) and *M. fusiformis* (10.2 ± 3.2%, $N = 15$ plants)—resulting in $RI_{viability} = 0.36$ for *M. brevistyla* and < 0 for *M. fusiformis*. However, pollen viability did not differ significantly among the three plant ecotypes (GLMM, type: $\chi^2 = 1.6$, $P = 0.44$).

Discussion

Reproductive isolating barriers were weak overall for *M. brevistyla* and *M. fusiformis* at Kebler Pass, but they were stronger for *M. brevistyla* than for *M. fusiformis*. First, there were significant differences in the vegetation assemblages associated with each parental species'

habitat and the overlapping region where putative hybrids were found, but close proximity between parental individuals in the overlapping region led to weak habitat isolation (at a 50 m scale, *M. brevistyla*: 0.25; *M. fusiformis*: 0.0). There were also vegetative and reproductive trait differences among *M. brevistyla*, *M. fusiformis*, and putative hybrids, but the parental species shared similar flowering phenology (phenological isolation: *M. brevistyla*: 0.40; *M. fusiformis*: 0.024) and pollinator assemblages (pollinator isolation: *M. brevistyla*: 0.20; *M. fusiformis*: 0.10). Pollinators were more likely to transfer pollen analogues between conspecific plants than between heterospecific plants in a mixed array (floral isolation: *M. brevistyla*: 0.47; *M. fusiformis*: 0.23), suggesting that pollinators could distinguish the two species. Plants receiving conspecific pollen yielded higher seed set than those receiving heterospecific pollen (post-pollination isolation: *M. brevistyla*: 0.55; *M. fusiformis*: 0.40), suggesting some post-pollination processes preventing hybrid seed formation. However, pollen of the putative hybrids germinated at similar rates to that of the two parental species, resulting in a weak pollen viability barrier (*M. brevistyla*: 0.36; *M. fusiformis*: 0.0). Below, I discuss each barrier and how it influences reproductive isolation between *M. brevistyla* and *M. fusiformis* at Kebler Pass.

Habitat and phenological isolation barriers

Pre-zygotic barriers are generally expected to be more important than post-zygotic barriers, and among the pre-zygotic barriers, spatial and temporal separation between species forms the first barrier to gene flow (Losos and Glor 2003, Sobel 2014, Baack et al. 2015). My study did not investigate which factors limit the broad-scale geographic distributions of both *Mertensia* species, or if Kebler Pass represents a site of initial divergence or secondary contact. Nonetheless, I detected a significant difference in the local plant communities of *M. brevistyla* and *M. fusiformis* habitats at Kebler Pass, suggesting the existence of abiotic or biotic conditions

that allow different vegetation to establish and persist in each environment. The *M. fusiformis* population is located on a south-facing slope that is likely warmer and drier than the east-facing slope where the *M. brevistyla* population is found. Previous studies have found several environmental gradients that can exert divergent selection on different species and maintain species boundaries (Goulson 2009, Abbott 2017, Rajakaruna 2018). Furthermore, adaptation to specific edaphic conditions can reduce fitness of parental and/or hybrid plants in non-native soil and maintain hybrid fitness in intermediate habitats (McNeilly and Antonovics 1968, Burge et al. 2013, Melo et al. 2014, Wang et al. 2015, Abbott 2017). However, my measurement of habitat isolation is limited to a very small spatial scale at Kebler Pass (< 50 m), and this result should be interpreted with caution until reproductive isolation is examined across geographic ranges of both species (see Future Directions).

Flowering phenology can act as a strong pre-zygotic isolating barrier (McNeilly and Antonovics 1968, Antonovics 2006, Martin and Willis 2007, Briscoe Runquist et al. 2014, Melo et al. 2014). Both *Mertensia* species are spring ephemerals that share similar flowering phenology, which is likely constrained by the short growing seasons in montane environments. Based on my surveys in 2014, substantial phenological overlap suggests a strong possibility for heterospecific pollen transfer between *M. brevistyla* and *M. fusiformis*—particularly from the former to the latter. In years with early snowmelt, onset and peak flowering are likely to shift earlier and the flowering season is likely to be prolonged, with the opposite effects expected in late-snowmelt years (see Forrest and Thomson 2010). It is unclear whether interannual shifts in phenology would affect interspecific phenological overlap or whether phenologies of both species would shift in parallel. Overall, characterizing these pre-pollination barriers remains important given that geographical ranges and flowering phenology can shift with changing

climatic conditions and influence the amount of spatiotemporal overlap between related species (Duffy and Jacquemyn 2019).

Pollinator-mediated isolation barriers

Many studies have emphasized the importance of pollinators in mediating reproductive isolation between angiosperms with specialized morphologies (Fulton and Hodges 1999, Kay 2006, Schiestl and Schlüter 2009). However, pollinators are expected to play a lesser role for species with generalized pollination systems (Waser 1998). *Mertensia brevistyla* and *M. fusiformis* have generalized but distinct floral morphologies—*M. brevistyla* has short floral tubes and *M. fusiformis* has longer reproductive structures (see Fig. 2-1), suggesting a potential for floral divergence and reproductive isolation influenced by pollinators. However, both *Mertensia* species shared similar assemblages of floral visitors, resulting in weak pollinator isolation. When I examined dye (as a proxy for pollen) movement in mixed-species arrays, floral visitors consistently exhibited a preference for conspecific individuals, indicating that they were able to detect some interspecific differences in morphological or other traits. Although I did not detect significant differences in corolla colour between the two species, pollinators may have been sensitive to the marginal difference in dominant wavelength I observed, or to other attributes of the colour spectra. Pollinators may also have detected differences in floral odour, although I did not investigate that trait, and these flowers are not fragrant to a human nose. Regardless, interspecies visits still comprised approximately 35% and 43% of all visits involving *M. brevistyla* and *M. fusiformis*, respectively. This suggests that floral isolation (combining mechanical and ethological isolation) is stronger than other pre-pollination barriers but remains incomplete between the two *Mertensia* species. Overall, pollinators only contribute weakly to reproductive isolation in *Mertensia*, supporting other studies that have examined and found weak

pre-zygotic barriers in generalist angiosperms (e.g., in the genera *Chamaecrista*, *Gelsemium*, and *Helianthus*; Costa et al. 2007, Pascarella 2007, Ostevik et al. 2016).

Post-pollination barriers

For related species with weak pre-pollination barriers, post-pollination and post-zygotic barriers (e.g., pollen–pistil interactions, pollen-tube competition, and gamete incompatibility) may be critical in preventing interspecific fertilization and hybridization (Coyne and Orr 2004). This effect may be stronger in systems associated with generalized pollination because heterospecific pollen transfer by shared pollinators is more likely to take place (Fang and Huang 2013). For example, Scopece et al. (2008) found stronger post- than pre-zygotic barriers in food-deceptive orchids (that are more generalized), and the opposite trend in sexually-deceptive orchids (that are more specialized). Furthermore, Wang et al. (2015) documented strong post-zygotic barriers in two closely related *Arnebia* (Boraginaceae) species, plants with morphologically similar flowers to those of *Mertensia*. Indeed, I found that hand-pollination with conspecific pollen yielded significantly higher seed set than pollination with heterospecific pollen in both *M. brevistyla* and *M. fusiformis*. This may be due to conspecific pollen having greater adherence to the stigmatic surface, greater germination rate, or faster pollen tube growth than heterospecific pollen (reviewed by Howard 1999). Interspecific differences in pollen tube length and style length can also promote asymmetric reproductive isolation, as pollen from long-styled plants can grow and reach the ovules of short-styled plants, but not the opposite (Yost and Kay 2009, Brothers and Delph 2017). Following this reasoning, I would have expected differences in style length between the two *Mertensia* species to contribute to stronger reproduction isolation in the long-styled *M. fusiformis* than in the short-styled *M. brevistyla*. Contrary to this expectation, however, pollen from the short-styled *M. brevistyla* was more

successful in fertilizing *M. fusiformis* ovules (11.3% seed set) than that of long-styled *M. fusiformis* in fertilizing *M. brevistyla* ovules (5.7% seed set) in the hand-pollination experiment. Although reproductive isolating barriers following interspecific pollination and fertilization were not particularly strong between *M. brevistyla* and *M. fusiformis*, post-zygotic barriers in certain angiosperms with generalized pollination are very strong (e.g., genera *Chamaecrista* and *Helianthus*; see Lowry et al. 2008).

Future directions

Based on the barriers I examined, reproductive isolation is not complete but remains important in limiting gene flow between *M. brevistyla* and *M. fusiformis*. The relatively recent divergence and the weak reproductive barriers between the two *Mertensia* species are consistent with comparative studies that have found a positive relationship between divergence time or genetic distance and reproductive isolation across different taxa (Coyne and Orr 1989, Coyne and Orr 1997, Funk et al. 2006, Rabosky 2016). However, there are components of my study system that require further investigation. First, the small spatial scale of my study limits my ability to determine the strength of ecogeographical isolation across the ranges of these two *Mertensia* species (not to mention other *Mertensia* species that co-occur in parts of their ranges). Despite both species being documented in Colorado and Utah (also in Nevada for *M. brevistyla*; see Nazaire and Hufford 2014), there is still little knowledge about the geographical distribution of both species, which should be a primary objective in a future study. Second, in addition to using morphological characters to identity parental species and putative hybrids, future studies should use molecular genetic assays to confirm identities of parentals and hybrids. However, incomplete lineage sorting and possible polyploidy in *Mertensia* pose significant challenges in the genetic assessment of hybrids and even the taxonomic circumscriptions (Nazaire and Hufford

2014). Another challenge to carrying out these studies is that *Mertensia* seeds have an extremely low germination rate (~1.5% in the lab, J.R.K. Forrest, unpublished data; see also Pelton 1961) and take at least two years to flower (J.R.K. Forrest, unpublished data). Examination of other plants with generalized pollination systems, ideally ones that can more readily be cultivated, may allow us to broaden our knowledge of the relative importance of pre- and post-pollination barriers influencing speciation in angiosperms.

Table 2-1: Equations quantifying reproductive isolating barriers between *Mertensia brevistyla* and *M. fusiformis*.

