

Interpopulation variation and optimal mating in the perennial herb, *Polemonium brandegeei*

By

Jeremy Poortvliet

Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial
fulfilment of the requirements for the degree of
MASTER OF SCIENCE

Department of Biological Sciences
University of Manitoba
Winnipeg, Manitoba

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Abstract

Generalist pollinated plants receive pollination services from a wide range of animals, but pollinator effectiveness varies. More effective pollinators in generalist systems optimize pollen transfer by promoting outcrossing between more distant plants. This leads to increased pollinator mediated selection by these pollinators on floral traits and variation in pollinator abundance may therefore cause population genetic and phenotypic differentiation across the range of a species. However, variation may also be caused by plastic responses to variable environmental conditions. Here, I analyze the prevalence and potential causes of interpopulation variation as well as optimal mating distance in generalist pollinated *Polemonium brandegeei*. I compared morphology and floral scent (1) among four field populations and (2) between field and greenhouse grown plants sourced from the same populations. Populations varied in morphology and floral scent profile and the southernmost population was most distinct with floral traits consistent with increased selection by hawkmoths or hybridization events. All major among population differences were preserved in the greenhouse which suggests a genetic component to phenotypic variation but there was an overall increase in flower size and total scent emission in the greenhouse. Field hand crosses indicated that mating in *P. brandegeei* was optimized provided crosses were made between plants at least 1 – 3.5m apart, and that environmental variability influenced mating success. These findings may reflect differences in pollinator abundance and/or pollinator mediated selection among populations or hybridization events and offer support for past selection by more effective pollinators (hawkmoths and hummingbirds) which transport pollen over larger distances.

Acknowledgements

Land Acknowledgement

I acknowledge that my field research was conducted on the traditional lands and gathering places of numerous Indigenous nations including the Arapaho, Cheyenne, Crow, Shoshone, and Ute Nations and peoples in Colorado and Wyoming, and the Pueblo, Navajo, and Apache Nations and peoples in New Mexico. I also acknowledge that the University of Manitoba campus and facilities are located on the ancestral lands of the Anishinaabeg, Cree, Anisinew, Dakota, and Dene peoples, and on the homeland of the Red River Métis Nation. The water used to grow my research plants was sourced from Shoal Lake 40 First Nation. I honour the resilience and wisdom of Indigenous peoples. I commit to always respect and champion the land I work on and the plants and animals I work with.

Funding/Permit Acknowledgement

I gratefully acknowledge the numerous sources of funding that made this research possible and kept me alive during my MSc:

- The National Sciences and Engineering Research Council (NSERC) for a Canada Graduate Scholarship-Masters program and for additional support through Dr. Worley's NSERC Discovery Grant.
- The University of Manitoba for a University of Manitoba Graduate Fellowship and additional support.
- The Dalgarno family for their scholarship support.
- The Rocky Mountain Biological Laboratory (RMBL) for their support through a RMBL Graduate Fellowship.

Also, thank-you to the US National Park Service for providing permits to work at Deer Mountain and for the free accommodation at McGraw Ranch, to various US Forest Service offices for providing permits to work at Vedauwoo and Sandia Crest, and to RMBL for permission to use the RMBL GCMS equipment and to work at Taylor Canyon.

Personal Acknowledgements

This research was made possible through the assistance of numerous wonderful individuals. First, I would like to thank my supervisor, Dr. Anne Worley, for her incredible mentorship and guidance throughout my MSc. Anne, I value your academic guidance immensely, and our random discussions as much! I also would like to thank my committee members, Dr. Jake Stout and Dr. Richard Westwood for providing their time and useful feedback. Special thanks also to Dr. Rob Raguso for his enthusiastic support of the floral scent work and to Rob, Dr. Diane Cambell, and Dr. John Powers for a fantastic floral scent workshop. John also ran our samples on the GCMS and provided invaluable support as I analyzed the scent data. Thanks also to Dr. Jennifer Reithel at RMBL for facilitating the use of the RMBL GCMS equipment and to Dr. Alistair Brown and Dr. Annemieke Farenhorst at the MASS Lab for analyzing samples on their new GCMS equipment. Finally, thank you Jacalyn Grey for making our field trips fun and successful, to Avital Shalev for making our busy and intense 2023 field trip a success, and to Chloé Buisse and Yantong Tan for their field and lab support.

Lastly, thank you to my friends and family for their support during my MSc era! Special thanks to Karlo for putting up with me during the thesis writing process.

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Chapter 1: General Introduction

Background

Pollination services are essential to the successful reproduction of most angiosperm species (Ollerton et al. 2011). While some plant-pollinator relationships are highly specialized, pollination is best considered to exist on a continuum from highly specialized to highly generalized (Waser et al. 1996). In fact, most pollination systems are at least somewhat generalised, and most plants are visited by multiple different functional groups of pollinators (Waser et al. 1996; Brosi 2016). Plant species occupying highly variable environments are especially likely to have more generalized pollination systems due to temporal and spatial fluctuations in pollinator abundances (Waser et al. 1996). Generalization under such conditions reduces the risk associated with reliance on one pollinator species and increases the likelihood that pollination services are available when needed (Waser et al. 1996; Burns et al. 2022). Despite their prevalence, generalised pollination systems have received considerably less attention than highly specialized systems and the relative role different pollinators play in such systems needs further attention (Fenster et al. 2004; Jaeger et al. 2023).

Understanding the role all pollinators play in generalist systems is critical as pollinator effectiveness in generalist systems varies. While some pollinators may be highly effective (i.e., transfer increased amounts of high-quality pollen), others may be considerably less effective (Aigner 2003; Larsson 2005). For example, large-bodied pollinators such as hawkmoths and hummingbirds often transfer more pollen over longer distances which may increase their effectiveness relative to smaller pollinators with smaller foraging ranges such as bees and flies (Kulbaba & Worley 2008; Skogen et al. 2016; Rhodes et al. 2017).

The role different pollinators play in generalist systems is especially important when considering the effects of pollen limitation. Pollen limitation, or the reduction in fruit or seed set due to insufficient pollen deposition, is prevalent across most plant families and has major effects on plant reproductive output (Knight et al. 2005). In a generalist system, pollen limitation may increase or decrease depending on the relative abundance of more effective pollinators (Cisternas-Fuentes et al. 2023). Less effective pollinators may cause pollen limitation

through the transfer of less pollen or through the transfer of poor-quality pollen, both of which can have equally detrimental effects on plant fitness. Of particular concern, especially for self-incompatible plants, is the receipt of pollen from a close genetic relative because greater homozygosity can lead to inbreeding depression due to increased expression of deleterious alleles (Charlesworth & Willis 2009). Inbreeding depression can have widespread negative effects on seed set and offspring survival (Charlesworth & Willis 2009).

Inbreeding depression may be more prevalent in plant populations with spatial genetic structure in which limited seed and pollen dispersal prevent gene flow throughout the population (Ayre et al. 2019). Plants in proximity to each other in structured populations are likely to be closely related and pollen transfer between more distant plants is therefore necessary to reduce inbreeding pressures (Waser & Price 1989). The minimum distance of pollen transfer over which fitness is maximized in a population with spatial genetic structure is referred to as the optimal mating distance of a species (Waser & Price 1989). In generalist systems, the most effective pollinators not only transfer large amounts of pollen, but also transfer pollen between plants at the optimal mating distance (Ayre et al. 2019). A better understanding of optimal mating distances and their relationship to specific pollinators in generalist systems is important, especially due to ongoing shifts in pollinator abundances which may affect pollen transfer (Rhodes 2018).

Optimized pollen transfer that limits inbreeding depression will confer a fitness advantage to plants. Therefore, the more effective pollinators in a generalist system may exert stronger selective pressures on plant and floral traits than other pollinators in the system (Stebbins 1970). According to Stebbins' "most effective pollinator principle", plant traits will evolve to best suit their most effective pollinator (Stebbins 1970; Rosas-Guerrero et al. 2014). As a result, consistent differences in the relative abundance of effective pollinators temporally or across the range of a species can lead to variation in plant traits among populations (Chapurlat et al. 2018). Indeed, variation in floral traits among populations is associated with variation in pollinator assemblage (Gomez et al. 2008). For example, floral scent, which is essential to the attraction and orientation of pollinators, varied among populations of *Gymnadenia conopsea* (Orchidaceae) with different major pollinators (Chapurlat et al. 2018).

Variation in antagonists (i.e., herbivores) across the range of a plant species can have similar effects (Agren et al. 2013). Analysis of variation in floral traits can, therefore, offer insights into past and current selection on floral traits by pollinators and antagonists (e.g., Schueller 2007; Szenteczki et al. 2021).

Although natural selection and adaptation to local pollinators and/or antagonists contribute to variation among plant populations, phenotypic plasticity, by which plants respond to environmental variation without genetic change, is another important mechanism through which plants respond to local conditions (Palacio-Lopez et al. 2015). Phenotypic plasticity enables plants to respond quickly to temporal and spatial environmental variation and consequently causes among population variation if environmental conditions vary across the range of a species (Palacio-Lopez et al. 2015). While plasticity is generally adaptive, especially in variable environments, it may be nonadaptive if it causes traits to shift away from their optima because such shifts may have negative effects on plant fitness (Palacio-Lopez et al. 2015).

Most plant traits are at least somewhat plastic (Palacio-Lopez et al. 2015). Traits such as leaf and flower/display size and shape, for example, are affected by environmental conditions and display considerable plasticity (Martin & Puech 2001; Vitasse et al. 2010; Palacio-Lopez et al. 2015). Floral scent chemistry is also affected by environmental conditions; studies have shown that floral scent chemistry is affected qualitatively and quantitatively by environmental and climate conditions such as temperature (Hu et al. 2013), soil moisture (Burkle & Runyon 2016), and nutrient availability (Luizzi et al. 2021). For example, due to enzyme kinetics and variable compound volatility, floral scent emissions often increase as temperature increases until a species-specific optimum is reached, after which emissions decrease (Hu et al. 2013). Thus, differences in environmental conditions among populations may cause among population variation in floral traits due to phenotypic plasticity.

Various techniques have been developed to determine the relative contribution of phenotypic plasticity and genetic change through local adaptation to variation in floral morphology and scent. Common garden environments are often an important first step in making that determination as they can be used to disentangle the relative contributions of

phenotypic plasticity or local adaptation to among population phenotypic variation (de Villemereuil et al. 2020). However, common garden experiments have not often been used for plants from higher elevations, to study the relative causes of among population variation in floral scent, or to study variation in floral morphology and scent simultaneously (Majetic et al. 2009; Berend et al. 2019; Eisen et al. 2022). A better understanding of the relative contributions of phenotypic plasticity and local adaptation to among population variation can provide insights into plant responses to environmental change (Paiano et al. 2012). Further, it can provide insights into past and current selection by pollinators on floral traits and may help explain the effects of ongoing pollinator shifts on floral phenotypes and mating success (Szenteczki et al. 2021; Eisen et al. 2022). In this thesis, I examine optimal mating and interpopulation variation in floral morphology and scent (and its potential causes) in *Polemonium brandegeei*.

Study System

P. brandegeei (A. Gray) Greene (Brandegee's Jacob's-ladder or Honey Polemonium) is a perennial herb native to mountainous areas from Arizona and New Mexico to South Dakota and Montana, USA. Documented populations of *P. brandegeei* range in elevation from about 2500 – 3400 m, thus characterizing it as a sub(alpine) species. Plants are often found on shaded southwest facing cliffs or steep scree slopes with shallow soil. Flowers range in colour from bright yellow to white (many with a purple tinge) and are variable in size; this variation is especially pronounced within and among some populations which made further study of interpopulation variation appropriate (Kulbaba & Worley 2008; Figure 1.1). Further, *P. brandegeei* emits many volatiles from its skunky-scented foliage and contrasting sweet-scented flowers, but the floral scent profile of this species had yet to be characterized.

P. brandegeei is a generalist pollinated species visited by hummingbirds, hawkmoths, and large and small bees and flies (Kulbaba & Worley 2008; pers. obs.). Hawkmoths and hummingbirds were thought to be more effective pollinators than bees and flies and contribute most to seed set, but more recent work found that small visitors also play an important role in pollination (Kulbaba & Worley 2008, 2012, 2013; Pond 2017). Given that smaller pollinators have smaller foraging ranges, the effects of short vs. long distance pollination were of interest and made study of optimal mating distance suitable.



Figure 1.1: Collage of pictures from four field populations of *P. brandegeei* showcasing plant growth habit and natural variation in flower size, shape, and colour. Clockwise from top left: SC, SC, TC, DM, VD.

Thesis Objectives

My thesis has 4 main objectives.

1. Characterize the floral scent profile of *P. brandegeei* for the first time.
2. Describe variation in morphology and floral scent chemistry across four populations of *P. brandegeei*.
3. Determine the relative contributions of genetic variation and phenotypic plasticity to among population phenotypic variation using a common garden experiment.
4. Determine if mating in *P. brandegeei* is optimized at a certain physical distance and if environmental variation within populations affects mating success.

Thesis Overview

The above objectives were addressed through field and greenhouse work from May 2022 – July 2023. To meet objectives 1-3, I collected floral scent samples and controls and morphological measurements from field plants in four populations of *P. brandegeei* and compared these data to floral scent samples and morphological measurements collected from greenhouse grown plants from the same populations. These data are discussed in Chapter 2. To meet objective 4, I collected environmental data in two populations of *P. brandegeei*, performed hand crosses between field and greenhouse plants at a range of distances, and analyzed various metrics of crossing success, including seed germination and survival. These data are discussed in Chapter 3. Chapter 4 provides a final summary and discussion of the full thesis.

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Chapter 2: Interpopulation variation in *P. brandegeei* floral morphology and scent

INTRODUCTION & RATIONALE

Background

Population-level variation in floral morphology and scent is common across the range of many plant species (Nattero et al. 2011; Chapurlat et al. 2018; Soteras et al. 2020). Due to their combined importance to the attraction and orientation of pollinators, any variation in these traits can affect plant fitness; it is therefore important to understand why and how floral morphology and scent vary among populations (Eisen et al. 2022). In general, interpopulation variation in floral traits has two major causes that occupy opposite ends of a spectrum (Palacio-Lopez et al. 2015). On the one hand, among population phenotypic variation can result from among population genetic variation. Natural selection can lead to the evolution of new floral traits and cause populations to differentiate genetically, especially when gene flow is limited and there is standing genetic variation and/or new mutations (Arafeh et al. 2002; Barrett & Schluter 2007; Stocklin et al. 2009; Agren et al. 2013; Chapurlat et al. 2015, 2018; Wang et al. 2020; Opedal et al. 2023). Alternatively, phenotypic plasticity, which occurs when a phenotypic trait associated with a specific genotype is expressed differently under different environmental conditions, can cause populations to differentiate phenotypically with little to no genetic change (Gratani 2013; Palacio-Lopez et al. 2015; Laitinen & Nikoloski 2019). These two major processes may interact to produce a range of floral phenotypes among different populations (Paiano et al. 2012; Chapurlat et al. 2018).

It is important to understand the relative role genetic change and phenotypic plasticity play in causing interpopulation variation. This knowledge can, for example, inform our understanding of plant evolutionary history and the ability of plants to respond to environmental heterogeneity and environmental change, the latter of which is especially relevant due to global climate change (Nattero et al. 2011; Paiano et al. 2012; Soteras et al. 2020; Eisen et al. 2022). Common garden experiments, in which plants from different populations are grown under constant environmental conditions, can serve as a useful tool to

study the underlying causes of among population phenotypic variability (Berend et al. 2019). Common environments serve to disentangle the relative effects of phenotypic plasticity and genetic variation because among population phenotypic variation with a genetic basis will theoretically be preserved in a common environment, while variation due to phenotypic plasticity will not (de Villemereuil et al. 2020).

Common environments have been employed to understand interpopulation variation in various plant traits, including morphology (Berend et al. 2019). First, studies have shown that interpopulation variation in morphological traits can arise due to among population genetic variation and differential selection by pollinators and antagonists. For example, populations of *Erysimum mediohispanicum* (Brassicaceae) with more bee (Hymenoptera) pollinators have a different corolla shape and narrower petals than populations with more beefly (Bombyliidae) pollinators (Gomez et al. 2008, 2009). Similarly, variation in scape length among populations of *Primula farinosa* (Primulaceae) is caused by differences in grazing intensity between populations, as mammalian grazers select for short-scaped morphs while pollinators select for longer-scaped morphs (Agren et al. 2013). However, plasticity in morphological traits is also common (Valladares et al. 2007) including phenotypic plasticity in various leaf traits (Sultan 2000; Matos et al. 2009; Vitasse et al. 2010) and fruit and floral display size (Martin & Puech 2001). More common garden studies, especially those involving plant species from higher elevations, are needed to understand the relative role of phenotypic plasticity and evolutionary processes in among population variation in morphology (Berend et al. 2019).

The underlying causes of among population variation in whole plant and/or floral VOC (volatile organic compound) synthesis and emission (i.e., floral scent) have seldom been analyzed (Majetic et al. 2009; Eisen et al. 2022). Plant VOC emissions are highly complex and dynamic and require specialized methods and technology to analyze relative to many other traits which has limited their study (Raguso et al. 2008; Delle-Vedove et al. 2017). For example, over 1700 distinct floral VOCs divided into several compound classes, have been recorded in the literature (Knudsen et al. 2006) and the analysis of these diverse VOCs requires access to reliable and expensive gas chromatography – mass spectrometry (GCMS) equipment (Raguso et al. 2008). Furthermore, choosing a floral scent sampling and analysis method can be challenging

due to the wide range of options and limited method comparisons (Raguso & Pellmyr 1988; Raguso et al. 2015). However, floral VOCs serve diverse roles and are used to attract pollinators (Farré-Armengol et al. 2020), deter antagonists (Galen et al. 2011), attract herbivore parasitoids (Schiestl et al. 2014), inhibit the growth of phyllosphere microbiota (Farré-Armengol et al. 2013), and communicate with other plants (Caruso & Parachnowitsch 2016). Logistical challenges should not deter study of this important plant trait, as a better understanding of the extent, causes and effects of variation in plant VOC profiles is needed (Eisen et al. 2022).

The growing body of available research suggests that floral scent displays extensive variation both within and among populations in multiple species. This variation is often attributed to differential selection by pollinators and antagonists, and populations or individuals with different pollinator assemblages might also differ in their floral scent emissions (Mant et al. 2005; Sun et al. 2014; Gross et al. 2016; Chapurlat et al. 2018). For example, scent emissions of a population of *Gymnadenia conopsea* (Orchidaceae) pollinated by flies differed from the scent emissions of populations pollinated primarily by moths (Chapurlat et al. 2018). Similarly, antagonists can also exert strong selective pressures on plant VOCs, often in parallel or opposition to pollinator mediated selection (Farré-Armengol et al. 2020). Populations or individuals experiencing increased herbivory may have different scent emissions than other populations to either deter herbivores directly or to limit their attraction (Delle-Vedove et al. 2017). For example, Galen et al. (2011) found that emission of 2-phenylethanol (2PE; phenylethyl alcohol), a defensive compound in *Polemonium viscosum* (Polemoniaceae), varies with floral shape and broadly flared flowers more vulnerable to ant antagonists emit more 2PE than others.

Further, plant VOC synthesis and emission is also affected both qualitatively and quantitatively by numerous environmental factors. Examples include temperature (Sagae et al. 2008; Hu et al. 2013), drought stress (Burkle & Runyon 2016; Campbell et al. 2019), light levels (Hendel-Rahman et al. 2007; Chuang et al. 2017), nutrient availability (Luizzi et al. 2021), and soil type (Cna'ani et al. 2021). Due to these environmental effects, there is potential for phenotypic plasticity in VOC emissions. Indeed, rearing *Hesperis matronalis* (Brassicaceae) plants from different populations in a common garden significantly reduced interpopulation

differences in floral scent emissions, suggesting an important role for plasticity in among population variation in VOC emissions in wild populations (Majetic et al. 2009). However, the role of phenotypic plasticity in interpopulation variation in floral scent emissions remains unclear as other research indicates that interpopulation variation in scent has a largely genetic component. For example, little evidence for phenotypic plasticity in floral scent emissions was found in *G. conopsea* (Orchidaceae) from multiple populations (Chapurlat et al. 2018), and among population variation in VOC emissions has a major genetic component in other species also (e.g., *Lithophragma bolanderi* (Saxifragaceae), Friberg et al. 2017; *Oenothera cespitosa* (Onagraceae), Eisen et al. 2022).

Thus, it is apparent that more research, including common environment studies, are needed to fully understand the scope of interpopulation variation in floral morphology and scent and its relative causes (Majetic et al. 2009; Berend et al. 2019; Eisen et al. 2022). Furthermore, an increased number of integrated studies that analyze variation in both floral morphology and scent are necessary; pollinators use a combination of many floral traits to find and orient themselves on flowers and may select for multiple traits in tandem which makes a holistic approach appropriate (Junker & Parachnowitsch 2015). Further, variation in one floral trait may be related to variation in another and traits should therefore be studied together (Eisen et al. 2022). In this research, I compare morphological and chemical traits among flowers from different populations of *P. brandegeei* and use a common garden experiment as the first step to determine the contributions of genetic divergence and phenotypic plasticity to among population variation.

Rationale

P. brandegeei provides a good model system for an analysis of interpopulation variation in both morphological and chemical traits and its relative causes. Populations are apparently quite separated geographically, vary in environmental conditions (pers. obs.), and likely experience differences in pollinator mediated selection on floral morphology and scent due to the variable spatial and temporal abundance of hawkmoths, hummingbirds, and other visitors (Kulbaba & Worley 2008, 2014; Brunet 2009; Pond 2017). Thus, interpopulation variation is expected and has been recorded for some other traits including corolla tube length and width

and stigma/anther length (Kulbaba & Worley 2008). For the current study, the addition of a new study population in New Mexico at the southerly edge of the range of *P. brandegeei* and the addition of floral scent analysis will allow for a better understanding of the scale of morphological variation in this species. Finally, *P. brandegeei* lends itself well to common garden studies as I was able to grow numerous plants to flowering in the greenhouse, which is atypical of higher elevation species (de Villemereuil et al. 2020).

Finally, the use of different methods of VOC sampling and analysis for this study provides an opportunity for a method comparison. *Polemonium brandegeei* VOCs were sampled using the relatively uncommon full evaporative technique (FET) with gas chromatography-mass spectrometry (GCMS) and the more common dynamic headspace sampling with thermal desorption GCMS. Due to their major differences (FET requires heating of fresh samples while dynamic headspace sample occurs non-destructively on live plants), each method may provide unique insights, and together they may provide a more comprehensive picture of the scent profile of *P. brandegeei*.

OBJECTIVES & HYPOTHESES

Objectives

- Characterize the floral scent profile of *P. brandegeei*.
- Describe interpopulation variation in *P. brandegeei* floral scent profiles and/or morphological traits.
- Determine, using a common garden, if interpopulation variation in *P. brandegeei* chemical and morphological traits is more likely to have a genetic component or is caused by phenotypic plasticity.
- Compare two methods of collecting *P. brandegeei* scent samples (FET-GCMS vs. dynamic headspace TD-GCMS).

Hypotheses + Predictions

- As a generalist pollinated plant, the floral scent profile of *P. brandegeei* largely consists of common floral VOCs but also contains hawkmoth specific attractants.
- Populations of *P. brandegeei* have variable morphology and scent profiles.
- Interpopulation variation in *P. brandegeei* morphological and chemical traits is primarily heritable, and variation among populations in the field will largely be preserved in a common environment.
- Overall patterns of VOC emissions are preserved when different sampling and analysis methods are used.

METHODS & MATERIALS

Study Design

I collected seeds at random from four populations of *P. brandegeei* at Vedauwoo, Wyoming (VD), Deer Mountain, Colorado (DM), Taylor Canyon, Colorado (TC), and Sandia Crest, New Mexico (SC) in 2022 (Table 2.1); these populations represent much of the latitudinal range of *P. brandegeei* (Grant 1989). I then grew plants from three populations (DM, TC, SC) to flower in the greenhouse in 2023 (plants from the VD population did not flower in time for analysis). I collected floral, leaf, and control scent samples from greenhouse plants using two sampling and analysis methods (FET-GCMS and dynamic headspace sampling with TD-GCMS) which allowed me to characterize the scent profile of *P. brandegeei* for the first time. I also collected 11 floral measurements from greenhouse plants. I then returned to the field in summer 2023 to collect scent samples and morphological measurements from all four populations. This enabled me to compare field and greenhouse plants from three populations (not VD) for these important plant traits and determine if there is a genetic basis to observed interpopulation variation or if this variation is more likely to be caused by phenotypic plasticity. I also compiled images from iNaturalist to visualize the latitudinal range of variation in *P. brandegeei* floral morphology.

Table 2.1: Locations and descriptions of the four populations of *P. brandegeei* used in this study. The Vedauwoo, WY (VD) population was only used for field data collection while the Deer Mountain, CO (DM), Taylor Canyon, CO (TC) and Sandia Crest, NM (SC) populations were used for both greenhouse and field data collection.

Population	Coordinates	Elevation	Description
Vedauwoo, Wyoming (VD)	41.15438 N, 105.37386 W	2519m	Plants found on/at the base of a large rock formation that divides the (accessible) population into distinct sides; plants growing in gravel, shallow soil, or in rock crevices, often sheltered by rock overhangs; high winds common; plants relatively exposed; habitat variable
Deer Mountain, Colorado (DM)	40.38673 N, 105.59737 W	2880m	Plants found on steep, rocky, SW facing slope; plants growing in shallow soil or in rock crevices, sheltered by trees; plants less exposed; habitat homogenous
Taylor Canyon, Colorado (TC)	38.72829 N, 106.76393 W	2600m	Plants largely on or at the base of sheer, SW facing rock walls; soil very shallow; plants quite exposed or sheltered by trees; habitat largely homogenous
Sandia Crest, New Mexico (SC)	35.20793 N, 106.44771 W	3100m	Plants largely on or at the base of sheer, SW facing rock walls; soil shallow; plants sheltered by trees or quite exposed; gusty winds common; habitat variable

Seed Germination + Plant Care

I used 100 mm x 15mm plastic petri dishes (Fisher Scientific) lined with number 1 qualitative filter paper (Whatman) as germination “chambers”. I placed seeds on the filter paper after it was wetted with deionized water and then placed the petri dishes in a dark, 4°C fridge for about two weeks. I kept filter papers moist and replaced them as needed. I moved any seeds that germinated in the fridge or incubator to 72 cell plug trays (Deep-Root Seed Starters; Lee Valley) one or two days after germination was noted (radicle emergence). I moved any remaining ungerminated seeds to soil at the end of the two-week cold stratification period. I grew plants in a soil mix consisting of 10g 15-9-12 fertilizer (Osmocote Plus; Scotts-Sierra Horticultural Products) per 1L soil (Pro-Mix High Porosity with Mycorrhizae; Premier Tech Horticulture) in the Crop Technology Centre greenhouse at the University of Manitoba.

Germination rate varied among populations. SC and TC seeds generally germinated very well under the above-described two-week cold stratification. DM and VD seeds did not respond well to this treatment, so seeds were subjected to physical scarification using fine grain sandpaper and cold stratified for four weeks. Further discussion of germination trials and results is included in Chapter 3 and Appendix 1.

I transferred plants to 800-ml conical Deepots once they had reached an appropriate size (approximately 1 month after emergence). After about one month of growth, I placed the Deepots (in racks) into 50 L plastic bins to facilitate bottom watering. I left plants in the bins until experiments ended, misted them regularly to increase RH, and spaced them as needed. I was able to grow plants from SC, TC and DM to flower in the greenhouse, but no VD plants flowered in time for me to collect greenhouse data.

Morphological Measurements

I obtained 11 floral measurements from field and greenhouse plants. For greenhouse plants, I removed two randomly selected fresh flowers (one in male phase and one in female phase) from the first inflorescence 10 days after first flowering and measured them to 0.01 mm using digital calipers. Floral measurements included flower length, corolla width, corolla tube length, basal tube diameter, apical tube diameter, style length, anther height, stigma-anther

separation (i.e., herkogamy), anther exsertion and stigma exsertion (Table 2.2, Figure 2.1). I dried measured flowers in a plant dryer set to 25°C overnight and then weighed them using an analytical balance to obtain a dry mass measurement. In the greenhouse, measurements were performed on 64 flowers from SC plants, 46 flowers from TC plants, and 22 flowers from DM plants. VD plants did not flower in the greenhouse in time for data collection.

I also collected the same 11 floral measurements (as in Table 2.2, Figure 2.1) on field plants. I measured 30 flowers at TC, DM, and VD, and 40 flowers at SC. In general, two flowers per plant (one male, one female phase) were measured in the field as well. Plants that were measured were randomly selected from across the populations and all measured flowers appeared fresh at time of collection. Flowers were removed from plants for measuring, then dried and weighed.

Finally, to anecdotally determine the extent of morphological variation across the range of *P. brandegeei* I compiled images from iNaturalist observations (inaturalist.org). I used “research grade” observations with clear images of flowering plants only. All suitable observations and images were from 2018 and later and images included in the figure are representative of all available images.

Table 2.2: List and description of the 11 floral measurements taken on *P. brandegeei* field and greenhouse flowers. All measurements in mm or mg.

Measurement	Description
Flower Length	Base of flower to tip of tallest corolla lobe
Corolla Width	Tip of corolla lobe to tip of corolla lobe across widest section
Corolla Tube Length	Base of corolla tube to corolla lobe join point
Basal Corolla Tube Diameter	Diameter of corolla tube just above receptacle
Apical Corolla Tube Diameter	Diameter of corolla tube just below corolla lobe join point
Style Length	Base of flower to base of stigma
Anther Length	Base of flower to middle of anther
Stigma-Anther Separation	Difference between style length and anther height
Stigma Exsertion	Difference between flower length and style length
Anther Exsertion	Difference between flower length and anther height
Dry Mass	Mass of flower after drying about 1 month (field) or about 1 day in plant dryer (greenhouse)

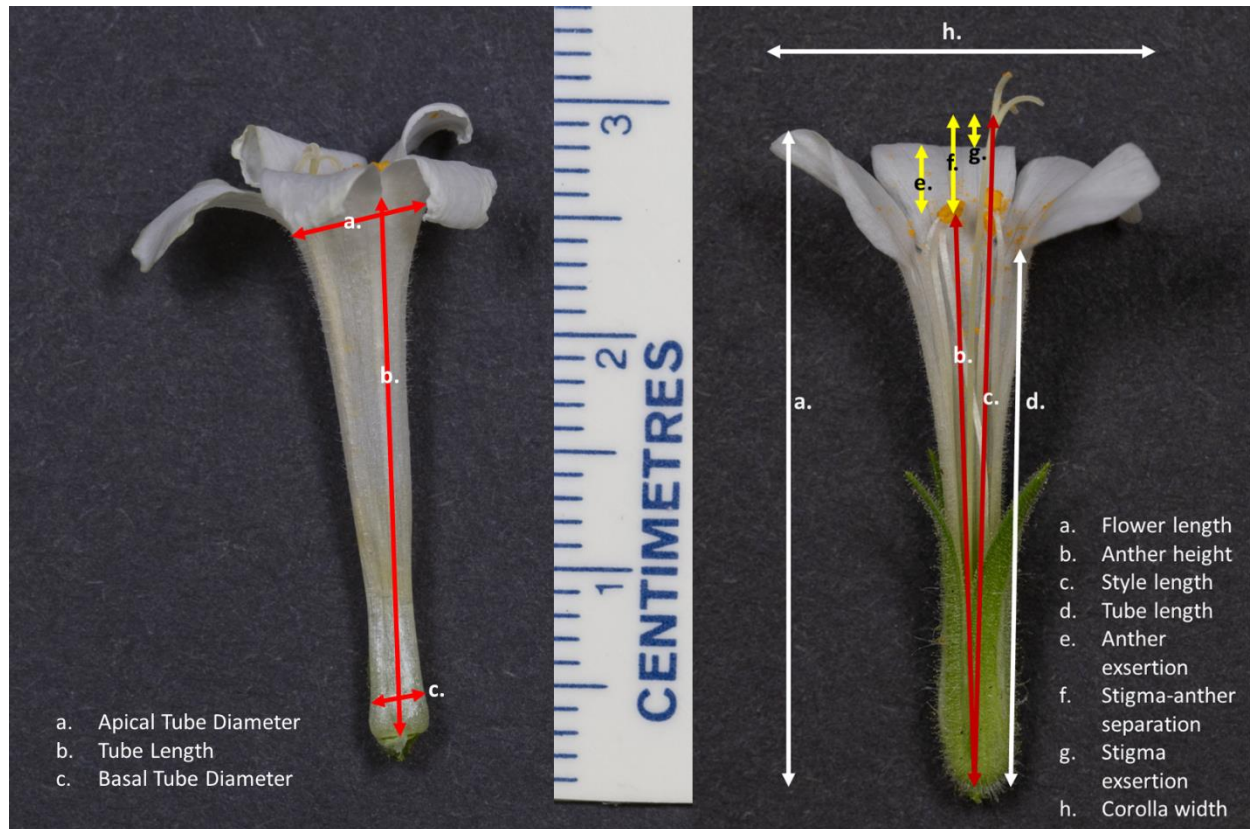


Figure 2.1: Visual representation of the 10 floral measurements taken on field and greenhouse grown *P. brandegeei* flowers. All measurements to 0.01 mm.