Barriers	Equation
Habitat	1 – [number of heterospecific plots/number of heterospecific and conspecific plots combined]
Phenology	1 – [number of open flowers counted during the overlapping days/number of open flowers counted during the entire flowering season]
Pollinators	1 – [proportion of shared visitor taxa/proportion number of total visitor taxa]
Floral	1 – [proportion of plants receiving interspecific dye deposition/proportion of plants receiving intraspecific dye deposition]
Post-pollination	1 – [mean seed set from interspecific crosses/mean seed set from intraspecific crosses]
Viability	1 – [pollen germinability of putative hybrids/pollen germinability of *M. brevistyla* or *M. fusiformis*]

Table 2-2: Number of floral visitors observed visiting *M. brevistyla* and *M. fusiformis* flowers at Kebler Pass between 2014 and 2017. Below each year, the first number represents the number of observation hours, and the second number represents the number of different observers.

	M. brevistyla					*M. fusiformis*				
	2014	2015	2016	2017		2014	2015	2016	2017	
Visitor taxa	(12, 2)	(11, 3)	(7, 1)	(0.25, 1)	TOTAL	(12, 2)	(12, 3)	(4, 1)	(1.75, 1)	TOTAL
Bombus	0	9	0	1	10	0	13	2	1	16
Halictidae	2	8	6	16	32	11	16	1	4	32
Megachilidae	3	7	1	8	18	1	11	1	0	13
Syrphidae	3	4	1	1	9	1	8	1	0	10
Dipteran	1	11	0	0	12	0	2	0	0	2

Table 2-3: Indicator values of plant species found in the habitats of *M. brevistyla* and *M. fusiformis* and the overlapping region. Indicator value ranges from 0 to 1, with greater values indicating that the species comprises a larger proportion of the community in that habitat. Significant indicator species are bolded ($P < 0.05$).

M. brevistyla habitat			Overlapping region			*M. fusiformis* habitat		
Species	Indval	*P*	Species	Indval	*P*	Species	Indval	*P*
Elymus trachycaulus	0.429	0.059	***Salix* sp.**	**0.500**	**0.025**	***Helianthella* sp.**	**0.667**	**0.004**
Potentilla fruticosa	0.381	0.12	***Bistorta* sp.**	**0.417**	**0.039**	***Thalictrum fendleri***	**0.667**	**0.002**
Erythronium grandiflorum	0.375	0.066	*Trifolium* sp.	0.333	0.12	***Lathyrus lanszwertii***	**0.533**	**0.013**
Claytonia lanceolata	0.333	0.11	*Carex* spp.	0.250	0.30	***Carex egglestonii***	**0.500**	**0.022**
Fragaria sp.	0.333	0.10	*Caltha leptosepala*	0.222	0.32	*Senecio atratus*	0.410	0.067
Hymenoxys hoopesii	0.333	0.30	*Cymopterus lemmonii*	0.222	0.34	*Ligusticum porteri*	0.356	0.18
Mertensia brevistyla	0.333	0.11	*Mertensia ciliata*	0.222	0.31	*Bromus carinatus*	0.333	0.13
Taraxacum officinale	0.250	0.40	*Valeriana edulis*	0.167	0.36	*Osmorhiza occidentalis*	0.333	0.16
Allium sp.	0.190	0.48	*Deschampsia cespitosa*	0.150	0.77	*Vicia americana*	0.333	0.111
Ranunculus alismifolius	0.190	0.51	*Carex hoodii*	0.0833	1.0	*Delphinium nuttallianum*	0.222	0.398
Veratrum californicum	0.190	0.50	*Carex pellita*	0.0833	1.0	*Androsace septentrionalis*	0.167	0.49
Achillea millefolium	0.167	0.74	*Equisetum* sp.	0.0833	1.0	*Erythranthe guttata*	0.167	0.476
Chrysothamnus viscidiflorus	0.167	0.48	*Erigeron* sp.	0.0833	1.0	*Geranium richardsonii*	0.167	0.502
Collomia sp.	0.167	0.51	*Juncus filiformis*	0.0833	1.0	*Linum lewisii*	0.167	0.525
Danthonia intermedia	0.167	0.53	*Phleum pratense*	0.0833	1.0	*Trisetum wolfii*	0.167	0.513
Pedicularis bracteosa	0.167	0.52	Poaceae	0.0833	1.0	*Aconitum columbianum*	0.111	0.869
Phleum alpinum	0.167	0.74	*Sedum* sp.	0.0833	1.0			
Melica sp.ectabilis	0.148	0.89	*Senecio* sp.	0.0833	0.79			
Viola sp.	0.148	0.81						
Antennaria sp.	0.111	0.88						
Cerastium arvense	0.111	0.84						
Lewisia pygmaea	0.111	0.86						
Castilleja sulphurea	0.0667	1.0						

Table 2-4: Vegetative and reproductive traits (mean ± SE) of the five ecotypes: *M. brevistyla* (inside and outside the overlapping region), *M. fusiformis* (inside and outside the overlapping region), and putative hybrids. These values are also represented graphically in Figure 2. Superscript letters indicate significant differences among the five ecotypes based on parametric analyses of variance (ANOVA) or non-parametric Kruskal-Wallis tests (depending on the normality and homoscedasticity of the variables), followed by post-hoc pairwise contrasts using the function TukeyHSD in the stats package (for ANOVA) and the function dunnTest in the FSA package (for Kruskal-Wallis tests; Ogle 2017).

	Ecotypes				
Traits	*M. brevistyla* outside (*N* = 51 plants)	*M. brevistyla* inside (*N* = 15 plants)	Putative hybrids (*N* = 15 plants)	*M. fusiformis* inside (*N* = 15 plants)	*M. fusiformis* outside (*N* = 57 plants)
Plant height (cm)	10.7 ± 0.6 ^{A}	13.3 ± 0.9 ^{B}	9.9 ± 0.7 ^{A}	14.2 ± 0.8 ^{B}	10.4 ± 0.4 ^{A}
Leaf area (cm^2)	3.8 ± 0.3 ^{B}	2.9 ± 0.2 ^{B}	1.9 ± 0.2 ^{A}	3.6 ± 0.3 ^{B,C}	5.1 ± 0.3 ^{C}
Number of flowers	30.1 ± 3.3 ^{A}	36.3 ± 4.1 ^{A,B}	41.1 ± 8.1 ^{A,B}	43.8 ± 6.6 ^{A,B}	52.3 ± 5.3 ^{B}
Floral limb width (cm)	0.90 ± 0.01 ^{D}	0.75 ± 0.03 ^{C}	0.68 ± 0.02 ^{B,C}	0.58 ± 0.02 ^{A}	0.61 ± 0.01 ^{A,B}
Floral limb length (cm)	0.62 ± 0.01 ^{B}	0.55 ± 0.02 ^{A}	0.55 ± 0.02 ^{A}	0.54 ± 0.01 ^{A}	0.57 ± 0.01 ^{A}
Floral tube width (cm)	0.19 ± 0.003 ^{B}	0.17 ± 0.004 ^{A}	0.19 ± 0.004 ^{A,B}	0.19 ± 0.004 ^{A,B}	0.19 ± 0.003 ^{A,B}
Floral tube length (cm)	0.32 ± 0.01 ^{A}	0.31 ± 0.01 ^{A}	0.38 ± 0.01 ^{B}	0.42 ± 0.01 ^{B}	0.48 ± 0.01 ^{C}
Stigma height (cm)	0.22 ± 0.005 ^{A}	0.21 ± 0.01 ^{A}	0.34 ± 0.02 ^{B}	0.49 ± 0.02 ^{C}	0.53 ± 0.01 ^{C}
Anther height (cm)	0.32 ± 0.005 ^{A}	0.30 ± 0.01 ^{A}	0.45 ± 0.01 ^{B}	0.58 ± 0.01 ^{C}	0.68 ± 0.01 ^{D}