Floral Scent Collection

Thermal Desorption (TD) – GCMS (Greenhouse and Field)

I collected scent samples using dynamic headspace methods and analyzed them using thermal desorption and GCMS. To do so, I made scent traps out of melting point capillary tubes (Kimble) cut to 30 mm lengths with a glass cutter. I plugged each tube on one end using a small amount of silanized glass wool (Sigma) and used a vacuum pump to insert 5mg of 60/80 mesh Tenax TA (Sigma) sorbent into the trap. I inserted another piece of glass wool into the other side of the tube to keep the Tenax in place. I then cleaned traps and vials by soaking them in ethanol (30 mins) and acetone (30 mins). After drying them in a clean fume hood, I baked cleaned traps at 180 °C for 15 minutes to remove any remaining volatiles.

For sampling, I enclosed single flowers (*P. brandegeei*) in 5cm x 7cm oven bags (Reynolds Inc.) sealed on three edges with an impulse heat sealer (Figure 2.2). I attached bags to the plants and closed them loosely using plastic twist ties to create a headspace chamber (Figure 2.2). After a 40-minute equilibration period, a small slit was cut into the bag using metal scissors. I then inserted a scent trap connected by Tygon tubing to a vacuum pump (PAS-500 Personal Air Sampler; Supelco/Spectrex) into the slit and vacuumed headspace air through the trap for 20 minutes at a flow rate of about 200 ml/min (Figure 2.2). Following the 20-minute sampling period, I removed scent traps and placed them into clean 1ml amber vials (Sigma). I then removed sampled flowers from plants to determine dry mass. I stored samples at room temperature until GCMS analysis, which occurred about two - three weeks after sample collection. At least one ambient control sample and one leaf control sample were collected per sampling day. In total, 22 floral samples were collected from SC plants (10 greenhouse, 12 field), 23 from DM plants (10 greenhouse, 13 field), 17 from TC plants (6 greenhouse, 11 field), and 5 from VD plants in the field only. Greenhouse samples were collected in May 2023 and field samples were collected in June 2023.

I collected temperature, relative humidity, and windspeed data using a Kestrel (Kestrel Instruments) before and after the sampling period. I also noted soil type and moisture, as well as cloud cover and co-flowering species. Following collection, samples were stored at room temperature prior to analysis.

For GCMS analysis, I used the method of Campbell et al (2019). Briefly, a Markes Ultra autoloading system connected to a Markes Unity-xr was used to introduce volatiles to a Shimadzu GCMS QP2020 at RMBL. Each scent trap was placed inside a Markes sampling tube and loaded into the autosampler to undergo a two-stage desorption. The primary desorption was for 5 minutes at 250°C. The sample was then trapped at 25°C on a Tenax-filled cold trap, followed by heating rapidly to 200°C for 3 minutes. The GC oven cycle was held for 2 minutes at 40°C to re-trap volatiles, then ramped at 10°C / minute to 250°C and at 30°C / minute to 275 °C, with a final hold for 3 minutes. Ultra-high purity helium was used as the carrier gas, and an Rtx-5MS column (30 m x 0.25 mm internal diameter, 0.25 µm film thickness).

Full Evaporative Technique (FET) – GCMS (Greenhouse Samples)

To determine optimal FET parameters, I performed some initial test runs using greenhouse grown flowers. As FET requires heating of the plant/floral samples, I ran 2 whole flower samples at 80°C and 2 whole flower samples and 1 leaf sample at 30°C to see if headspace analysis under more natural conditions were possible. Samples analyzed at 30°C contained significantly fewer floral compounds than those run at 80°C, however (5 compounds total vs. 10 compounds total), and I thus decided to use the higher temperature method to get more comprehensive results.

I then ran 22 samples using the higher temperature method. I removed 10 flowers per floral sample from *P. brandegeei* plants using forceps and approximately two whole leaves for leaf samples. Nine out of 22 samples were whole flower samples, nine were flowers with calyxes removed, and four were leaf samples. One empty vial was also run as an ambient control. *P. brandegeei* samples were from plants from three populations (DM, TC, SC) and all flowers in each sample were fresh (not senescent) and from the same plant.

All samples were run at the Manitoba Analytical Solutions (MASS) Lab at the Richardson Centre for Food Technology and Research. Headspace sampling was conducted using an Agilent 8697 headspace analyzer. I placed whole flowers or leaves into 20ml glass headspace vials and crimp sealed the vials with caps containing PTFE/silicon septa (Agilent; Figure 2.2). The headspace analyzer parameters were as follows: oven temperature of 80°C, loop temperature of 80°C, and transfer line temperature of 140°C. Vials were equilibrated for eight minutes at 80°C to allow for the maximum volatilization of compounds. Vials were shaken at 25 shakes/min at 90 cm s² for two minutes at a fill pressure of 15 psi. A single extraction with a purge time of 3 minutes and a purge flow of 200 ml/min was used to transfer to the GCSM.

Headspace contents were transferred to an Agilent Intuvo 9000GC system through a multimode inlet set at 200°C and a septum purge flow of 3 ml/min in split mode at a 150:1 ratio at 210 ml/min. Dual columns (Agilent DB-Select 624HI 30m x 250 µm x 1.4 µm) were run with backflush through a linear temperature gradient starting at 35°C (hold one minute) to 250°C at 10°C per minute, then held at 250°C for 10 minutes before a four minute post-run bakeout at

260°C, for a total run time of 36.5 minutes. The transfer line to the MS (Agilent 5977B MDS) was set at 280°C and the MS scanned from 28.5 to 350 m/z at a frequency of 4.4 scans/sec with a gain factor of 10. The source was set at 230°C and the MS quad was set at 150°C.

Chromatograms were analyzed using MassHunter Unknowns Analysis 10.0 software (Agilent). Peaks were subjected to deconvolution and tentatively identified by comparing mass spectra to those of known standards in the NIST mass spectral library (version 20). Compounds were assigned a similarity to each possible match (minimum match factor of 50%). I manually integrated peaks using MassHunter to determine relative peak areas.

Pollinator Observations

In June 2023, I completed a total of 12.5 hours of pollinator observations in all four populations (Table 2.3). I performed observations on 4-7 patches of plants per population and selected patches for observation based on the number of plants in flower and on the number of open flowers available to pollinators in each patch. I typically observed patches for 10-minute intervals, but observation times per patch were sometimes up to one hour long. I identified visitors on the wing and assigned them IDs according to seven broad classes: small bee, small fly, large bee, large fly, butterfly or moth, hummingbird, and other insect. I caught voucher specimens of common insect visitors, killed them in ethyl acetate, preserved them in ethanol, and pinned them for formal identification. I collected temperature, windspeed, and relative humidity data before and after each observation period. I also collected microsite data, including co-flowering species, soil type (organic, gravel, or mix), and soil moisture (dry, damp, moist) for each observation patch. All observations were performed during the day (9:00 am – 4:00 pm).

Table 2.3: Total time spent on pollinator observations and the number of observation sites for each of three populations of *P. brandegeei*. In total, 12.5 hours of pollinator observations were completed during the 2023 field season.

Population	# Patches Observed	Total Observation Time (min)
SC	6	198
TC	4	208
DM	4	256
VD	7	104
TOTAL		766



Figure 2.2: Images depicting methods used in Chapter 2. **A:** leaf sample enclosed in headspace chamber with sorbent trap inserted; **B:** plant being sampled in the field (TC); **C:** plants being sampled in the field (SC); **D:** flower enclosed in oven bag "headspace chamber"; **E:** vials containing flower and leaf samples after FET analysis.

Statistical Analyses

All analyses were performed in R version 4.3.2 (R Core Team 2023) with floral scent analyses based on a review of methods by Eisen et al. (2022a).

Morphological Measurements

I removed flowers for which measurements were incomplete from the analysis (2 field flowers from SC). To determine if flower age affects morphology, I pooled the measurement data from all populations (field and greenhouse separately) and compared male (younger) and female (older) phase flowers using three methods. First, I used paired t-tests (by individual plant) to test for differences between male and female phase flowers in both the greenhouse and field for the 11 floral traits described above. I then used Spearman's correlations (which make fewer assumptions about the distribution of the data) to test for significant correlations between male and female phase flowers in both the greenhouse and field for the same 11 floral traits. I also compared the average difference between male and female flowers to the range of values for a given morphological trait to determine the relative scale of the difference.

Correlations between male and female phase flowers were significant for all 11 floral traits for both greenhouse and field flowers (see results) and the mean difference was always much smaller than the range. I therefore took the average of the male and female flower measurements and used this for analysis. In some cases, I measured two female or two male phase flowers from the same plant; those were averaged as well (5 plants total). Finally, I also included all measurements from plants where only one flower was measured (24 plants total). This resulted in a sample size of 76 flowers (from 15 DM plants, 15 TC plants, 15 VD plants, 31 SC plants) for the field and a sample size of 66 flowers (from 11 DM plants, 32 SC plants, 23 TC plants) for the greenhouse.

To determine if floral morphology varies among populations of *P. brandegeei* I used multivariate methods. First, to understand general patterns in the data, I used a PCA. Then, I used a redundancy analysis (RDA) with a Euclidean distance matrix to visualize differences among populations in ordinal space. As a constrained ordination method, RDA maximally separates groups (i.e., populations). I removed highly correlated variables ($r > 0.80$) prior to computing the RDA which left nine response variables in the RDA (corolla width, tube length,

basal diameter, apical diameter, stigma anther separation, style length, stigma exertion, anther exertion and dry mass). I conducted three RDA analyses: greenhouse data only, field data only, and combined field and greenhouse data. For greenhouse and field data only analyses, I checked the significance of “population” in the RDA using the “anova.cca” function in R with 999 permutations (permutation test similar to PERMANOVA). For the analysis of the combined data, I included both population and growth location (field or greenhouse) as effects. Then, to determine which morphological traits contributed to population differentiation in the greenhouse and field RDAs, I determined which traits were correlated with RDA axis one or two using Pearson’s correlation tests. All highly correlated traits had more positive or more negative RDA loadings along RDA1 or RDA2 than uncorrelated traits and therefore contributed most to population differentiation.

Floral VOCs

Using the “bouquet” package in R (Eisen et al. 2022a), the following filters were applied to the floral VOC dataset to filter out contaminants. To be included in the final list of compounds, VOCs had to 1) have retention times between 5 and 19 minutes (this excludes known contaminants); 2) have a minimum NIST mass spectral database match score of 80%; 3) be found in at least 10% of floral samples; and 4) have a maximum peak area of 1,000,000 counts. Compounds were also excluded if the mean amount in floral samples was not 4x or more the mean amount in ambient controls or if they failed a t-test with False Discovery Rate correction between floral and control samples ($\alpha = 0.05$). I also checked for the presence of all identified compounds in an authoritative floral scent database (Knudsen et al. 2006; www.pherobase.com). Known contaminants were manually excluded (see Eisen et al. 2022a). Compounds found in leaf samples were not excluded if they passed these filters. A full list of compounds found in leaf samples can be found in Appendix 2 for reference.

Kovat’s retention indices for all confirmed compounds were calculated using a C7-C25 alkane ladder and compound identifications were confirmed by comparing calculated Kovat’s retention indices to those in the NIST database. Compound identifications that could not be confirmed using Kovat’s RI are clearly marked in the compound list with an asterisk.

Quantification of VOCs was performed using a dilution series of standards (e.g., Bischoff et al. 2014). Six standards in methanol (representing the major compound classes) were run on the GCMS during the summer 2023 season at RMBL. Emission rates were calculated from calibration curves (all with $r^2 > 0.77$) created from several replicates of each standard pipetted onto traps. Emission rates for benzenoids were calculated using the calibration curve for methyl-2-hydroxybenzoate, for aliphatics using (Z)-hex-3-en-1-ol, for nitrogen containing compounds using indole, for sesquiterpenes using beta-caryophyllene or beta-farnesene, for cyclic monoterpenes using α -pinene, and for acyclic monoterpenes using linalool. Emission rates were standardized by sampling time (1 hour total) and by dry mass of flowers. Emission rates are presented in ng/hr/g in this analysis.

Differences in floral scent profiles among populations were visualized using constrained ordination in the form of a CAP (Canonical Analysis of Principal Components) with Bray-Curtis distances using the function “capscale” in the R package “vegan” (Okansen et al. 2023). CAP analysis is similar to RDA in that it is a constrained ordination, but it allows for the use of non-Euclidean distance matrices. A permutation test (using the “anova.cca” function) was used to determine if population has a significant effect on qualitative VOC profiles (see above). Three CAP plots were created, one with field data only, one with greenhouse data only, and one with combined data. VOC emission rates were square root transformed before analysis to reduce skew.

To determine which VOCs contributed most to population differentiation in the greenhouse and field CAP analyses, I determined which traits were correlated with CAP axis one or two using Pearson’s correlation test (as above). I also performed an additional CAP analysis after removing the top three most abundant compounds from the dataset to see if the pattern of population differentiation remained after removing the most abundant compounds that appeared to drive the differences among populations.

Lastly, to determine if environmental and experimental conditions explained any variation in the floral scent data, I analyzed the floral scent environmental and experimental data using an NMDS. The NMDS separated the populations along NMDS axis 1. Relative humidity contributed most strongly to population differentiation, but this was caused by high

humidity at time of sampling for three DM samples only (Appendix 3). Temperature, windspeed and percent cover were correlated with NMDS2 and contributed to within population variation (Appendix 3). As this study is primarily concerned with overall differences among populations, and since populations separated approximately equally in the NMDS, I did not include environmental variables in further analyses.

To compare results of the FET – GCMS sampling and analysis method to the dynamic headspace sampling with TD – GCMS analysis method I performed another CAP analysis of the FET data. I performed similar filtering steps to the FET data as I did to the TD data but was unable to quantify the FET data due to the lack of standards for calculation of emission rates. Instead, I used relative percentages for the FET analyses. I then used a permutation test to determine if population had a significant effect on the FET data.

Pollinator Observations

I did not perform statistical analyses on my pollinator observation data due to small sample sizes and limited observation time.

RESULTS

General Summary

Four populations of *P. brandegeei* differed in morphology and floral scent emissions according to multivariate analyses. The New Mexico population (SC) was distinct from all other populations in all analyses. The Colorado (TC, DM) and Wyoming (VD) populations were more similar overall, although there were some differences in morphology and floral scent between them. All major patterns of population differentiation were maintained in the greenhouse which suggests a heritable component to the observed interpopulation variation. However, there was a clear greenhouse effect; greenhouse flowers were consistently larger and produced consistently more VOCs than field flowers which suggests that floral traits are also plastic (Figure 2.3). Lastly, two different VOC sampling and analysis methods produced similar results.