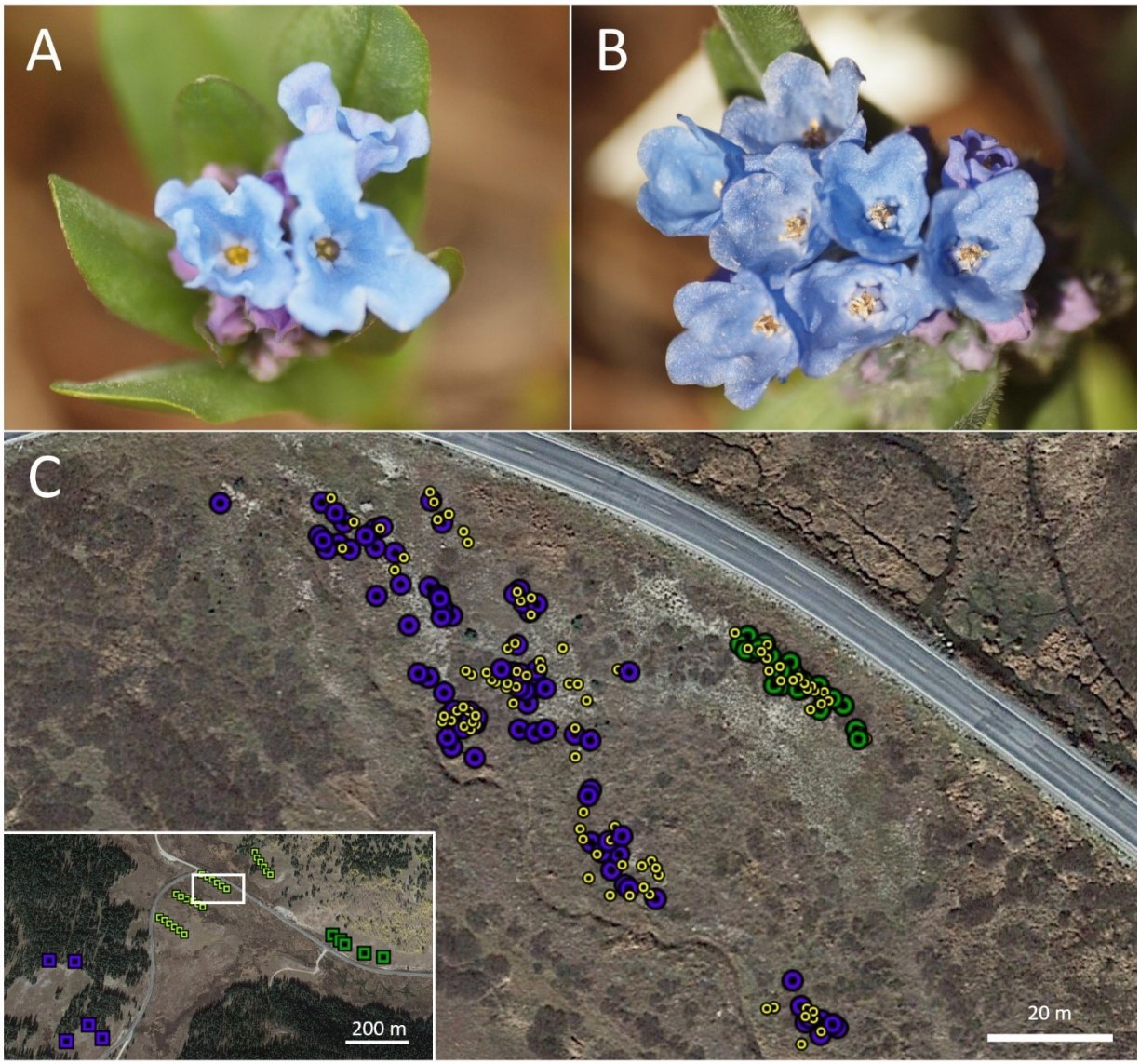

Figure 2-1: Panels A and B show close-up images of *M. brevistyla* (with inserted reproductive structures inside the corolla tube) and *M. fusiformis* (with exserted reproductive structures) flowers, respectively. Panel C shows the distribution of *M. brevistyla* (large blue circles), *M. fusiformis* (large green circles), and putative hybrids (small yellow circles) inside the overlapping region. Inset map shows the larger landscape at Kebler Pass. The large blue and green squares represent the sites where morphological measurements of *M. brevistyla* and *M. fusiformis* individuals took place, respectively (see Methods). The small yellow squares represent plots along transects where vegetation surveys took place (see Methods).

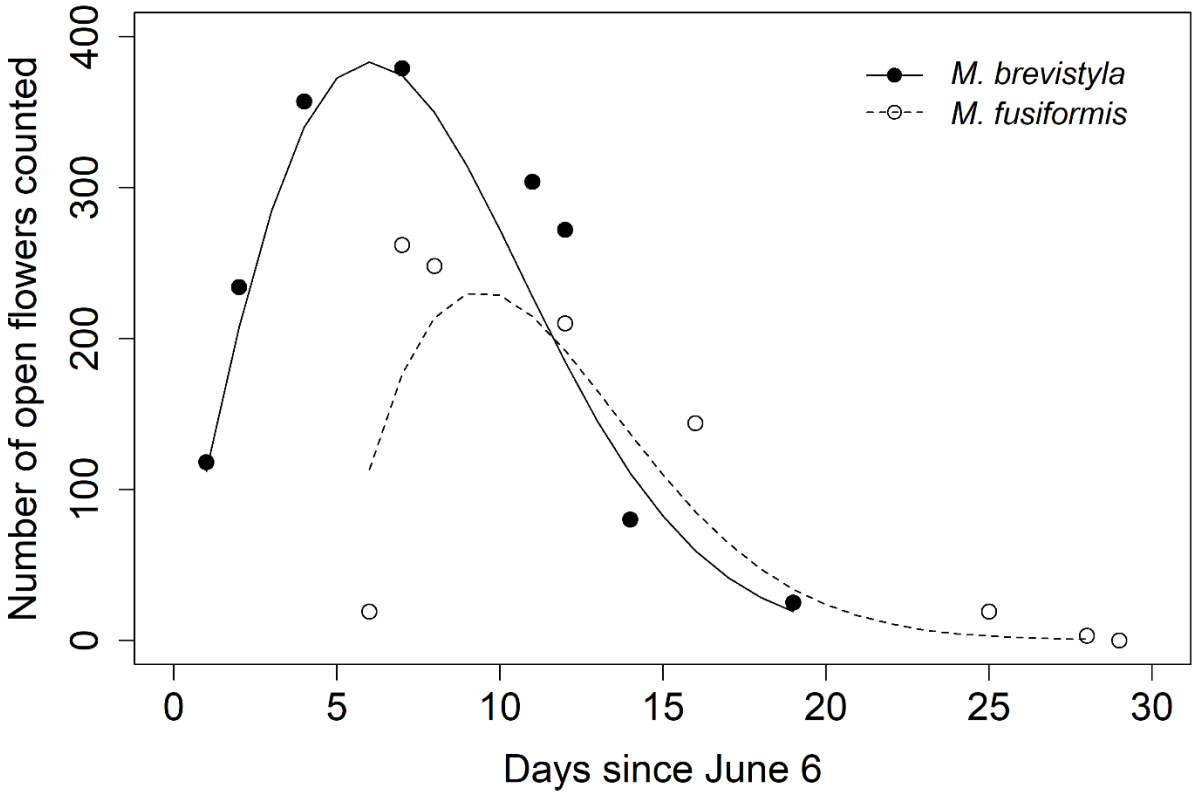

Figure 2-2: Number of open flowers counted between 6 June and 5 July 2014 for 42 *M. brevistyla* and 50 *M. fusiformis* plants. The curves are fitted Weibull distribution using the nls2 function in the nls2 package (Grothendieck 2013). The Weibull distribution for *M. brevistyla* phenology has shape parameter = 1.95 and scale parameter = 8.70; for *M. fusiformis*, the shape parameter = 1.75 and scale parameter = 7.18.

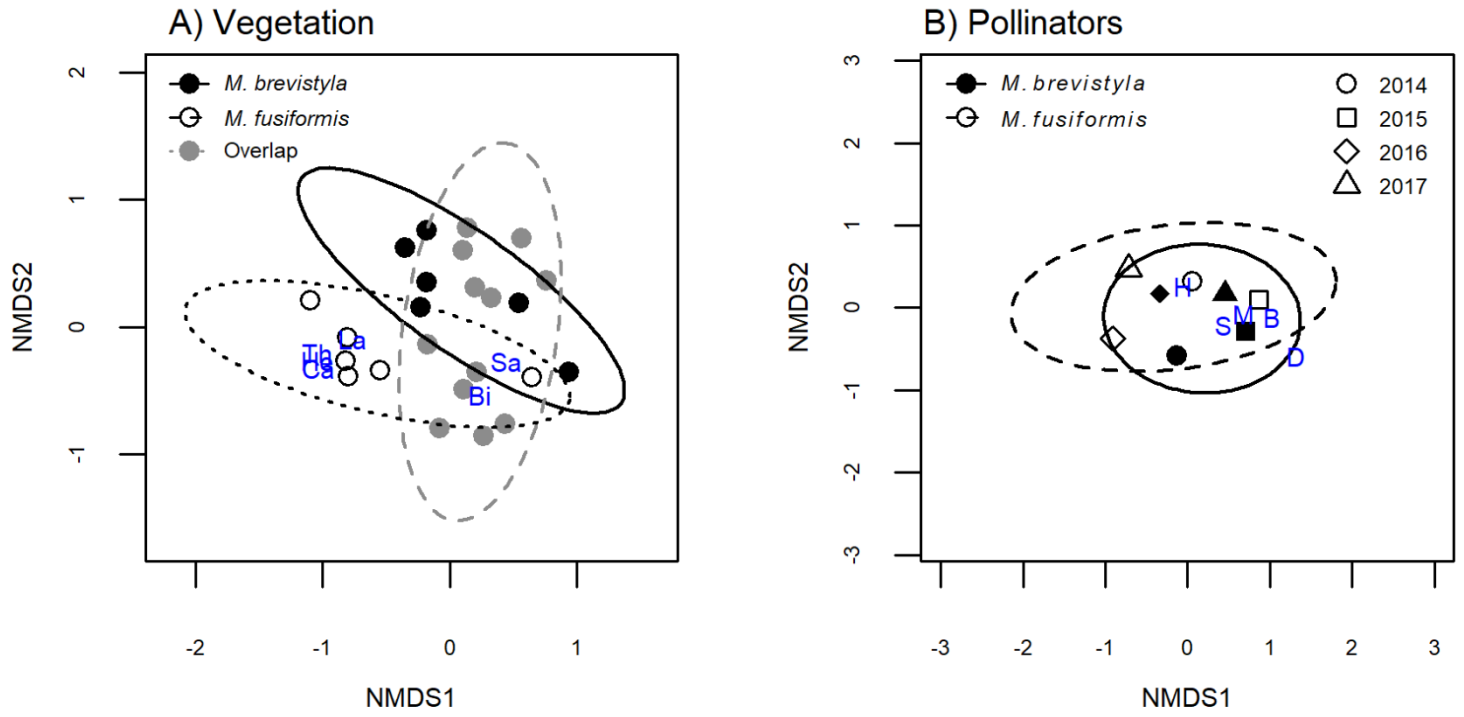

Figure 2-3: Nonmetric multidimensional scaling (NMDS) of (A) vegetation community (stress = 0.19) among *M. brevistyla* habitat, *M. fusiformis* habitat, and overlapping region and of (B) pollinator assemblages (stress < 0.01) of *M. brevistyla* and *M. fusiformis* at Kebler Pass in 2014–2017. In panel A, the 2-letter codes represent the significant indicator species documented in each habitat (*M. brevistyla*: none; *M. fusiformis*: *Carex egglestonii* [Ca], *Helianthella* sp. [He], *Lathyrus lanszwertii* [La], and *Thalictrum fendleri* [Th]; overlapping region: *Bistorta* sp. [Bi] and *Salix* sp. [Sa]). Refer to Table 2-3 for a complete list of indicator species found in each habitat. The circles represent the sampling quadrats. In panel B, the letters represent pollinator taxa: B = bumblebees; H = halictid bees; M = megachilid bees; S = syrphid flies and D = other Diptera. NMDS is based on Bray–Curtis similarities, and the ellipses indicate a 95% confidence interval.

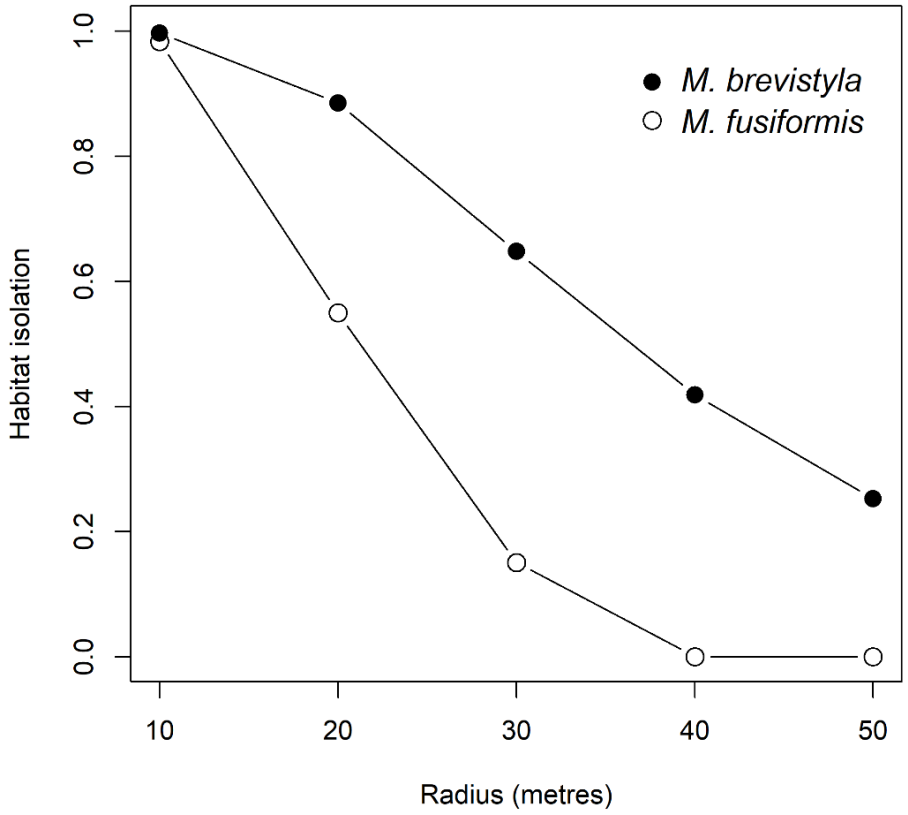

Figure 2-4: Habitat isolation for *M. brevistyla* and *M. fusiformis* across five spatial scales (i.e., virtual plots with different radii). Using coordinates of plants in the field, I generated 10000 virtual plots and calculated number of plots containing both species divided by the number of plots containing both or just one species. I then subtracted this value from one to determine habitat isolation.

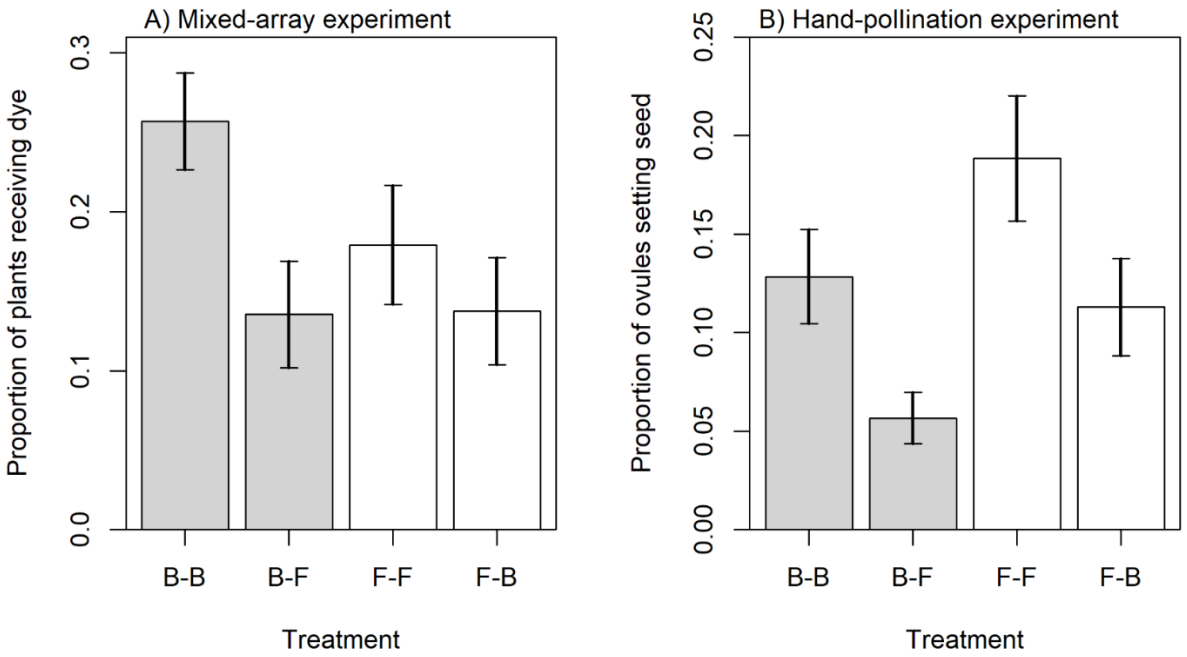

Figure 2-5: Barplots showing differences among treatments in (A) proportion of plants receiving dye in the mixed-array experiment and (B) seed set in hand-pollination experiment. In both panels, B-B and B-F represent conspecific and heterospecific interactions in *M. brevistyla*, respectively; and F-F and F-B represent conspecific and heterospecific interactions in *M. fusiformis*, respectively. Error bars are ± 1 SE.

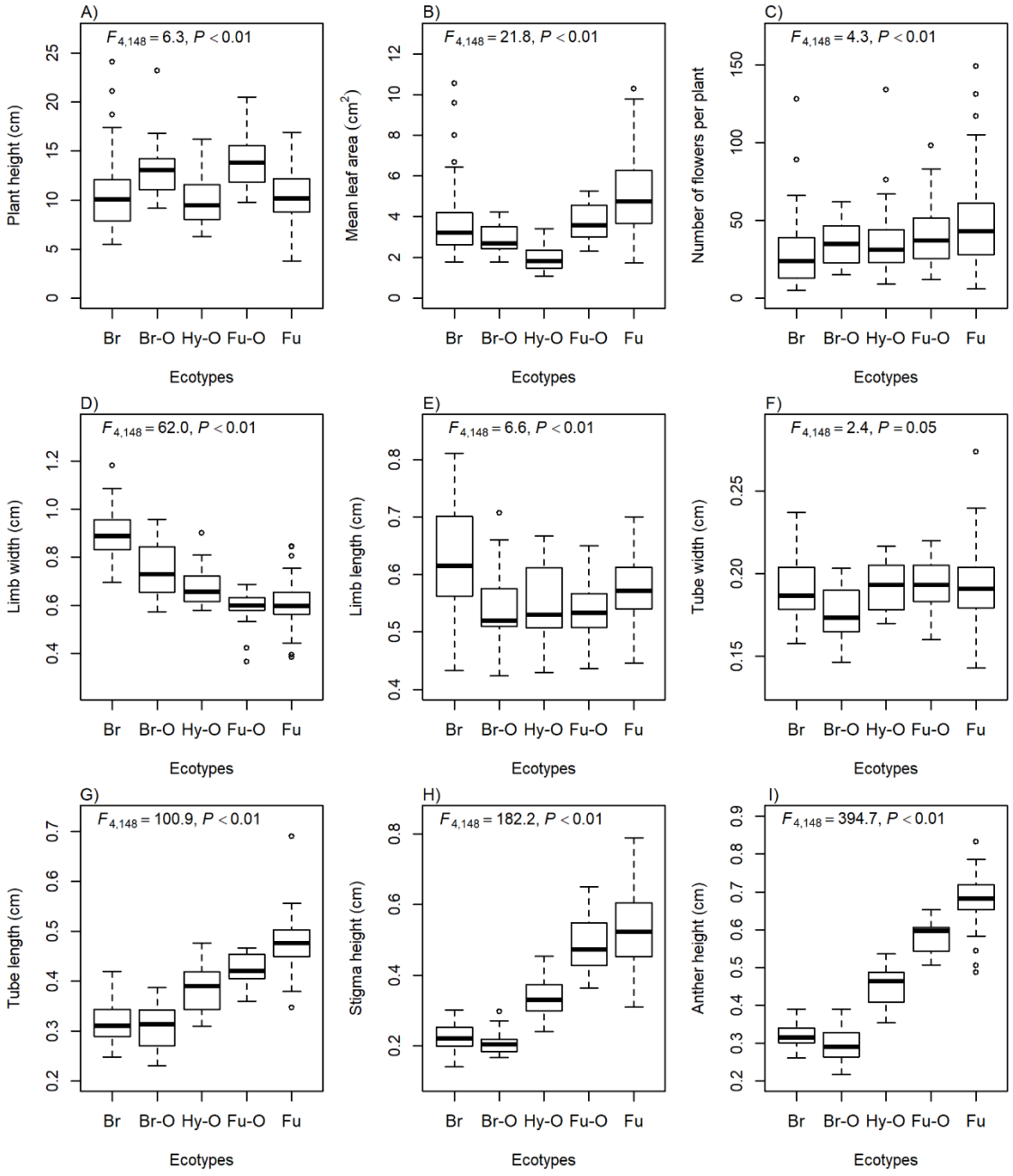

Figure 2-6: Boxplots showing morphological differences among the five ecotypes: *M. brevistyla* in its natural habitat (Br), *M. brevistyla* inside the overlapping region (Br-O), putative hybrids (Hy-O), *M. fusiformis* inside the overlapping region (Fu-O), and *M. fusiformis* in its natural habitat (Fu). The traits examined are: (A) plant height, (B) mean leaf area, (C) number of flowers per plant, (D) floral limb width, (E) floral limb length, (F) floral tube width, (G) floral tube length, (H) stigma height, and (I) anther height. Test statistics from MANOVA are provided in each panel (see Methods). These data are reported numerically in Table 2-4.