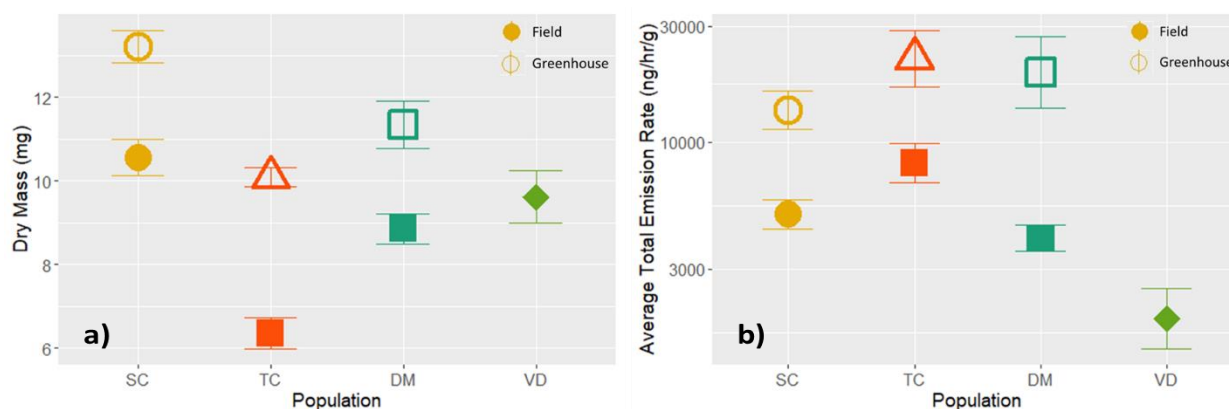


Figure 2.3: Mean (+ SE) (a) dry mass (mg) of flowers and (b) average total emission rate (ng/hr/g) of flowers in four field populations of *P. brandegeei* in New Mexico (SC), Colorado (TC, DM) and Wyoming (VD) and three populations grown in the greenhouse (SC, TC, DM).

Morphological Measurements – Effects of Flower Age

There was a significant correlation between male and female phase flowers for all floral measurements in both the greenhouse and field ($r_s = 0.49-0.95$; $P < 0.001$; Appendix 4). Also, the mean difference between male and female phase flowers was <10% of than the range of values for all traits (Appendix 4). For example, the mean difference in stigma-anther separation for female and male phase flowers was 0.57 mm (F: 0.71 ± 0.39 mm; M: 1.28 ± 0.31 mm) while the range was -7.39 – 5.70 mm across all measured flowers. Based on this, I averaged the measurements of male and female phase flowers for analysis.

Is there variation in floral morphology across four populations of *P. brandegeei* and is this variation retained in the greenhouse?

As predicted, the RDA analyses showed distinct clustering of most populations in the field data (Figure 2.4a). SC separated from VD, TC, and DM along RDA1 in the field and RDA1 explained 24 % of the variation in the field floral measurements (Figure 2.4a). TC and DM separated along RDA2 which explained 11% of the variation in the field floral measurements (Figure 2.4a). VD measurements did not separate distinctly from DM in the field data (Figure 2.4a). The separation of populations in the RDA of the field data represents a significant effect of population ($F_{3,72} = 15.34$, $P < 0.001$; Table 2.4) which explained 39% of the variation in floral morphology in the field.

A similar pattern emerged in the greenhouse data, as predicted (Figure 2.4b). SC also separated from DM and TC along RDA1 which explained 23% of the variation in the greenhouse floral measurements (Figure 2.4b). Although the orientation of populations shifted, TC and DM also separated along RDA2 in the RDA of greenhouse data and RDA2 explained 6% of the

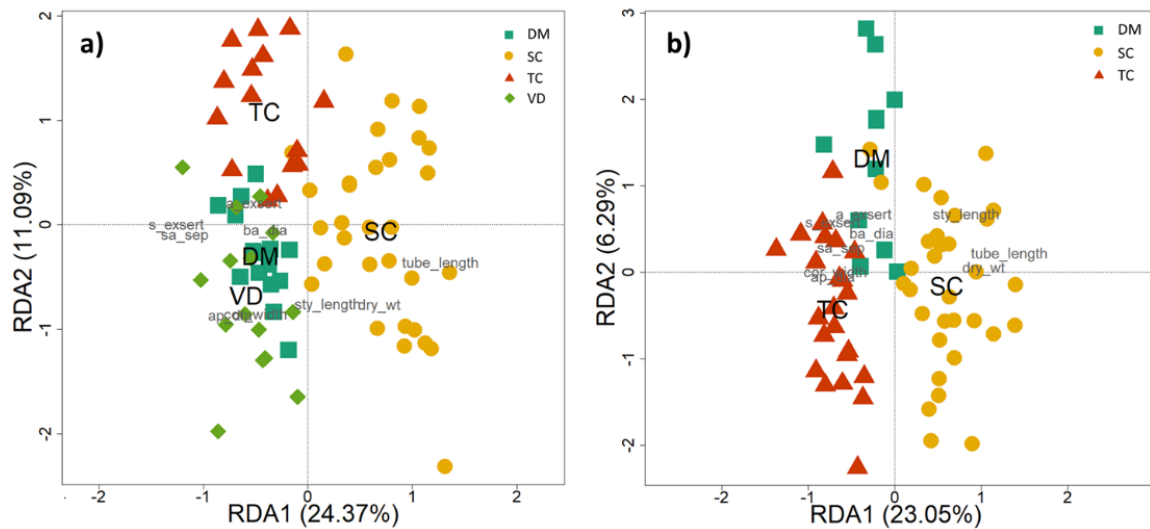


Figure 2.4: RDA biplots of nine morphological measurements for (a) flowers in field populations of *P. brandegeei* in New Mexico (SC), Colorado (TC, DM), and Wyoming (VD) and (b) greenhouse grown flowers from three populations of *P. brandegeei* (SC, TC, DM). (*tube_length* = corolla tube length, *dry_wt* = dry mass, *sty_length* = style length, *ap_dia/ba_dia* = apical/basal corolla tube diameter, *sa_sep* = stigma-anther separation, *cor_width* = corolla tube diameter, *a_/s_exsert* = anther/stigma exertion.)

variation in the data (Figure 2.4b). Finally, there was also a significant population effect on the greenhouse data ($F_{2,63} = 13.08$, $P < 0.001$; Table 2.4) in which population explained 29% of the variation.

Further, these patterns of population differentiation were conserved in the RDA analysis of the combined greenhouse and field data (Figure 2.5). SC again separated from TC and DM along RDA1, which explained 20% of the variation in the data, and TC and DM separated along RDA2, which explained 12% of the variation (Figure 2.5). There was also a significant population effect in the combined data ($F_{2,123} = 22.76$, $P < 0.001$; Table 2.5) and population explained 24% of the variation. There was a significant greenhouse effect in the combined data ($F_{1,123} = 19.02$, $P < 0.001$; Table 2.4; Figure 2.5) and growth location explained 10% of the variation in floral morphology. Flowers in the greenhouse were larger (Figure 2.3a) and there was a significant increase in size for 6/11 traits in the greenhouse. Of the remaining five traits, all showed a (non-significant) increase in size; the exception was corolla width which decreased non-significantly in the greenhouse due to increased recurving of corolla lobes on SC flowers in the greenhouse.

Table 2.4: Statistics from “anova.cca” permutation test for the effects of population and/or growth location on nine morphological measurements taken on *P. brandegeei* flowers in field populations in New Mexico (SC), Colorado (TC, DM), and Wyoming (VD) and the same three populations grown in the greenhouse (SC, TC, DM). Separate field and greenhouse and a combined analysis were performed. R^2 = proportion of the variance explained by factor of interest (= variance/total variance).

Location	Term	df	Variance	F	<i>p</i>	R^2
Field	Population	3	3.51	15.34	<0.001	0.39
	Residual	72	5.49			
Greenhouse	Population	2	2.64	13.08	< 0.001	0.29
	Residual	63	6.36			
Combined	Population	2	2.14	22.76	<0.001	0.24
	Growth Location	1	0.92	19.02	<0.001	0.10
	Residual	123	5.93			

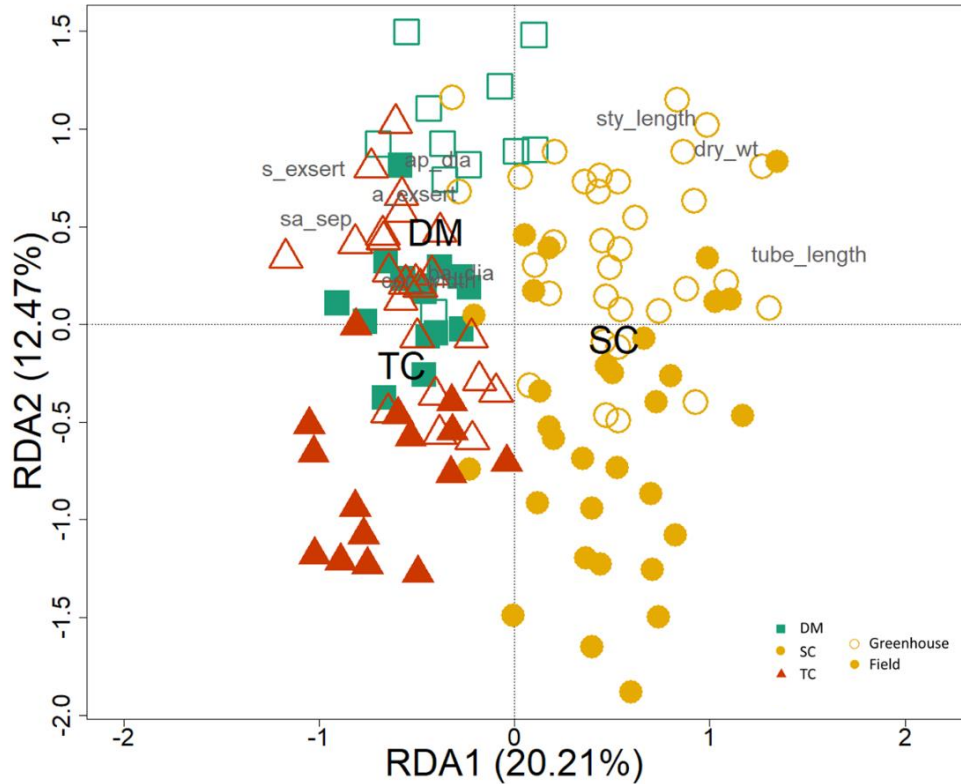


Figure 2.5: RDA biplot of nine morphological measurements from field grown and greenhouse grown flowers from three populations of *P. brandegeei* in New Mexico (SC) and Colorado (TC, DM). (*tube_length* = corolla tube length, *dry_wt* = dry mass, *sty_length* = style length, *ap_dia/ba_dia* = apical/basal corolla tube diameter, *sa_sep* = stigma-anther separation, *cor_width* = corolla tube diameter, *a/s_exsert* = anther/stigma exsertion.)

The field, greenhouse, and combined data were also similar in the correlation analyses, in further support of predictions. Tube length and dry mass were most strongly negatively correlated with RDA1 in all ordinations (tube length: all $r \leq -0.86$; dry mass: all $r \leq -0.51$; Table 2.5). These strong correlations relate to a general trend towards longer flowers at SC compared to DM, VD and TC (Figure 2.6a). Indeed, corolla tubes and dry mass of SC flowers were at least 1.3x greater than the same traits in all other populations (Figure 2.6a). On the other hand, stigma exsertion and stigma-anther separation were most strongly positively correlated with RDA1 in all ordinations (stigma exsertion all $r \geq 0.61$; stigma-anther separation: all $r \geq 0.57$; Table 2.5). This pattern corresponds to more recessed stigmas and reverse herkogamy (stigma exserted below anthers) at SC and to more exserted stigmas and approach herkogamy (stigma exserted beyond anthers) at DM and TC (Figure 2.6b). Stigmas on flowers from other populations were at least 2.7x more exserted than stigmas on flowers from SC.

Table 2.5: Correlations of the nine floral traits with RDA1 and RDA2 in the analysis of field data (four populations in New Mexico (SC), Colorado (TC, DM) and Wyoming (VD)), greenhouse data (three populations - SC, TC, DM), and combined field and greenhouse data (three populations – SC, TC, DM).

Measurement	Field Correlation with RDA1/RDA2	Greenhouse Correlation with RDA1/RDA2	Combined Correlation with RDA1/RDA2
Tube Length	-0.86 / 0.46	-0.92 / -0.17	-0.92 / 0.30
Dry Mass	-0.51 / 0.79	-0.67 / 0.02	-0.65 / 0.56
Apical Diameter	0.33 / 0.73	0.39 / 0.20	0.06 / 0.58
Anther Exsertion	0.41 / -0.26	0.19 / -0.67	0.31 / 0.37
Stigma/Anther Separation	0.84 / 0.00	0.57 / -0.41	0.72 / 0.42
Stigma Exsertion	0.92 / -0.12	0.61 / -0.75	0.76 / 0.54
Style Length	-0.14 / 0.73	-0.53 / -0.67	-0.37 / 0.80
Corolla Width	0.20 / 0.70	0.40 / -0.13	0.14 / 0.22
Basal Diameter	0.24 / 0.30	0.27 / -0.35	0.12 / 0.33

Dry mass, style length, and apical diameter (all $r \geq 0.73$; Table 2.5) were most strongly correlated with RDA2 for the field data. This further emphasizes the general trend of flower size differences among populations, with TC flowers being smaller than flowers at both DM and SC (Figure 2.3a, 2.6a). Dry mass of flowers from other populations was at least 1.3x higher than dry mass of TC flowers. The combined data displayed a similar pattern to the field data, and style length, apical diameter, and dry mass were also most strongly correlated with RDA2 (all $r \geq 0.56$; Table 2.5). In the greenhouse data, style length, anther exsertion, and stigma exsertion were strongly correlated with RDA2 (all $r \leq -0.67$; Table 2.5). Due to different orientations of the populations in the greenhouse RDA, these negative correlations correspond to a similar trend regarding larger flower size at DM and SC, as well as to an increase in stigma and anther exsertion on DM flowers compared to flowers from TC or SC (Figure 2.6a). Stigmas on DM flowers were more than 2.9x more exerted than stigmas on flowers from other populations.

Finally, iNaturalist records from several additional populations support my findings of significant latitudinal variation in morphology in *P. brandegeei*. There is apparently a geographic cline in flower colour and shape ranging from long, bright yellow flowers in southern populations (including SC) to shorter, cream-coloured flowers in central populations to wider, pure white flowers in the most northern populations (Figure 2.7).

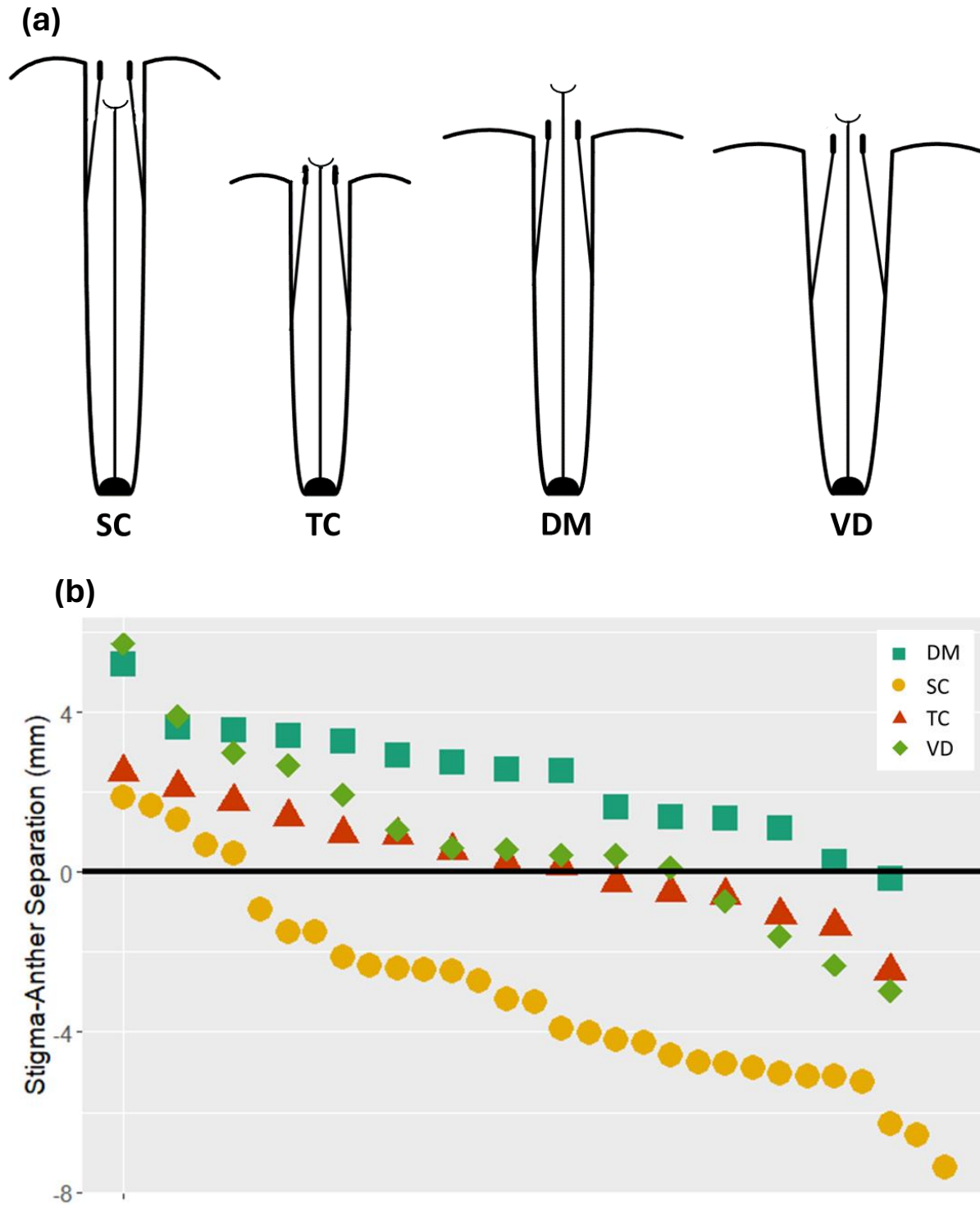


Figure 2.6: (a) To scale line drawings (by average measurement in mm) and (b) ranked plot of stigma-anther separation (herkogamy) of flowers from four field populations of *P. brandegeei* in New Mexico (SC), Colorado (TC, DM) and Wyoming (VD). Negative values for (b) indicate stigmas recessed below anthers while positive values indicate anthers exerted beyond stigmas.

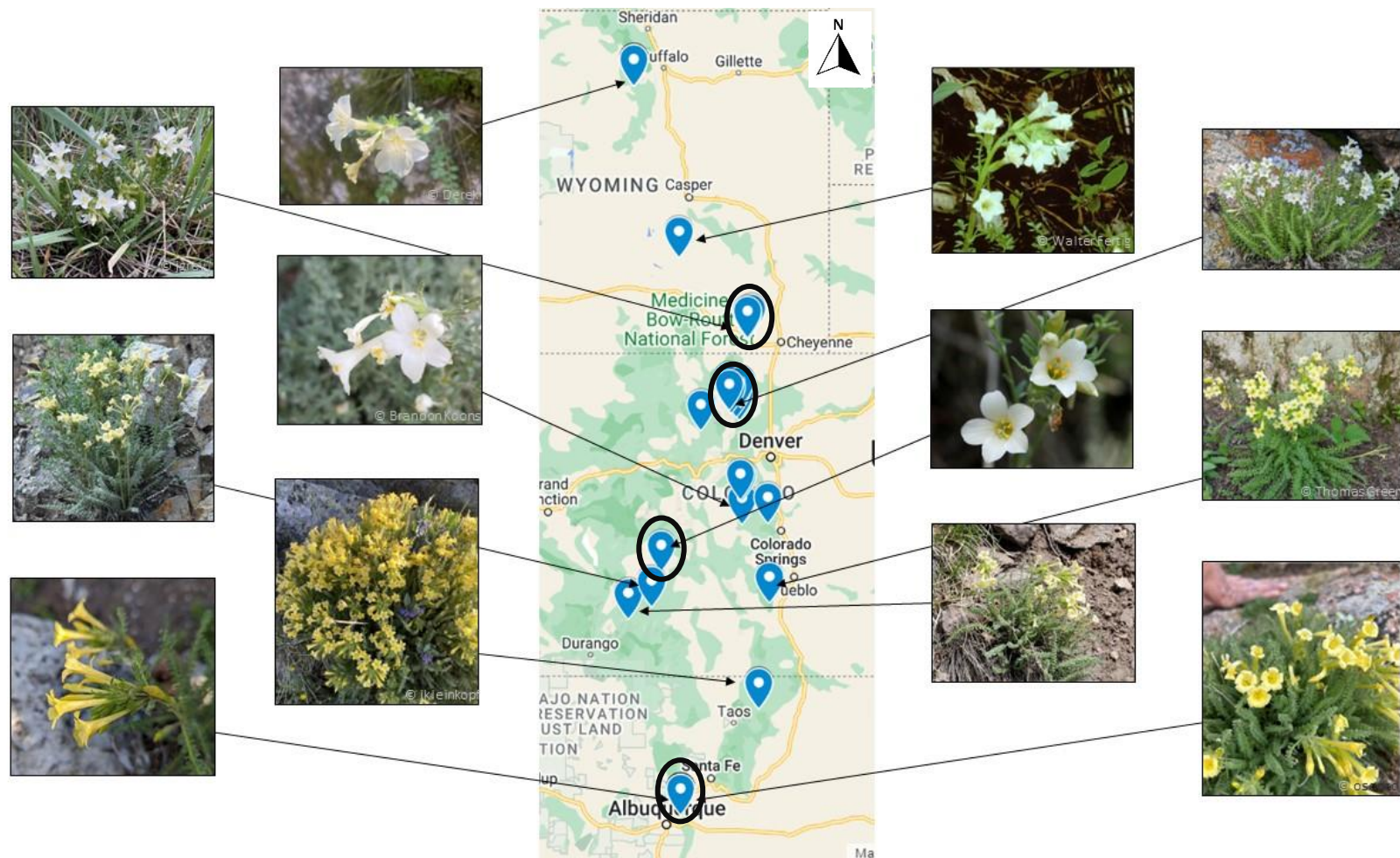


Figure 2.7: Compilation of (research grade) photos from iNaturalist displaying a latitudinal gradient in flower colour (and shape) across the range of *P. brandegeei*. Study sites circled in black (from south to north: SC, TC, DM, VD).

Floral VOCs - Overview

34 VOCs were identified in the floral scent of *P. brandegeei* after the filtering steps were applied (Table 2.6). The VOC profile was dominated by benzenoid compounds (18/34; 75% of all field emissions), with the remaining compounds divided among aliphatics (5/34; 8% of all field emissions), monoterpenes (5/34; 12% of all field emissions), sesquiterpenes (4/34; 3% of all field emissions), and nitrogen-containing compounds (2/34; 2% of all field emissions). Average total emission rate (mean + SE) in the greenhouse samples ($22,658 \pm 3124$ ng/hr/g; Figure 2.2b) was considerably higher than in the field ($5,876 \pm 646$ ng/hr/g; Figure 2.3b).

Is there variation in floral scent emissions among four populations of *P. brandegeei*, and is this variation maintained in the greenhouse?

First, populations differed in average total emission rate (Figure 2.3b). In the field, TC flowers had the highest total emission rate (9621 ± 1696 ng/hr/g) followed by SC (5580 ± 718 ng/hr/g), DM (4388 ± 513 ng/hr/g), and VD (2214 ± 660 ng/hr/g). In the greenhouse, TC flowers also had the highest emission rates, but SC flowers had lower emission rates than DM flowers (Figure 2.3b).

In support of predictions, CAP analysis of the 34 floral VOCs detected in the 41 field samples showed some separation of populations (Figure 2.8a). SC samples separated from DM and TC samples along CAP1 which explained 18% of the variation in the data (Figure 2.8a). VD samples separated from all other samples and DM and TC samples separated somewhat along CAP2 which explained 8% of the variation (Figure 2.8a). These visual differences were confirmed by the permutation test which found a significant effect of population in the CAP analysis of the field data ($F_{3,37} = 5.38$, $P < 0.001$; Table 2.7). Population explained 30% of the variation in the field VOC data (Table 2.7).

As predicted, this separation of populations was retained in the CAP analysis of the greenhouse data (Figure 2.8b). SC samples also separated from TC and DM samples along CAP1 in this analysis and CAP1 also explained a higher proportion of the variation in the data (31%; Figure 2.8b). There was a stronger separation of TC and DM samples along CAP2 but CAP2 only explained 4% of the variation in the greenhouse samples (Figure 2.8b). As in the

Table 2.6: List of 34 VOCs making up the floral scent profile of *P. brandegeei*. CAS# = CAS registry number; match % = compound match; Kovat's RI = Kovat's retention index. Compounds with an asterisk (*) could not be confirmed with Kovat's RI.

Class	Compound	CAS#	Match %	Kovat's RI	Mean Relative Content (%)		Mean Emission Rate (ng/hr/g)	
					Field	Greenhouse	Field	Greenhouse
Aliphatic	3-Hexen-1-ol	928-96-1	90%	855.21	0.97 ± 0.36	0.83 ± 0.36	64 ± 29	126 ± 67
	Hexenyl Acetate	1708-82-3	96%	1006.07	3.33 ± 0.88	3.62 ± 1.17	222 ± 60	455 ± 137
	Hexyl Acetate	142-92-7	87%	1012.17	0.03 ± 0.01	0.04 ± 0.02	3 ± 1	7 ± 3
	2-Nonadecanone*		84%	1137.37	2.23 ± 1.09	0.23 ± 0.15	98 ± 62	56 ± 48
	Tetradecanoic Acid	544-63-8	89%	1759.72	1.33 ± 0.62	0.20 ± 0.10	70 ± 39	44 ± 24
Benzenoid	Benzaldehyde	100-52-7	98%	962.05	4.41 ± 0.41	2.25 ± 0.28	215 ± 20	496 ± 96
	Benzyl Alcohol	100-51-6	96%	1035.202	13.84 ± 1.54	8.03 ± 1.57	800 ± 121	2400 ± 664
	Phenylacetaldehyde	122-78-1	91%	1044.74	0.49 ± 0.10	0.22 ± 0.04	26 ± 6	42 ± 7
	Methyl Benzoate	93-58-3	98%	1094.62	11.67 ± 2.48	14.75 ± 4.23	655 ± 182	2285 ± 759
	Phenylethyl Alcohol	60-12-8	95%	1115.18	22.15 ± 2.70	33.85 ± 4.98	1320 ± 275	9554 ± 2234
	Benzyl Nitrile	140-29-4	82%	1137.31	0.51 ± 0.13	0.04 ± 0.04	29 ± 7	8 ± 8
	Benzyl Acetate	140-11-4	92%	1161.83	0.15 ± 0.05	0.14 ± 0.03	7 ± 2	42 ± 11
	Methyl Phenylacetate	101-41-7	93%	1175.45	0.62 ± 0.16	0.87 ± 0.24	37 ± 9	197 ± 53
	3-Phenylpropanol	122-97-4	87%	1229.58	0.23 ± 0.06	0.26 ± 0.12	13 ± 3	64 ± 35
	Benzeneacetic Acid	103-82-2	97%	1247.99	2.22 ± 0.39	1.43 ± 0.61	95 ± 17	318 ± 149
	Cinnamaldehyde	14371-10-9	95%	1270.93	1.51 ± 0.28	2.47 ± 0.66	89 ± 18	592 ± 207
	Cinnamyl Alcohol	104-54-1	91%	1304.92	4.20 ± 0.94	3.88 ± 1.28	325 ± 110	846 ± 235
	Methyl Anthranilate	134-20-3	93%	1346.06	0.04 ± 0.02	0.54 ± 0.20	4 ± 2	88 ± 31
	Eugenol	97-53-0	87%	1360.47	3.03 ± 0.58	0.77 ± 0.19	168 ± 30	264 ± 82
	Methyl Cinnamate	103-26-4	96%	1388.27	5.96 ± 1.52	1.29 ± 0.79	410 ± 120	313 ± 187
	2-Amino Benzaldehyde*		87%	1413.03	0.44 ± 0.12	0.55 ± 0.16	28 ± 7	113 ± 37
	Cinnamyl Acetate	103-54-8	93%	1449.72	0.13 ± 0.03	0.28 ± 0.08	9 ± 3	55 ± 16
	Benzyl Benzoate	120-51-4	94%	1782.77	3.31 ± 0.68	3.35 ± 1.13	169 ± 29	661 ± 234
Monoterpene	α-Pinene	80-56-8	96%	636.78	8.80 ± 1.83	3.48 ± 1.12	441 ± 98	464 ± 108
	Sabinene	3387-41-5	94%	976.42	0.62 ± 0.13	0.92 ± 0.49	36 ± 7	92 ± 32
	β-Pinene	127-91-3	86%	980.68	1.95 ± 1.05	2.42 ± 1.02	154 ± 85	433 ± 181
	β-Myrcene	123-35-3	93%	991.65	1.18 ± 0.36	1.74 ± 0.38	63 ± 16	246 ± 37
	Linalool Oxide (Furanoid)	34995-77-2	89%	1087.80	0.08 ± 0.03	0.39 ± 0.17	5 ± 2	56 ± 15
N-Compound	Methyl Nicotinate	93-60-7	91%	1137.53	0.20 ± 0.08	1.24 ± 0.25	17 ± 9	241 ± 55
	Indole	120-72-9	96%	1293.80	1.60 ± 0.28	3.37 ± 0.50	117 ± 26	827 ± 140
Sesquiterpene	Longifolene	475-20-7	93%	1425.10	0.06 ± 0.04	0.11 ± 0.07	7 ± 5	22 ± 14
	α-Copaene*		87%	1431.28	2.31 ± 0.86	1.27 ± 0.59	150 ± 58	223 ± 105
	Caryophyllene	87-44-5	92%	1436.73	0.05 ± 0.03	0.44 ± 0.38	4 ± 2	83 ± 74
	β-Farnesene	28973-97-9	92%	1460.23	0.33 ± 0.11	4.74 ± 1.18	24 ± 7	944 ± 298

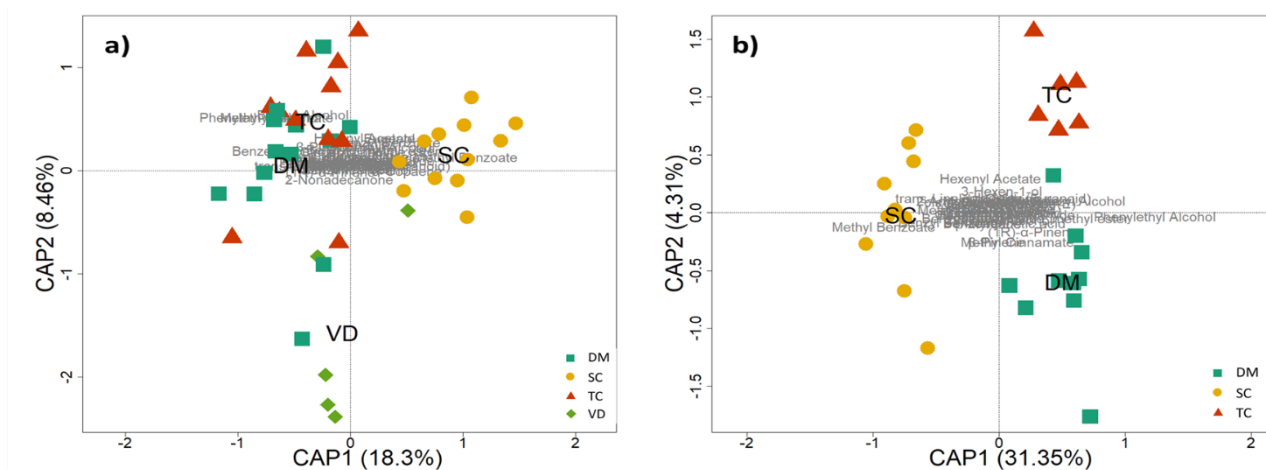


Figure 2.8: CAP biplots of the emission rate (ng/hr/g) of 34 floral VOCs in (a) field populations of *P. brandegeei* in New Mexico (SC), Colorado (TC, DM) and Wyoming (VD); and (b) three populations of *P. brandegeei* grown in the greenhouse (SC, TC, DM).

field data, there was a significant effect of population in the CAP analysis of the greenhouse data ($F_{2,23} = 6.38$, $P < 0.001$; Table 2.7). Population explained 36% of the variation in the greenhouse data (Table 2.7).