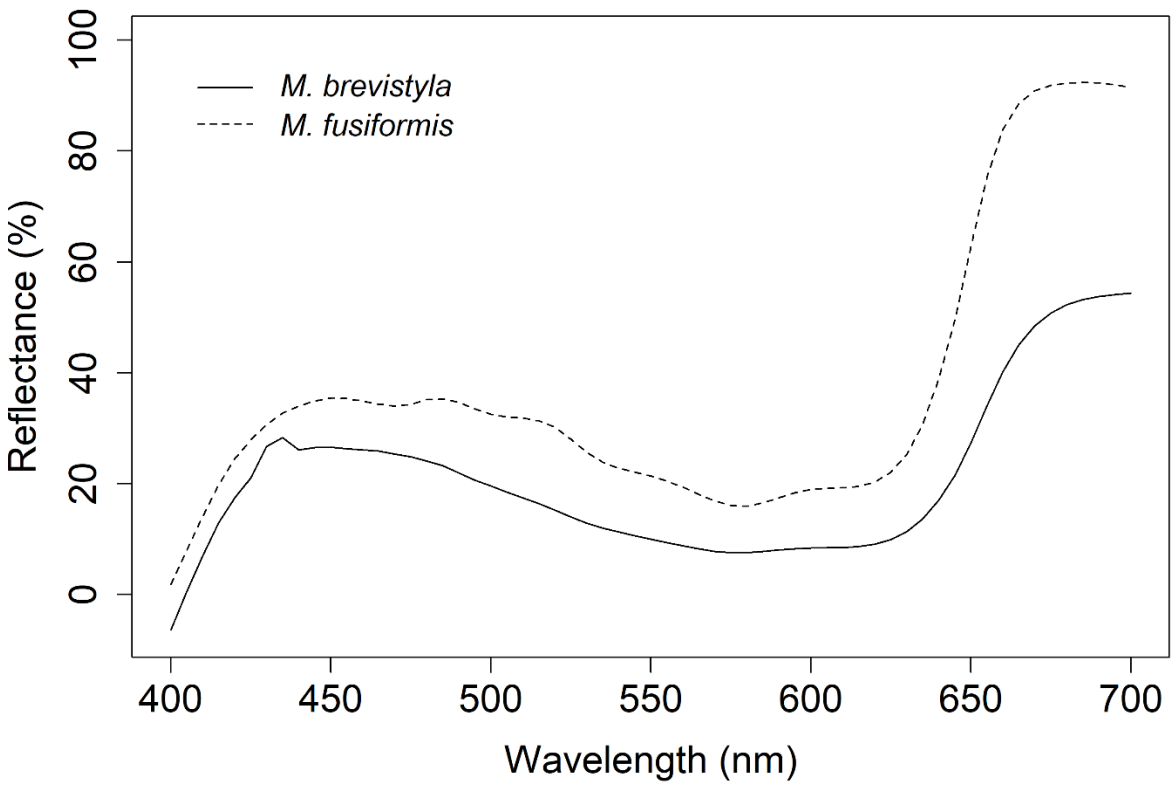

Figure 2-7: Mean corolla reflectance spectra of *M. brevistyla* (*N* = 45 individuals) and *M. fusiformis* (*N* = 57 individuals).

Chapter 3

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

This chapter formed the basis of the following publication:

Lin, S.-Y. and J. R. K. Forrest. 2019. The function of floral orientation in bluebells: interactions with pollinators and rain in two species of *Mertensia* (Boraginaceae). *Journal of Plant Ecology* 12: 113–123.

Abstract

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. I measured and compared floral orientation and visitor assemblages between *M. brevistyla* and *M. fusiformis* populations in western Colorado, USA. I 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. I examined pollinator- and rain-mediated selection on floral orientation by manipulating orientation, conducting supplemental pollinations, applying watering treatments, and measuring subsequent seed set. I also experimentally tested the likelihood of rain contact with anthers, and the effect of rainwater on pollen germinability. *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. I 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.

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 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 (Holtsford and Ellstrand 1992, Elle and Hare 2002), 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 Chen et al. 2013, Aronne et al. 2015), 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 versus effectiveness) and rain (influencing male versus 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, I 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 I 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. I 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), I 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, I expect stronger selection from rain for pendant floral orientation in *M. fusiformis* than *M. brevistyla*, given the exserted floral organs in *M. fusiformis*.

I tested these expectations using several field observations and experiments. First, I measured and compared floral orientation and visitor assemblages between *M. brevistyla* and *M. fusiformis.* Second, I 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, I 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, I 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, I 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, my results help to explain the functional significance of floral orientation in *M. brevistyla* and *M. fusiformis*, while still leaving some questions unanswered.

Materials 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 my study area near Crested Butte, CO. Both species occur at my Kebler Pass site (KP; 38°51'9.26"N, 107°6'4.72"W); only *M. fusiformis* occurs at my Rocky Mountain Biological Laboratory site (RMBL; 38°57'28.66"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. 3-1). The

flowers are clustered on a determinate cymose inflorescence. At KP, measured individuals were similar in plant height above ground (mean ± 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), I 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 straight edge of the protractor (Fig. 3-1). I 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. 3-1). I 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*, I observed any insects visiting *Mertensia* flowers on warm, calm, dry days

during the flowering season between 2014 and 2017 at KP and RMBL. I identified these floral visitors as: bumblebees (*Bombus* spp., Apidae), megachilid bees (Megachilidae), halictid bees (Halictidae), syrphid flies (Syrphidae), or other flies (Diptera). I pooled my observations with data from Forrest et al. (2011) on *M. fusiformis* visitors at KP and RMBL sites (Table 3-1). I performed a non-metric multidimensional scaling (NMDS) on proportion of observed individual visitors per site and year using the 'metaMDS' function in the 'vegan' package (Oksanen et al. 2018). I then conducted a permutational multivariate analysis of variance (perMANOVA with 10000 permutations) using the 'adonis' function, accompanied by a permutational multivariate homogeneity of group dispersions (PERMDISP with 10000 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, I 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. I 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, I 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), I 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, I spent 7 hours in 2016 observing pollinator visits to *M. brevistyla* and *M. fusiformis* plants. For each flower visitor, I 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). I examined floral orientation of the visited and unvisited flowering stems for each species separately using linear mixed-effects models, in which I considered stem status (visited or unvisited) as the fixed factor, and individual pollinator as the random factor. I 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, I 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). I manipulated floral orientation by fixing one flowering stem per plant around a bent and looped metal wire inserted into the ground. In 2014, I 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 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, I compared seed set among the three floral orientation treatments and between the two pollination treatments (open-pollinated versus pollen-supplemented) by fitting a generalized linear model (GLM) with a binomial error distribution using the 'stats' package. In each model, I considered floral orientation, pollination treatment, and interaction between orientation and pollination treatments as the predictor variables. I tested the significance of the predictor variables using likelihood-ratio χ^2 statistics (Morrell 1998). I 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, I conducted the same experiment as above but I used only 15 plants per orientation per species and I did not conduct pollen supplementation because pollinator-mediated selection was not detected in 2014. For each species, I 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, I considered study year, orientation, and interaction between year and floral orientation as predictor variables. I 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, I set up four 1.0 × 1.0 m plots in natural populations of each species in 2016. I 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 I 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, I 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. I 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, I selected 15 plants of each species and removed two flowers from each. I 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, I 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, I 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. I 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, I 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. I compared proportion of germinating pollen grains among treatments using a generalized linear mixed-effects model with a binomial error distribution. I considered treatment, species, flower, field of view, and interaction between treatment and species as fixed factors; plant was treated as a random factor. I 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 3-1). 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. 3-2).

Floral visitor preferences

Based on my 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).

I 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 (Fig. 3-3).

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. 3-4a), 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. 3-4b). 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% versus 14% mean seed set

in the pendant orientation and 18% versus 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% versus 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: $F_{2,76} = 2.1$, $P = 0.12$).

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. 3-5a). 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. 3-5b). On average, mean *M. fusiformis* seed set 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. 3-6a). Contrary to my 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. 3-6b).

Water accumulation in corolla tube

Water accumulation inside corolla tubes occurred in 3% and 10% of *M. brevistyla* and *M. fusiformis* flowers examined, 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. 3-1g), 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, I 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. 3-7). Pollen germination rates were generally higher in *M. brevistyla* than in *M. fusiformis*, except in the treatment where anthers were exposed to water for one hour first. Pollen from both species had a significantly reduced germination rate when exposed to air or water for one 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 versus exposure to air: $z = 7.5$, $P < 0.01$; control versus 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. I 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. My 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, my 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 (Fulton and Hodges 1999, Wang et al. 2014a), while other studies find no difference in visitor attraction between floral orientations (Tadey and Aizen 2001, Huang et al. 2002, Ushimaru and Hyodo 2005, Wang et al. 2010, Chen et al. 2013). In my field observations of *Mertensia* spp., I found no consistent floral visitor preference for a particular orientation; nor was there a difference in preference between the two plant species (although my 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, I 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, I may have failed to detect pollinator-mediated selection on floral orientation because I 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 my 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; Eisikowitch and Woodell 1974, Tadey and Aizen 2001, Huang et al. 2002, Aizen 2003, 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 my study species, other factors must explain the observed differences in seed set between species and orientations. First, it is important to note that I 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, I 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. I 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 I 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; Hodges et al. 2002, Campbell et al. 2016). 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, I 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 I did not consider (e.g., herbivores, although these are rarely observed on my 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, my 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 I did observe, they add to the challenge of understanding the adaptive significance of floral orientation and may have limited my ability to detect pollinator- or rain-mediated selection on this trait.

Conclusion

I 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, my 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.

Table 3-1: Proportions of floral visitors observed visiting *M. brevistyla* and *M. fusiformis* flowers in 2010 and 2014-2017 at KP and RMBL (see Methods). The numbers below each site represent the sample sizes for total number of observed floral visitors and the number of observation-hours spent at each site, respectively.