The major population differences observed in the separate field and greenhouse CAP analyses were maintained in the CAP analysis of the combined greenhouse and field data (Figure 2.9). In the combined analysis, CAP1 explained 18% of the variation in the data and SC samples also separated from TC and DM samples along CAP1 (Figure 2.9). There was partial separation of TC and DM samples along CAP2, which explained 13% of the variation in the combined data (Figure 2.9). However, there was a clear greenhouse effect on floral scent emissions; average total scent emission rate in greenhouse samples were 3.9x higher than in field samples (Figure 2.2b). Growth location (greenhouse or field) had a significant effect on the combined data ($F_{1,58} = 10.94$, $P < 0.001$; Table 2.7) and growth location explained 13% of the variation in the data. Finally, there was also a significant effect of population on the combined data ($F_{2,58} = 8.86$, $P < 0.001$; Table 2.7) and population explained 20% of the variation in the combined data. There was also some evidence of within population variation in all analyses (Figure 2.8, 2.9).

The field and greenhouse analyses also showed similar patterns with respect to specific compounds. Three compounds, phenylethyl alcohol, methyl benzoate, and benzyl alcohol, made up an average total of 56.57% of field samples and 46.50% of greenhouse samples (Table 2.6; Figure 2.10). All three compounds are common floral volatiles, but methyl benzoate is often particularly abundant in the floral scent of hawkmoth pollinated plants (Knudsen et al. 2006; Dotterl & Gershenzon 2023), which supports the prediction that the floral scent of *P. brandegeei* contains both hawkmoth specific attractants and generalist compounds.

The three most abundant compounds were important to the separation of the populations in the CAP analyses of both the field and greenhouse data. All correlations are presented with field analysis first, greenhouse second, and combined last. Methyl benzoate was strongly correlated with CAP1 in all ordinations ($r = 0.76, -0.70, -0.63$, respectively; Table 2.8) which represents the higher emission of methyl benzoate by SC flowers (Figure 2.10). Phenylethyl alcohol was strongly correlated with CAP1 in the field and greenhouse analyses ($r = -0.50, 0.64$; Table 2.8) and with CAP2 in the field and combined analyses ($r = -0.48, 0.69$; Table 2.8). This represents the increased emission of phenylethyl alcohol by TC and DM flowers

Table 2.8: Statistics for correlation tests with CAP1/CAP2 for floral VOCs collected from field plants in populations of *P. brandegeei* in New Mexico (SC), Colorado (TC, DM) and Wyoming (VD), from greenhouse plants from three populations (SC, TC, DM), and field and greenhouse plants combined from three populations (SC, TC, DM). Only VOCs with $r > 0.60$ for one analysis included in the table.

Compound	Correlation with CAP1/CAP2 Field	Correlation with CAP1/CAP2 Greenhouse	Correlation with CAP1/CAP2 Combined
Methyl Benzoate	0.76 / 0.10	-0.70 / 0.06	-0.63 / -0.13
α -Copaene	0.65 / 0.18	-0.54 / -0.03	-0.57 / -0.05
Benzyl Benzoate	0.64 / 0.54	-0.56 / 0.00	-0.48 / -0.20
α -Pinene	-0.10 / 0.08	0.60 / -0.31	0.35 / -0.28
Eugenol	0.62 / 0.48	0.30 / 0.08	-0.11 / -0.32
Methyl Anthranilate	0.04 / 0.19	-0.65 / 0.18	-0.47 / -0.28
Benzaldehyde	0.00 / 0.71	0.47 / 0.49	0.18 / -0.60
Benzyl Acetate	-0.29 / 0.13	0.60 / 0.33	0.27 / -0.59
Phenylethyl Alcohol	-0.50 / -0.48	0.64 / 0.16	0.26 / -0.69
Benzyl Alcohol	-0.37 / 0.65	0.57 / 0.32	0.30 / -0.57
Methyl Phenylacetate	-0.46 / 0.51	0.60 / -0.03	0.33 / -0.54
Indole	0.47 / 0.59	-0.00 / 0.47	-0.22 / -0.74

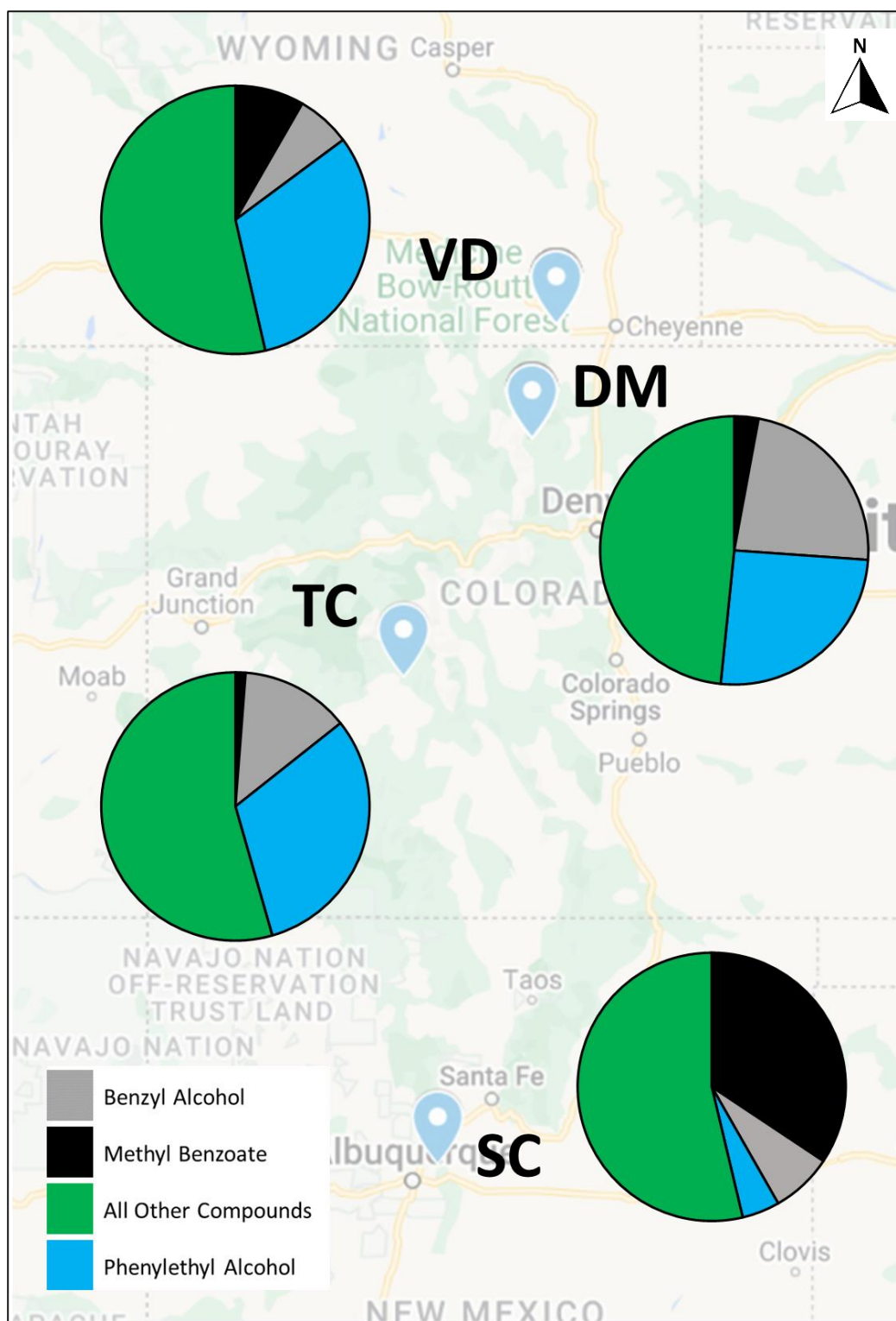


Figure 2.10: Pie charts of the three VOCs with the highest emission rates (benzyl alcohol, methyl benzoate, and phenylethyl alcohol; by average ng/hr/g) and all other compounds together for field populations of *P. brandegeei* in New Mexico (SC), Colorado (TC, DM), and Wyoming (VD).

compared to SC flowers; TC and DM flowers emitted at least 7.5x more phenylethyl alcohol than SC flowers (Figure 2.10). Finally, benzyl alcohol was strongly correlated with CAP2 in the field and combined analyses ($r = 0.65, -0.57$; Table 2.8), and with CAP1 only in the greenhouse analysis ($r = 0.57$; Table 2.8). This represents the higher amount of benzyl alcohol found in TC and DM flowers compared to SC flowers (Figure 2.10) and to the slightly higher amounts of benzyl alcohol in TC flowers compared to DM flowers.

Alongside differences in emission of these three major compounds among populations, several other VOCs also displayed strong correlations with CAP1 and/or CAP2 in all analyses. For example, α -copaene was strongly correlated with CAP1 in all analyses ($r = 0.65, -0.55, -0.57$; Table 2.8) due to the complete lack of α -copaene in TC and DM flowers. Methyl phenylacetate was strongly correlated with CAP1 in the greenhouse analysis ($r = 0.60$; Table 2.8) and with CAP2 in the field and combined analyses ($r = 0.51, -0.54$; Table 2.8). This corresponds to increased emission of methyl phenylacetate by DM and TC flowers. Indole was strongly correlated with CAP2 in all analyses ($r = 0.59, 0.47, -0.74$) which is related to 1) lower indole emission by VD flowers (only in 1/5 flowers) and 2) higher overall indole emissions in the greenhouse compared to the field. Benzyl benzoate emissions were higher in SC flowers than TC and DM flowers which explains the strong correlation with CAP1 in all analyses ($r = 0.64, -0.56, -0.57$; Table 2.8). Finally, benzaldehyde was strongly correlated with CAP1 and CAP2 in the greenhouse analyses (both $r \geq 0.47$; Table 2.8), and with CAP2 in the field and combined analysis ($r = 0.71, -0.60$; Table 2.8) which corresponds to a higher benzaldehyde emission by TC flowers compared to both DM and SC flowers in the greenhouse (but not the field), to especially low benzaldehyde emission in VD flowers in the field and to significantly lower overall benzaldehyde emissions in the field. These compounds represent a mixture of common floral volatiles (α -copaene, benzaldehyde) and hawkmoth specific compounds (indole), as predicted (Dotterl & Gershenzon 2023).

Conversely, other VOCs were strongly correlated with CAP1 or CAP2 in the field or greenhouse analyses only. Eugenol was strongly correlated with CAP1 in the field analysis only ($r = 0.62$; Table 2.8) which is due to higher eugenol emissions by SC flowers in the field only. Similarly, α -pinene, methyl anthranilate, and benzyl acetate were strongly correlated with CAP1

in the greenhouse analyses only ($r = 0.60, -0.65, 0.60$, respectively; Table 2.8). This relates to decreased α -pinene emissions by SC flowers in the greenhouse (but not field), a pronounced decrease in benzyl acetate emissions by SC flowers in the greenhouse only, and to more greenhouse flowers containing methyl anthranilate than field flowers (9/26 greenhouse flowers vs. 5/41 in the field).

Removal of the three most common compounds from the field dataset further illustrated the role less abundant compounds play in the separation of populations in the CAP analysis (Figure 2.11). Although population explained less of the variation in the analysis of the minor compounds (CAP1: 10.97%, CAP2: 6.33%; Figure 2.11), the pattern of population differentiation remained similar. Several different compounds were especially highly correlated with either CAP1 or CAP2 in the reduced analysis such as eugenol ($r = 0.68$), α -copaene ($r = 0.68$), and benzyl benzoate ($r = 0.66$) with CAP1 and benzaldehyde ($r = 0.57$) and methyl cinnamate ($r = 0.51$) with CAP2. These represent more subtle population differences in emission of these compounds among the respective populations.

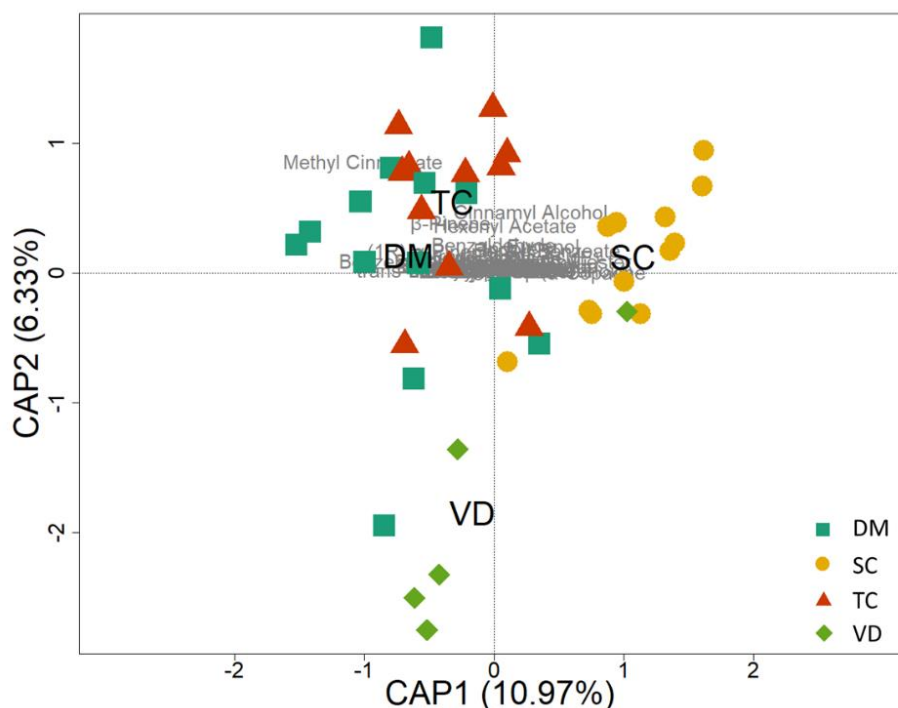


Figure 2.11: CAP biplot of the 31 least abundant VOCs in 41 field samples (by emission rate in ng/hr/g) from field populations of *P. brandegeei* in New Mexico (SC), Colorado (TC, DM), and Wyoming (VD); the most abundant VOCs (phenylethyl alcohol, methyl benzoate, benzyl alcohol) were removed to determine if less abundant volatiles also contribute to population differentiation.

Do results from FET-GCMS match results from TD-GCMS?

To facilitate a comparison of two floral VOC sampling and analysis techniques, I compared the results of the rarely used full evaporative technique (FET) – GCMS to the commonly used dynamic headspace sampling with thermal desorption (TD) – GCMS. The FET technique yielded similar results overall, and major patterns in the TD-GCMS data were preserved, as predicted (Figure 2.12). For example, SC flowers separated from TC and DM flowers along CAP1 in the FET data as well, and this differentiation was also driven by higher amounts of methyl benzoate in the SC flowers; CAP1 explained 29.04% of the variation in the data (Figure 2.12). The TC and DM flowers were largely distinguishable along CAP2; however, CAP2 only explained 5.3% of the variation in the data (Figure 2.12).

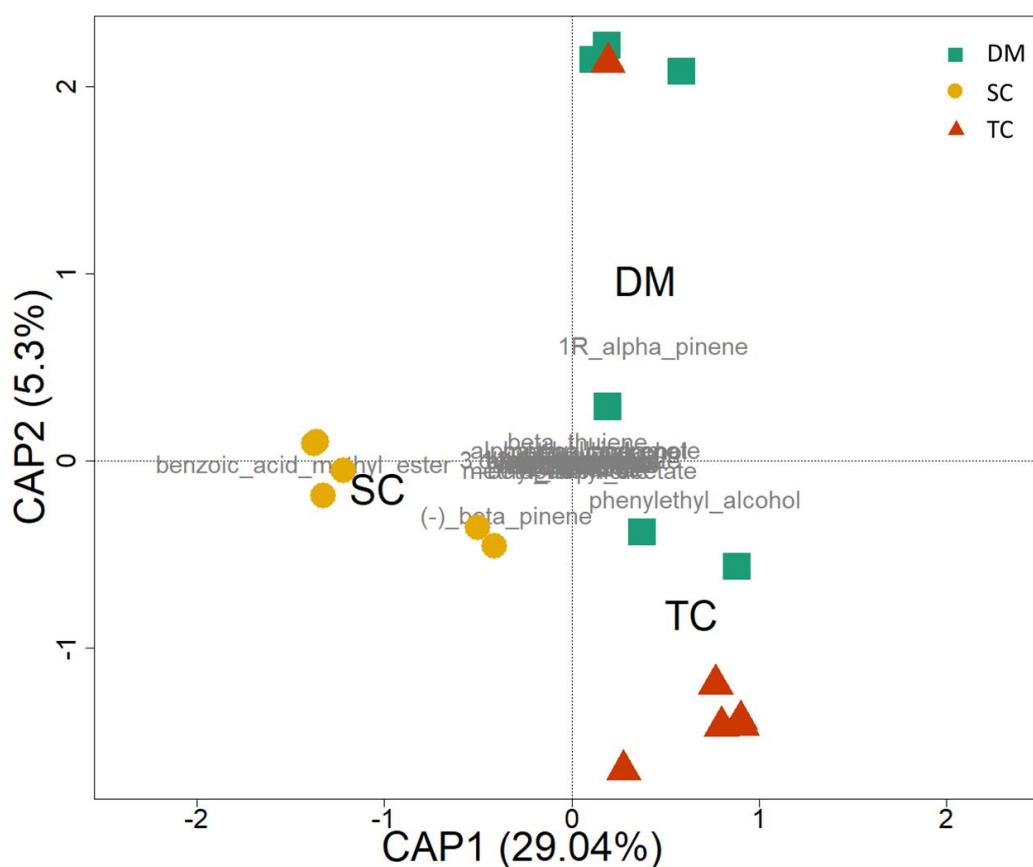


Figure 2.12: CAP biplot of the relative abundance of 26 VOCs collected from greenhouse grown plants from populations of *P. brandegeei* in New Mexico (SC) and Colorado (TC, DM) and analyzed using FET-GCMS.

Flowers analysed using FET-GCMS differed from flowers analysed using TD-GCMS in their overall composition, however, also in support of predictions. FET samples contained fewer VOCs overall (27 compared to 34 in the TD-GCMS samples). FET samples also contained higher relative amounts of terpenoid compounds (48% vs. 26%), lower amounts of benzenoid compounds (30% vs. 53%), and no nitrogen containing compounds. Also, one sulfur containing compound, dimethyl sulfide, was detected in FET samples which was not detected in TD-GCMS samples. There were similar amounts of aliphatic compounds in both sample types (19% vs. 15%).

While the top 10 compounds (by mean relative percent) reflect these differences in overall composition, major compounds in TD-GCMS samples were also found in FET samples (Table 2.9). Methyl benzoate and phenylethyl alcohol, which together made up 59.11% of the total floral scent profile in the TD-GCMS samples, also made up 60.63% of the total floral scent profile in the FET samples (Table 2.9). β -pinene, benzyl alcohol, and methyl cinnamate were also in the top 10 of both samples (Table 2.9).

Table 2.9: Comparison of the 10 most abundant compounds (by mean nonzero relative %) in greenhouse grown *P. brandegeei* floral samples from populations in New Mexico (SC) and Colorado (TC, DM) from dynamic headspace sampling coupled with thermal desorption GCMS (27 samples total) and full evaporative technique coupled with GCMS (18 samples total). Compounds shared between both lists in bold.

<u>Thermal Desorption</u>		<u>Full Evaporative Technique</u>	
Compound	Mean Relative % (non-zero)	Compound	Mean Relative % (non-zero)
Phenylethyl Alcohol	35.16%	(1R)- α -Pinene	41.54%
Methyl Benzoate	23.95%	Methyl Benzoate	36.74%
Benzyl Alcohol	8.34%	β-Pinene	32.66%
α -Copaene	6.58%	Phenylethyl Alcohol	26.60%
β-Pinene	6.25%	α -Thujene	14.61%
Benzeneacetic Acid	6.19%	1-Hexanol	14.16%
β -Farnesene	6.14%	β -Terpinene	9.21%
Hexenyl Acetate	5.86%	Methyl Cinnamate	9.82%
Methyl Cinnamate	5.57%	Benzyl Alcohol	8.52%
Benzyl Benzoate	5.44%	Linalool	6.71%

Are there differences in pollinator visits among populations?

The pollinator observation data reveals some potential trends in pollinator visits in the four populations. Small flies were the most common visitors at SC and DM while small and large bees were the most common visitors at TC and VD, respectively (Table 2.10). Hummingbirds visited the most flowers per foraging bout while large bees visited the most plants per foraging bout (Table 2.10). No hawkmoth observations were recorded during our observation periods.

Table 2.10: Summary of pollinator observations performed in populations of *P. brandegeei* in June 2023 in New Mexico (SC), Colorado (TC, DM) and Wyoming (VD) . Observations presented as total visits / (visits/hr). Most common visitors for each population in bold.

Population	Total Observation Minutes	Small Fly	Large Fly	Small Bee	Large Bee	Butterflies/ Moths	Other Insect	Hummingbird
VD	104	3/3.44	0/0.00	2/2.30	7/8.05	0/0.00	0/0.00	0/0.00
DM	256	13/3.04	3/0.70	4/0.94	3/0.70	0/0.00	0/0.00	2/0.47
TC	208	3/0.86	0/0.00	24/6.92	6/1.73	0/0.00	0/0.00	3/0.86
SC	198	36/10.9	2/0.61	8/2.42	0/0.00	0/0.00	9/2.73	0/0.00
	Avg. Flowers Visited	1.53	0.79	1.45	1.97	0.00	0.25	6.04
	Avg. Plants Visited	1.08	0.58	1.01	1.47	0.00	0.25	1.25

DISCUSSION

Four populations of *P. brandegeei* differed in morphology and floral scent profiles. The most southerly population from New Mexico (SC) was most distinct in floral morphology and scent compared to the Colorado populations (TC, DM), which were more similar to each other. The most northerly population from Wyoming (VD) appeared distinct in scent profile from the other populations but was more comparable to DM in morphology. Three VOCs dominated the scent profile of *P. brandegeei* and contributed most to the separation of the populations, but less abundant compounds also played an important role in population differentiation. General patterns of population differentiation were preserved in the greenhouse which suggests that differences among populations are largely heritable. However, flowers from greenhouse grown plants were larger and had higher total emission rates of floral VOCs than field flowers which indicates that *P. brandegeei* floral traits are also plastic. Finally, two different analysis methods produced similar results.

The floral scent of *P. brandegeei* contained 34 VOCs, including hawkmoth specific attractants.

The floral scent profile of *P. brandegeei* contained 34 VOCs. Fifty-three percent of these VOCs were benzenoid compounds, with the remainder spread among other compound classes, particularly terpenoids (26%). Many compounds are biosynthetically related and were derived from similar pathways or precursor compounds. For example, many of the benzenoid compounds are derivatives of the amino acid phenylalanine, such as phenylacetaldehyde, benzyl nitrile, phenylethyl alcohol, benzaldehyde, and eugenol (Dotterl & Gershenzon 2023). Other compounds, such as indole, 2-aminobenzaldehyde, and methyl anthranilate are derived from the amino acid tryptophan (Dotterl & Gershenzon 2023).

Among the compounds, many were VOCs common to the floral scent of various plant species. For example, benzaldehyde, myrcene, phenylethyl alcohol, α -/ β -pinene, benzyl alcohol, methyl benzoate, and caryophyllene are some of the most common floral VOCs found in a wide range of plant families and species (Knudsen et al. 2006). Several of these are known to be attractive to hawkmoths, and VOCs such as methyl benzoate, benzaldehyde, and benzyl alcohol are often dominant in the scent of hawkmoth pollinated plants (Raguso et al. 1996; Riffell et al.

2013; Dotterl & Gershenzon 2023). Some of the less abundant VOCs in *P. brandegeei* are also known hawkmoth attractants. Indole, for example, is known to attract *Hyles lineata* to *Polemonium* relative *Ipomopsis tenuituba* (Bischoff et al. 2014, 2014a). The floral scent profile of *P. brandegeei* is, therefore, made up of a mixture of general and more hawkmoth-specific VOCs, as predicted.

This mix of general and more specific hawkmoth attractants matches the documented pollination system of *P. brandegeei* (Kulbaba & Worley 2008; Pond 2017). *P. brandegeei* is visited by a diverse array of pollinators, but the increased effectiveness of hawkmoths suggests that hawkmoths may exert increased selective pressures on floral traits (Kulbaba & Worley 2012). Although hummingbirds, which are also highly effective pollinators of *P. brandegeei*, were shown to exert contrasting selective pressures on morphological traits (Kulbaba & Worley 2013), hummingbirds are apparently less responsive to floral scent and therefore may contribute less to selection on specific scent compounds (Knudsen et al. 2004; Nunez et al. 2021).

Populations of *P. brandegeei* differed in floral morphology and scent.

Populations of *P. brandegeei* differed in morphology and floral scent according to the multivariate analyses. The most distinct pattern in both datasets was the difference between SC flowers and flowers from TC, DM, and VD. SC flowers were larger, had longer corolla tubes, and had more recessed stigmas (reverse herkogamy) than flowers from other populations (Figure 2.6). Also, the scent profile of SC flowers contained a much higher proportion of methyl benzoate and a lower amount of phenylethyl alcohol than flowers from other populations (Figure 2.11) and SC flowers were more likely to contain indole. Together, these traits suggest increased selection by hawkmoths on floral phenotypes at SC. Previous work on *P. brandegeei* by Kulbaba & Worley (2012) found that hawkmoths preferentially visit flowers with narrower corolla tubes and reverse herkogamy. Further, hawkmoth pollinated flowers often produce a large amount of methyl benzoate (Riffell et al. 2013; Dotterl & Gershenzon 2023) and indole is a known hawkmoth attractant (Bischoff et al. 2014, 2014a). Although we did not observe any hawkmoth visits during our daytime work at SC in 2023 and other research is limited, records of

several species of sphingid moths in the vicinity of the SC population, including those not found in Colorado or Wyoming, are abundant in various databases (iNaturalist; BAMONA). Hawkmoth visits in other populations (TC, DM, VD) were limited in previous years of study and displayed large year to year variation (Kulbaba & Worley 2008; Pond 2017). Thus, it is possible that hawkmoth mediated selection on floral traits at SC is or was stronger compared to other populations of *P. brandegeei*.

An alternative interpretation of population differences is that conflicting selection by hummingbirds, which prefer wider flowers and more exerted stigmas (approach herkogamy), may be less significant at SC than at VD, DM, and TC (Kulbaba & Worley 2013, 2014). Hummingbirds (*Selasphorus platycercus*) were relatively common visitors to *P. brandegeei* at TC, DM, and VD in other years (Kulbaba & Worley 2008; Pond 2017) and I observed hummingbird visits at DM and TC in 2023. I did not observe hummingbird visits to *P. brandegeei* at SC in 2023, but database records suggest that *S. platycercus* is present near the SC population (iNaturalist). More pollinator observations are required in this population to determine if hawkmoth visits are more common than hummingbird visits and if the SC population is visited by different hawkmoth species than the Colorado populations.

Additionally, based on the floral scent and morphological differences, and on the obvious visual differences (large, bright yellow flowers at SC vs. smaller white flowers at DM, TC, and VD), there is a possibility that the SC population represents a unique variety of *P. brandegeei* or a hybrid with another species. There is known to be a considerable amount of hybridization and genetic admixture within the genus *Polemonium* (Grant 1989; Rose 2021; Rose et al. 2021) and the potential for hybridization with other species exists at SC. For example, the SC population is geographically closer than the other populations to the range of *P. pauciflorum* which is a large, yellow flowered *Polemonium*; *P. foliosissimum*, which has yellow morphs, is also found at the SC site (pers. obs.; Rose 2021; Rose et al. 2021). The apparent geographic cline in floral traits (Figure 2.6) may be related to genetic admixture between yellow and white flowered populations but further work in additional populations and genetic analysis is required to confirm this.

The other populations were also distinct from each other, although the axis separating TC, DM, and VD explained less of the variation in both the scent and morphology data. VD appeared somewhat distinct from TC and DM in the CAP analyses. Morphologically, VD flowers had wider corollas but were otherwise quite similar to TC and DM flowers (Figure 2.4); this matches data collected by Kulbaba & Worley (2008). VD flowers separated more distinctly from TC and DM flowers in the floral scent analysis, and these differences were largely driven by an apparent lack of some VOCs such as β -pinene, methyl cinnamate, eugenol, or methyl nicotinate, in VD flowers. VD flowers also contained somewhat more benzyl alcohol than flowers from other populations. These differences may reflect different selection pressures at VD (e.g., increased selection by hummingbirds which prefer wider corollas; Kulbaba & Worley 2013). However, these differences, especially in scent profile, may be caused by the lower sample size at VD (5 flowers vs. at least 10 from other populations) and further sampling at VD is required to determine if these are true differences.

The major difference between TC and the other populations was the decrease in flower size. TC flowers were smaller overall and had a smaller separation between anthers and stigmas, which was also reported by Kulbaba & Worley (2008). While the difference in size may reflect the overall drier conditions at TC (Kulbaba & Worley 2008; Kuppler et al. 2021), TC flowers remained smaller in the greenhouse which suggests local selection for smaller flowers at TC. There was considerable overlap in floral scent profile between TC and DM with similar proportions of all major compounds. There was some variation in the relative amount of less abundant VOCs, such as an increase in cinnamaldehyde at TC and the presence of longifolene in TC flowers only. These minor differences likely reflect the relatively higher total emission rate at TC. The higher emission rates at TC can be explained by the relatively higher temperatures during VOC sampling; temperatures were at least 1.5°C higher at TC than at DM. An increase in temperature of even a few degrees can increase the volatility of compounds and cause changes to the emission of VOCs (Fu et al. 2017; Barman & Mitra 2021), as was found for *Polemonium* relatives *Ipomopsis aggregata* and *I. tenuituba* (Wu et al. 2023). Additional factors such as soil type, moisture levels, and light levels could also lead to variation in scent emissions (Farré-Armengol et al. 2020).

Population differences in floral morphology and scent were maintained in the greenhouse.

All major patterns of population differentiation were maintained in the greenhouse analyses. SC separated from the other populations by morphology and floral scent profile in greenhouse samples as well, and there was also a smaller distinction between TC and DM flowers in the greenhouse. All patterns regarding specific morphological traits were retained in the greenhouse as well and corolla tube length, dry mass, stigma exsertion, and stigma-anther separation also contributed most to population separation in the RDA of greenhouse morphology data. Similarly, all major patterns regarding specific compounds were also retained in the greenhouse; methyl benzoate, phenylethyl alcohol, and benzyl alcohol remained the most abundant compounds and contributed most to population separation in the CAP analysis of greenhouse data. Relative emission of most other compounds was also similar in greenhouse and field flowers (Table 2.6). This together suggests that variation in floral morphology and in floral scent emissions among populations of *P. brandegeei* has a strong genetic component and is not only caused by phenotypic plasticity (de Villemereuil et al. 2020).