	M. brevistyla				*M. fusiformis*						
	2014	2015	2016	2017	2010		2014	2015	2016	2017	
	KP	KP	KP	KP	KP	RMBL	KP	KP	KP	KP	RMBL
Visitors	(9/12)	(39/11)	(8/7)	(26/1.75)	(132/28)	(359/54)	(13/12)	(50/12)	(5/4)	(5/0.25)	(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

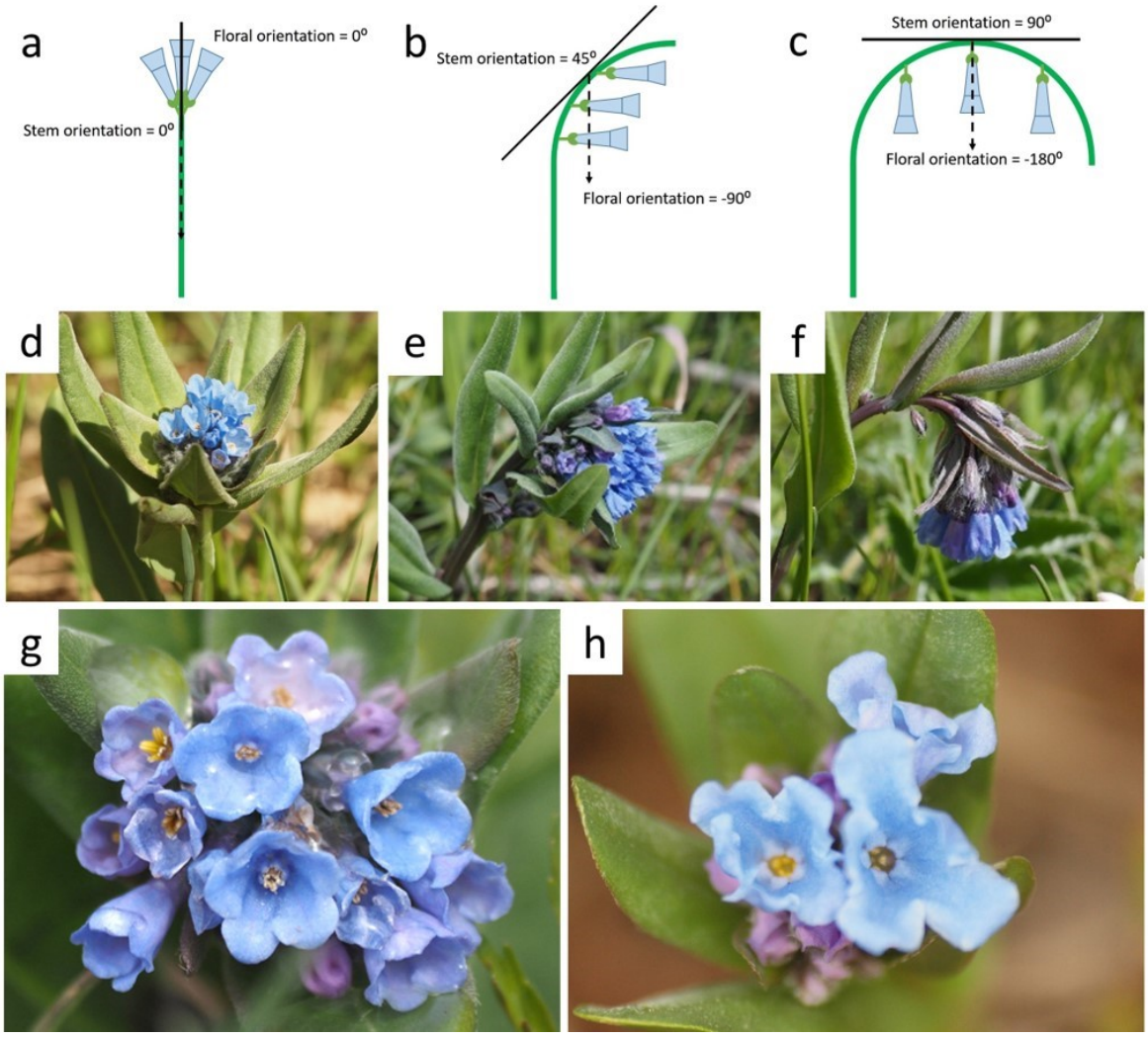

Figure 3-1: Diagrams illustrating the technique used to measure (a) upright, (b) horizontal, and (c) pendant floral orientations (the solid lines represent the edge of a protractor and the dashed arrows represent a suspended weight; see Materials and Methods). Examples of unmanipulated *M. fusiformis* 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.

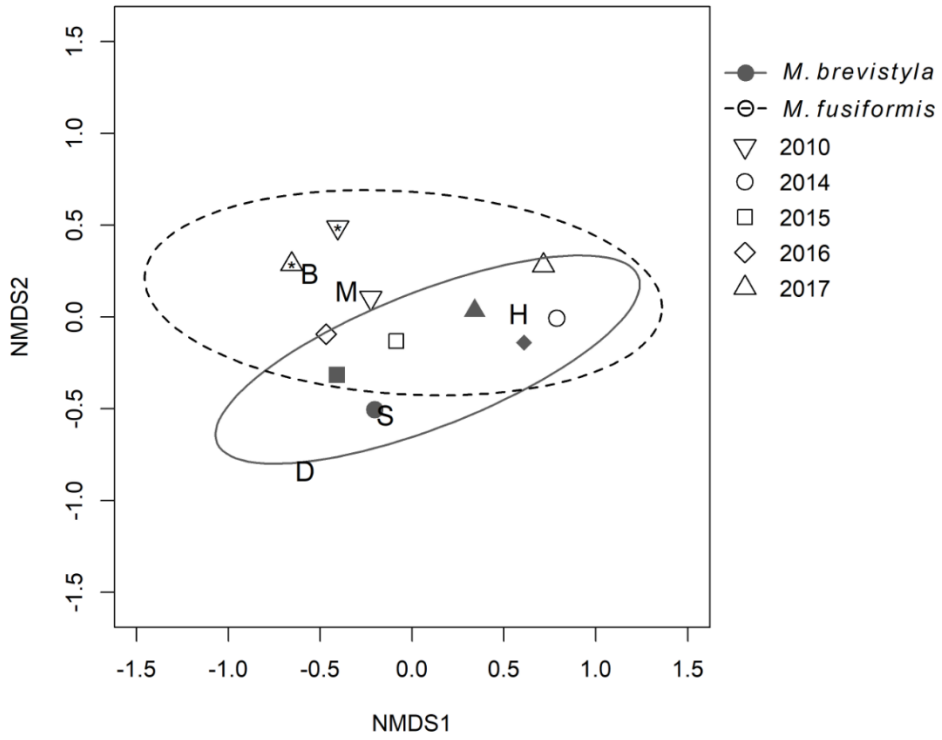

Figure 3-2: Non-metric multidimensional scaling (NMDS; stress = 0.08) of pollinator assemblages (represented by letters in the figure: B = *Bombus*; H = halictid bees; M = megachilid bees; S = syrphid flies; and D = other flies) of *M. brevistyla* and *M. fusiformis* in 2010 and 2014-2017. Asterisks 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.

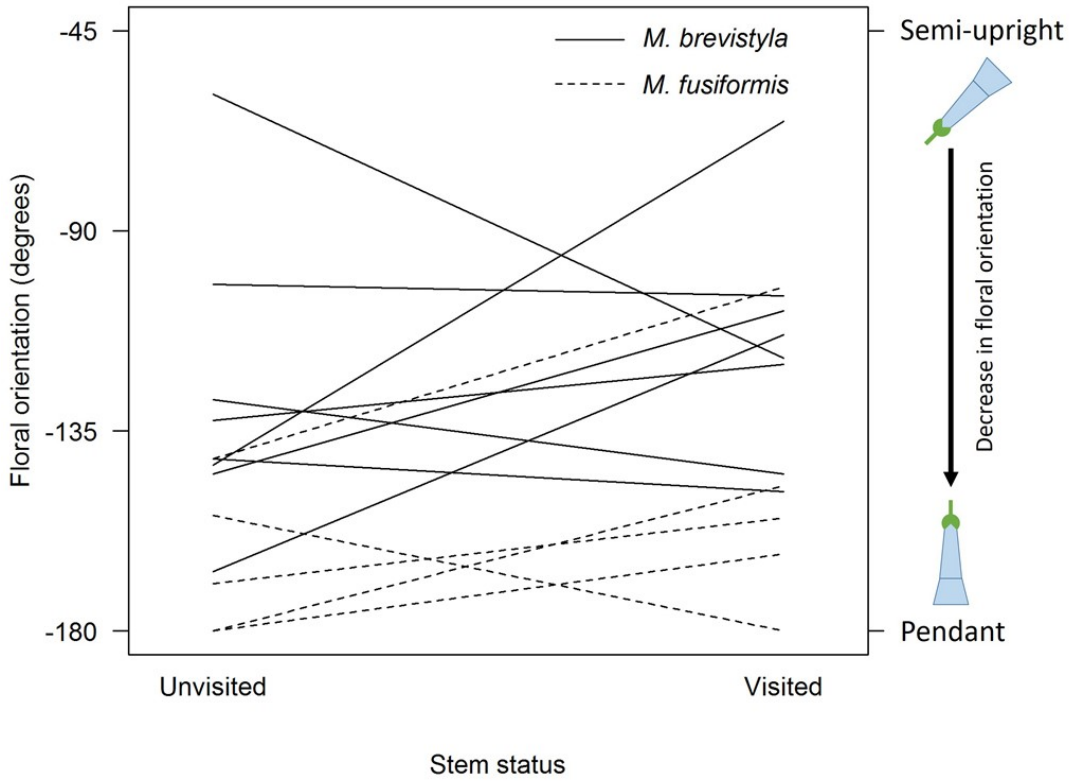

Figure 3-3: Interaction plot showing the floral orientation of stems visited by pollinators versus nearest unvisited stems (N = 8 *M. brevistyla* and 5 *M. fusiformis* pairs of plants).

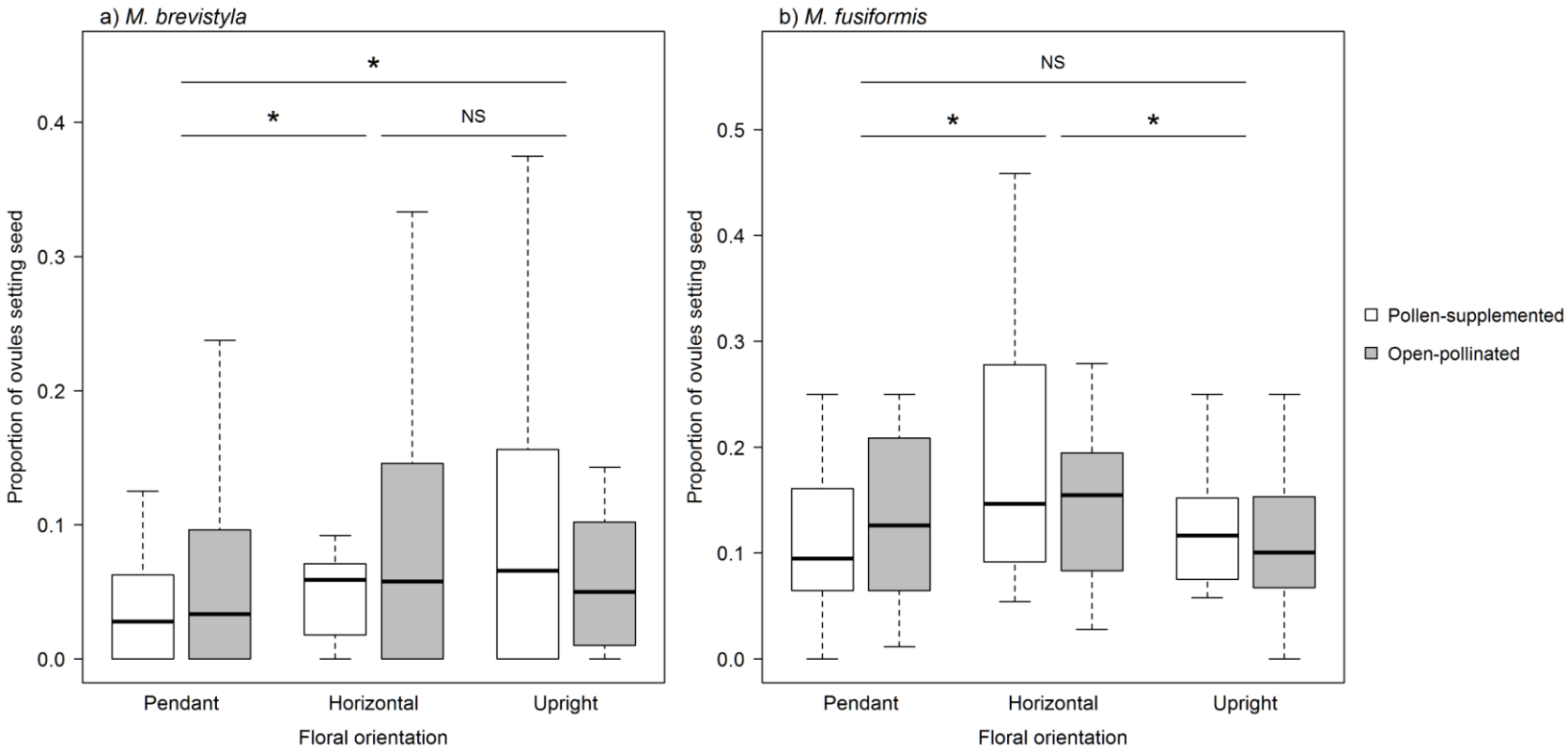

Figure 3-4: Boxplots showing the proportion of ovules setting seed for each supplementation and floral orientation treatment for a) *M. brevistyla* and b) *M. fusiformis* (N = 12 to 15 plants per treatment combination; total N = 118 *M. brevistyla* and 125 *M. fusiformis* plants). Boxes show the median and interquartile range. Lines above boxplots indicate Tukey's pairwise comparisons between orientations (* $P < 0.05$). Note difference in y-axis range between panels.

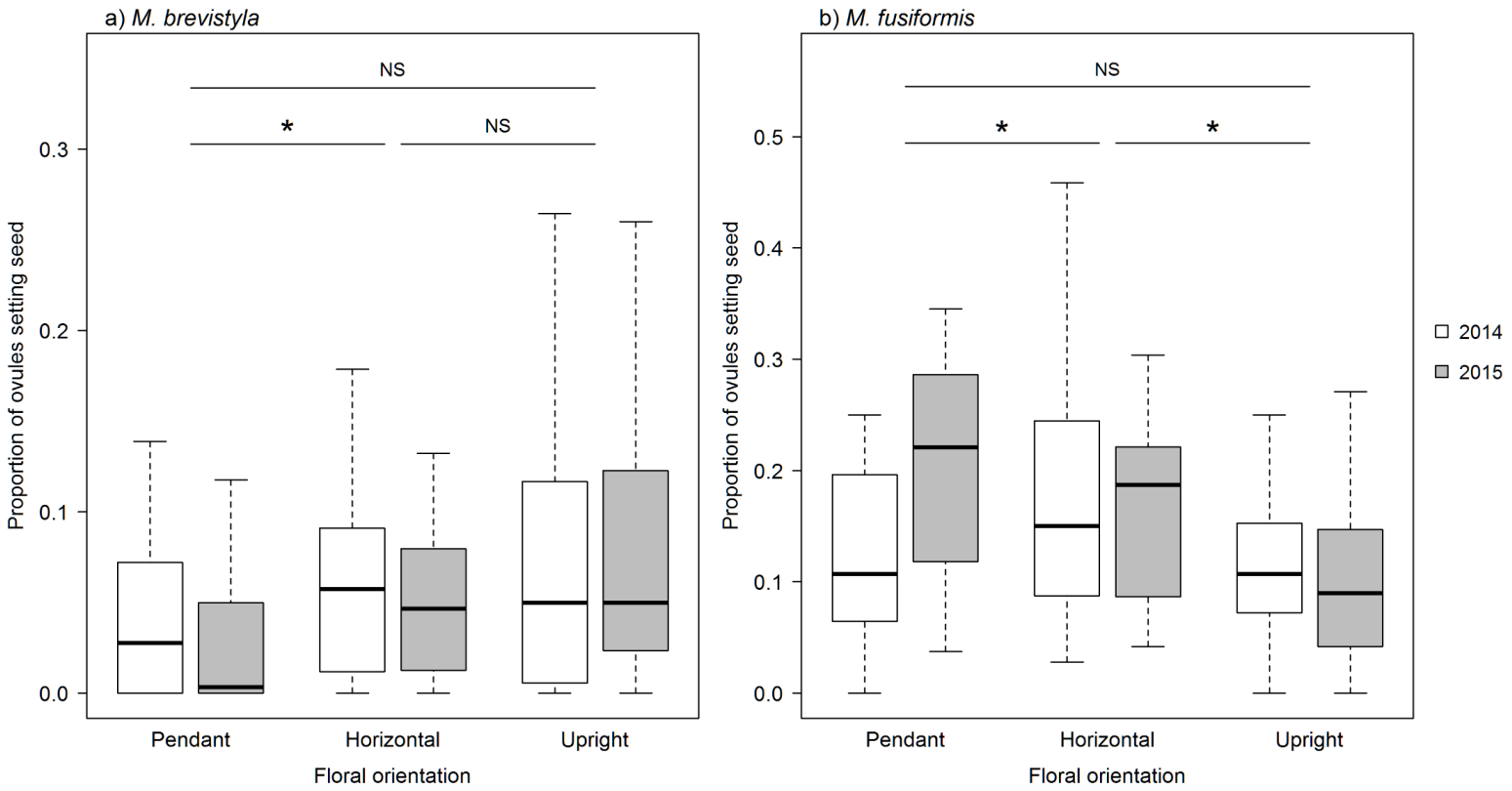

Figure 3-5: Boxplots showing the proportion of ovules setting seed for each orientation treatment in 2014 and 2015 for a) *M. brevistyla* and b) *M. fusiformis* (N = 10 to 28 plants per treatment combination; total N = 60 *M. brevistyla* and 80 *M. fusiformis* plants). Boxes show the median and interquartile range. Lines above boxplots indicate Tukey's pairwise comparisons between orientations (* $P < 0.05$). Note difference in y-axis range between panels.

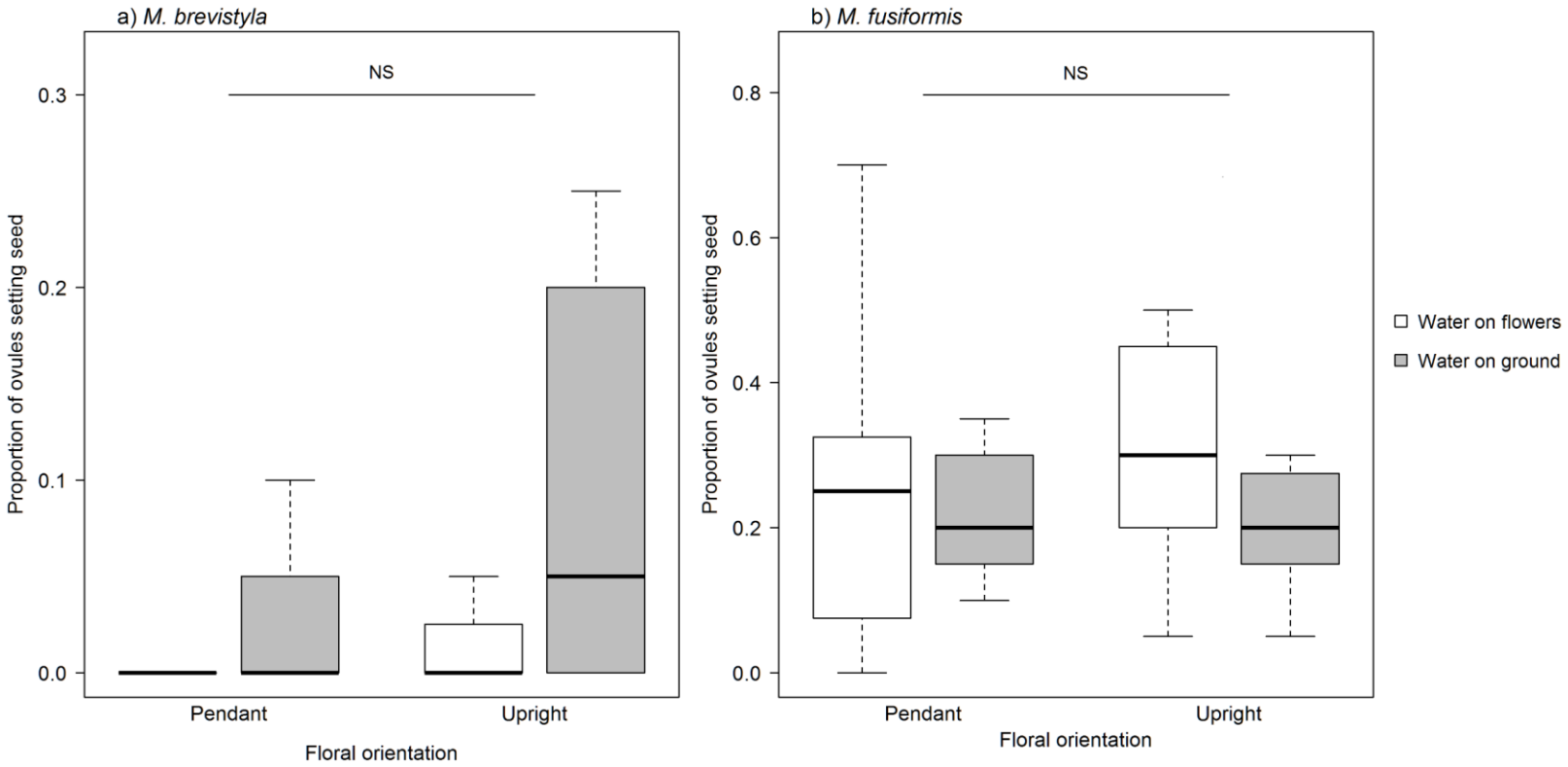

Figure 3-6: Boxplots showing proportion of ovules setting seed for each watering and floral orientation treatment for a) *M. brevistyla* and b) *M. fusiformis* (N = 5 to 8 plants per treatment combination; total N = 26 *M. brevistyla* and 25 *M. fusiformis* plants). Boxes show the median and interquartile range. Lines above boxplots indicate Tukey's pairwise comparisons between orientations. Note difference in y-axis range between panels.

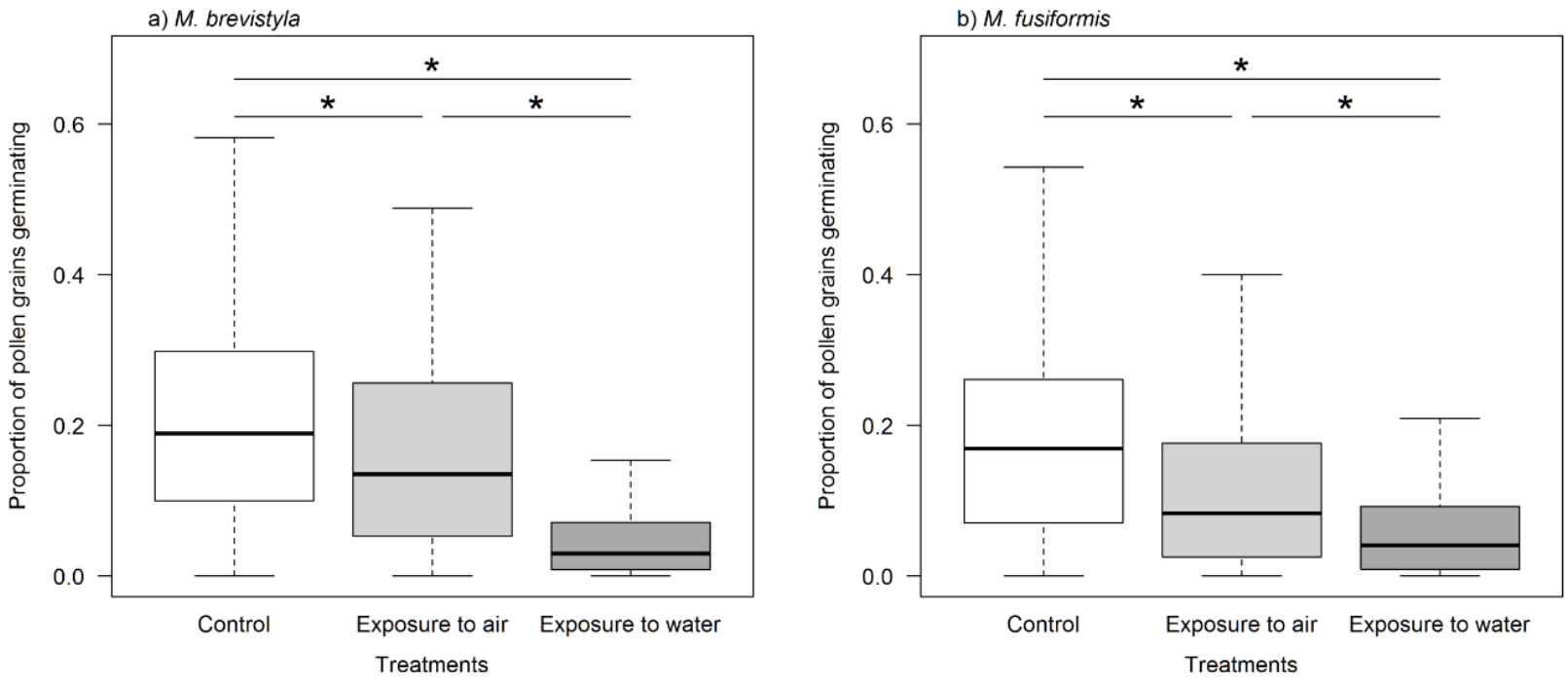

Figure 3-7: Boxplots showing proportion of pollen grains germinating in each treatment for a) *M. brevistyla* and b) *M. fusiformis* (N = 15 plants per treatment per species). Boxes show the median and interquartile range. Lines above boxplots indicate Tukey's pairwise comparisons between germination treatments (* $P < 0.05$).

General Discussion

In summary, my research demonstrates the importance of non-pollinator factors in influencing macroevolutionary patterns and in driving microevolutionary processes (i.e., selection on floral traits) in *Mertensia*; furthermore, I find that pollinators play a minor role in maintaining reproductive isolation between related *Mertensia* species. These results appear to contradict numerous studies that have shown pollinators to be key drivers of reproductive isolation and of selection on floral traits (Fulton and Hodges 1999, Ramsey et al. 2003, Whittall and Hodges 2007, Schiestl and Schlüter 2009, Sletvold and Ågren 2010, Sletvold et al. 2010, Sletvold et al. 2017). However, most of these results are based on a few well-studied taxa (e.g., *Aquilegia*, *Mimulus*, Orchidaceae) with specialized pollination systems or clear transitions in pollination syndrome. However, given that most angiosperms have generalized pollination systems (see Waser et al. 1996, Ollerton et al. 2007), my examination of *Mertensia* showcases a more representative system. Indeed, my results are in accordance with studies that have found pollinator assemblages to vary across different temporal and spatial scales and that have found selection on floral traits to be weak when multiple pollinator taxa are involved (e.g., in generalized pollination systems; see Herrera 1996, Ollerton 1996, Waser 1998). In addition, previous studies have identified non-pollinator agents (e.g., herbivores, pathogens, drought, heat stress, etc.) exerting selective pressures that may conflict with or outweigh pollinator-mediated selection (Strauss and Whittall 2006, Caruso et al. 2019, but see Sletvold et al. 2017).

Within the genus *Mertensia*, I detected an interspecific correlation between plant size traits (i.e., plant height, leaf area, and number of flowers) and flowering phenology, indicating that species flowering earlier in the growing season were smaller in stature than species flowering later in the season, after accounting for altitudinal variation in the onset and duration

of reproduction. This pattern also corresponded to these species occupying high-altitude habitats, likely because high-altitude environments select for smaller plant stature and early flowering (Chapter 1). In addition, this chapter required me to develop a novel approach to quantify flowering phenology from a single visit of a population. This approach relies on long-term phenological data to determine an averaged flowering curve, corrects for the altitudinal variation of different populations, and could be a useful tool in future phenological studies. Next, I found weak reproductive isolation between sympatric *M. brevistyla* and *M. fusiformis* populations, due to substantial local-scale spatial overlap between species, significant overlap in flowering phenology, shared pollinator taxa, and high pollen germination rates in putative hybrids. On the other hand, higher seed set from hand-pollination with conspecific pollen, and higher conspecific pollen transfer in mixed-species arrays, did contribute some reproductive isolation (Chapter 2). Lastly, I found that interspecific differences in floral orientation (more pendant orientation in *M. fusiformis* than *M. brevistyla* inflorescences) and reproductive structures (longer stamens and style in *M. fusiformis* than *M. brevistyla* flowers) may have been the result of selection imposed by rain and acting via paternal fitness (pollen germination); however, I was unable to detect pollinator- or rain-mediated selection on floral orientation based on maternal fitness (seed set; Chapter 3).

Further investigation is required to better understand adaptation and diversification in *Mertensia*. First, previous taxonomic studies based on morphological characteristics are inconsistent with the current phylogenetic hypothesis based on genomic data (see Nazaire and Hufford 2014); this makes *Mertensia* a challenging system to survey and to use for comparative analyses (Chapter 1). However, a future study could increase the sample size (i.e., multiple populations per species and/or more species), collect genetic material from each population, and

construct a phylogenetic tree based on the populations sampled. This would resolve the phylogenetic relationships among populations, especially if there is hybridization occurring, and could strengthen future comparative analyses. Second, *Mertensia* are relatively long-lived perennials with a long seed maturation stage and a low seed germination rate. These features make it difficult to assess the strength of post-zygotic barriers to hybridization between sympatric species (Chapter 2). A future study growing parental and hybrid seeds in the field, including the hybrid seeds in the habitats of the two parental species, and monitoring their maturation and germination might overcome this challenge. Last but not least, floral diversification between *M. brevistyla* and *M. fusiformis* may be influenced by the interaction between pollinators and rain (Chapter 3). A future study could examine the relative importance of rain- and pollinator-mediated selection on floral traits using a factorial design (e.g., Sletvold et al. 2017).

A comprehensive understanding of floral diversification and speciation requires examining selection processes across multiple scales, from local to landscape, seasonal to generational, and microevolutionary to macroevolutionary. Overall, my research shows that environmental conditions and life-history strategies are important factors shaping adaptation and diversification in *Mertensia*, a genus comprising species that primarily occupy alpine and montane habitats. These habitats exhibit pronounced variation in topography, climate, and edaphic conditions that likely contributed to the rapid diversification of *Mertensia* in North America (especially in the Rocky Mountains during the late Miocene and early Pliocene; see Nazaire et al. 2014). The biogeographic history of *Mertensia* in western North America suggests a framework of allopatric speciation associated with populations colonizing new habitats, diverging genetically, and adapting to different abiotic conditions, especially at high altitudes,

where non-pollinator agents likely play a greater role than pollinators in driving plant diversification.

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