Although research is somewhat limited, other studies have also detected a genetic basis to among population variation in scent and morphology using common garden experiments (e.g., Chapurlat et al. 2018; Eisen et al. 2022). For example, major differences in floral scent between field populations of *G. conopsea* were largely maintained when plants were grown in a common environment (Chapurlat et al. 2018). However, Majetic et al. (2009) determined that among population variation in floral scent is caused by phenotypic plasticity as among population differences in floral scent decreased significantly when plants from different populations were grown in a common environment. Further study, including reciprocal transplants in field populations which have more realistic environmental conditions, is required to understand interpopulation variation in floral scent and other traits and its causes and effects more fully (Berend et al. 2019).

There was also a clear greenhouse effect on both floral morphology and floral scent emissions which suggests that floral traits are also plastic and can change in response to environmental conditions. Greenhouse flowers were overall larger and had substantially higher

total VOC emission rates than field flowers. These differences were proportional, however, and all flowers from all populations were larger and produced more volatiles in the greenhouse. Thus, while floral traits in *P. brandegeei* are plastic, the underlying population differences in scent and morphology have a major genetic component, as discussed. Again, this differs from a study on *H. matronalis* by Majetic et al (2009) which found that a common environment homogenized differences in emission rates and VOC composition between populations.

The major increase in VOC emission rate of *P. brandegeei* flowers in the greenhouse may be temperature related, as temperatures in the greenhouse were consistently higher than in the field (up to about 15°C higher) which would have a direct effect on the volatility and emission of most VOCs (Fu et al. 2017; Barman & Mitra 2021). This variation may also be related to increased light levels, improved soil nutrient status and/or increased soil moisture in the greenhouse, all of which can affect VOC synthesis and emission (Hu et al. 2013; Campbell et al. 2019; Luizzi et al. 2021). Similarly, the proportional increase in average flower size across populations is likely related to better plant health and increased access to moisture and nutrients in the greenhouse (Munoz et al. 2005).

Analysis by FET-GCMS produced similar results to TD-GCMS.

Despite the major differences in sampling and analysis method, samples collected and analyzed by FET-GCMS were largely similar to those collected with dynamic headspace sampling and analyzed with thermal desorption – GCMS. The three major compounds in the TD-GCMS samples (methyl benzoate, benzyl alcohol, and phenylethyl alcohol) still made up a significant proportion of the FET samples, and major population differences were maintained (Figure 2.10, Table 10). However, there was a major increase in the relative amount of terpenoid compounds in the FET samples and α -pinene was the most abundant compound in the FET samples (Table 10). This may reflect changes to the volatility of compounds at the higher FET analysis temperatures (Hamm et al. 2003). Additionally, this may be related to an overall increase in the emission of defensive compounds such as α -pinene due to the tissue damage required for the FET method (Brosset & Blande 2022).

CONCLUSIONS & FUTURE DIRECTIONS

Populations of *P. brandegeei* differed in morphology and floral scent emissions. SC, the southernmost population, was most distinct from the other populations in both morphology and scent profile. Differences among populations may reflect differential selection by pollinators or hybridization events. Overall patterns of population differentiation were maintained in the greenhouse which suggests that major differences among populations are heritable. There were, however, changes to flower size and overall emission rate when plants were grown under common conditions which suggests that floral traits are also plastic in *P. brandegeei*. Different methods of VOC sampling and analysis produced similar results, further emphasizing the robustness of the identified patterns.

This study provides important baseline floral scent data that can be expanded on in future projects. Differences in scent profile between different floral parts, at different times of day, and/or floral scent comparisons between different *Polemonium* species are some examples of the many angles from which scent analysis could be approached in future work. Further, a closer look at the less abundant compounds and at the chiral compounds would offer insights into the nuances of scent emissions from *P. brandegeei*. Also, a detailed analysis of the different trichome types found on *P. brandegeei* floral surfaces and the volatiles produced in each would help explain patterns of VOC synthesis and emission. Genetic analysis could be used to explain observed population differences and answer questions regarding the genetic relatedness of populations of *P. brandegeei*. Finally, increased sample sizes and more populations would improve the robustness of this dataset.

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Chapter 3: Effects of crossing distance and environmental variation on mating success in *P. brandegeei*

INTRODUCTION & RATIONALE

Background

Pollen limitation, the reduction of fruit or seed set due to insufficient pollen deposition, is common in plants (Larson & Barrett 2000; Chacoff et al. 2003; Knight et al. 2005). Indeed, reviews by Burd (1994) and Knight et al. (2005) found that fruit set in at least some populations or years was pollen limited in more than 60% of the 250+ included plant species. Pollen limitation has important effects on plant populations through reduced reproductive output and through evolutionary changes in traits that influence pollen receipt (Ashman et al. 2004). Further, pollen limitation has larger community-wide effects through decreased resource availability for seed predators or changes to competitive interactions, for example (Ashman et al. 2004).

Although research has historically emphasized the receipt of a limited *quantity* of pollen as the most important contributor to pollen limitation, pollen *quality* is of similar importance (Aizen & Harder 2007; Cisternas-Fuentes et al. 2023). The receipt of a large amount of pollen does not necessarily alleviate pollen limitation as the quality of pollen grains deposited on stigmas is highly variable (Aizen & Harder 2007). Pollen can be of overall poor quality due environmental conditions and plant health during pollen production, development, and/or transport (Delph et al. 1997; Cisternas-Fuentes et al. 2023). Pollen can also be of poor quality genetically. The deposition of self-pollen, for example, is detrimental to plants with self-incompatibility mechanisms as it can interfere with the success of outcross pollen by preventing its germination, by disrupting outcross pollen tube growth, or by disabling ovules that would otherwise be available to outcross pollen (Aizen & Harder 2007; Alonso et al. 2011; Cisternas-Fuentes et al. 2023).

Additionally, pollen from closely or distantly related plants can be of lower quality due to inbreeding and outbreeding depression pressures, respectively (Ramsey & Vaughton 2000; Aizen & Harder, 2007; Chacoff et al. 2008). Inbreeding depression, the reduction in progeny fitness due to a high level of genetic *similarity* between parents, can result from the increase in homozygosity that arises when closely related individuals mate (Charlesworth & Willis 2009; Cheptou & Donohue 2010). Increased homozygosity can result in decreased progeny fitness through overdominance, in which heterozygotes are more fit than homozygotes, or more likely through (partial) dominance, in which deleterious alleles are fully expressed in homozygotes (Charlesworth & Willis 2009; Baskin & Baskin 2015). In either case, inbreeding depression can significantly reduce plant fitness through decreased seed set, decreased seed germination, and reduced seedling vigour (Carr & Dudash 1995; Forrest et al. 2011; Sletvold et al. 2012; but see Baskin & Baskin 2015).

By contrast, outbreeding depression is the reduction in offspring fitness as parents become more genetically *dissimilar* (Waser et al. 2000). Outbreeding depression is typically described as acting through two interacting mechanisms (e.g., Scheirup & Christiansen 1996; Waser et al. 2000). Through the “ecological” or “environmental” mechanism, matings between plants adapted to different local environments produce progeny maladapted to the maternal environment (Waser & Price 1994; Scheirup & Christiansen 1996; Waser et al. 2000). Through the “genetic” or “physiological” mechanism, crosses between genetically dissimilar individuals disrupt coadapted gene complexes (Waser & Price 1994; Waser et al. 2000; Frankham et al. 2011). Briefly, in highly isolated populations, genetic drift and limited gene flow result in the chance formation of gene complexes that interact through positive epistasis and are fixed by natural selection (Scheirup & Christiansen 1996; Waser et al. 2000). These coadapted gene complexes are disrupted when crosses between different populations occur. The disruption affects progeny fitness, especially in later generations due to multiple recombination and independent assortment events which further break up the coadapted gene complexes (Waser & Price 1989; Scheirup & Christiansen 1996; Etterson et al. 2007). Whatever the mechanism, outbreeding depression also results in a decrease in overall plant fitness through decreases in seed production (Forrest et al. 2011; Sletvold et al. 2012), lower percent germination and

germination rates (Waser et al. 2000; Etterson et al. 2007; Sletvold et al. 2012), and reduced progeny vigour and survival (Waser & Price 1994; Etterson et al. 2007; Forrest et al. 2011).

Because of their negative effects, mating is optimized when inbreeding and outbreeding pressures are minimized. Pollinators, as important pollen vectors for approximately 87% of angiosperm species (Ollerton et al. 2011), play an important role in pollen transfer and outcrossing. Not all pollinators are equally efficient, however, and certain pollinators within generalist pollination systems (i.e., plants pollinated by multiple functional groups of pollinators) are more likely to transfer high-quality pollen that minimizes inbreeding depression pressures and improves fitness outcomes (Mayfield et al. 2001). For example, hawkmoths and hummingbirds are often considered to be highly effective pollinators as they have larger foraging ranges and longer foraging times and are more likely to transfer outcross pollen between plants and between plants at greater distances from each other than small-bodied bees and flies (Kulbaba & Worley 2008; Skogen et al. 2016; Rhodes et al. 2017). Thus, in a generalist pollination system that includes hawkmoths, hummingbirds and diverse small-bodied pollinators, large-bodied pollinators are likely to contribute more to overall plant fitness than small-bodied pollinators as they are much more effective at transferring higher-quality pollen (Kulbaba & Worley 2008).

Effective transfer of pollen is especially important for plants with patchy distributions and for plants with limited seed and pollen dispersal as plants with these traits are susceptible to genetic structuring within their populations (Waser & Price 1989; Ayre et al. 2019). In genetically structured populations, spatial distance is likely to mirror genetic distance as plants within patches are more likely to be close genetic relatives than plants in different patches (Waser & Price 1989). Therefore, due to inbreeding pressures, matings between plants within patches might be less successful than matings between plants in different patches (Waser & Price 1989). Similarly, due to outbreeding pressures, matings between plants in very distant patches might be less successful than matings between plants in nearby patches (Waser & Price 1989). Thus, there may be an intermediate spatial distance or range of intermediate distances over which mating is optimized in genetically structured populations (Waser & Price 1989; Newport 1989; Ayre et al. 2019). In other words, mating within a population of patchily

distributed plants may be optimized between individuals that are just genetically and spatially distinct enough to avoid inbreeding depression but not genetically and spatially distinct enough to cause outbreeding depression (Palacio-Lopez et al. 2018). This phenomenon is referred to as the optimal mating or optimal outcrossing distance (Waser & Price 1989; Ayre et al. 2019).

Evidence for an intermediate optimal outcrossing distance has been reported for numerous species spanning different families and pollination systems. The exact distance(s) over which mating is optimized depends largely on the species in question. For example, some studies have identified an intermediate optimal mating distance at very small spatial scales in which the maximum distance between crosses is 100m or less (e.g., Waser & Price, 1979, 1989, 1991; Grindeland 2008). Ayre et al. (2019), for example, performed crosses between *Anigozanthos manglesii* (Haemodoraceae) plants at 0m (selfed), <1m, 1-3m, 7-15m, and 50m distances and found that seed set was highest for the 1-3m crosses. Other studies have determined an optimal intermediate mating distance at larger spatial scales using interpopulation crosses with crossing distances of up to 25 km (Fischer & Matthies 1997; Willi & van Buskirk 2005; Billingham et al. 2007; Forrest et al. 2011). For example, Fischer & Matthies (1997) performed crosses between *Gentianella germanica* (Gentianaceae) plants at 0m (selfed), 1m, 10m, and 25km (interpopulation) distances and found that germination rate and progeny vigour was highest for the 10m crosses.

Optimal outcrossing at intermediate distances such as those described above does not occur in all species, however. Numerous studies on species from various families have found no evidence of outbreeding depression and found that mating is optimized at any distance great enough to limit inbreeding depression (Redmond et al. 1989; Irwin 2001; Villegas et al. 2020). For example, crosses performed at 5, 15, and 60m in three populations of *Amianthium muscaetoxicum* (Liliaceae) all set statistically similar amounts of seed compared to self crosses (Redmond et al. 1989). Other studies have found no evidence of optimal outcrossing at any distance (e.g., Newport 1989; Li et al. 2020), or have found that mating between distant plants (e.g., Hauser & Loeschke 1994; Del Vecchio et al. 2019) or nearby plants (e.g., Quilichini et al. 2001; Heiser & Shaw 2006) is optimal. Conversely, mating may be optimized at any distance that avoids outbreeding depression (Ruane et al. 2015).

These seeming contradictions in optimal outcrossing distances can be caused by a variety of factors. For example, evidence of outbreeding and/or inbreeding depression may only become apparent in the F₂ generation and beyond and using simple measures of fitness such as fruit or seed set may be insufficient to the identification of an optimal outcrossing distance (Fischer & Matthies 1997). At the very least, germination rate of progeny should be assessed, as should various measurements of seedling vigour (e.g., Pelabon et al. 2005). Further, abiotic factors may influence the effects of inbreeding and/or outbreeding depression and evidence of an optimal mating distance may only be clear under certain environmental conditions (Sobrevila 1988) or only under stressful conditions (Wolfe 1993; Cheptou et al. 2001; Cheptou & Donohue 2010). Additionally, including more than one or two populations and a wider range of crossing distances within and between populations in optimal mating distance studies could potentially provide more conclusive results (Li et al. 2020). Finally, outbreeding depression may simply not occur in all species (Villegas et al. 2020).

Furthermore, microsite variation within a population could also affect mating success. Since microsite variation can have a significant effect on pollinator behaviours and foraging patterns (e.g., Herrera 1995; Arroyo-Correa et al. 2021), any microsite variation should be considered in optimal outcrossing studies. For example, certain microsite conditions such as increased shading or a higher concentration of co-occurring species might be favoured by pollinators which could increase the frequency of mating between plants at similar microsites (Herrera 1995; Janovsky et al. 2013; Arroyo-Correa et al. 2021). Similarly, plants at microsites with deeper or more nutrient-rich soil might be in better health overall than plants in other microsites and improved nutrient status may enhance pollinator attraction through effects on display size and pollen production; this could increase the likelihood of pollen transfer between these more productive microsites (Boerner & Koslowsky 1989; Haymes & Fox 2012). The potential effects of microsite variation are rarely, if ever, considered in optimal outcrossing studies and incorporating any microsite variation into experimental design and/or analysis could provide useful insights into plant mating and fine scale plant-pollinator interactions.

A more complete understanding of optimal mating distances and the factors contributing to them has numerous applications. First, understanding optimal mating in a

system can be important for plant conservation and restoration initiatives. Restoration programs, which often involve the transfer of plants or seeds from different populations or regions, have historically failed to take the potential effects of inbreeding and outbreeding depression into account (Edmands 2007; Ruane et al. 2015). A better understanding of the optimal mating distance for a given plant species could therefore improve plant conservation and restoration outcomes (e.g., Ruane et al. 2015; Del Vecchio et al. 2019; Thomas et al. 2021; Monks et al. 2022). Further, understanding how mating might be optimized in a system can provide more information on the potential effects of ongoing pollinator declines (Rhodes 2018). For example, knowledge that long-distance mating is optimal in a system could offer insights into what may happen if longer-distance pollinators are removed from a system due to climate or habitat changes. Using field and greenhouse crosses, I determined whether an optimal mating distance exists for *Polemonium brandegeei* and whether environmental variation affects mating success.

Rationale

P. brandegeei provides an excellent study system for a mating distance study due to its patchy distribution and limited dispersal mechanisms. Plants are typically found growing in dense patches of two to 30+ plants and the small, lightweight seeds are apparently primarily gravity dispersed (although there is potential for seed predation and animal dispersal; Pond 2017). Gravity dispersal is limited by the size of the plant and its location and is often not as efficient for long-distance seed dispersal as other dispersal mechanisms (Grasty et al. 2020); therefore, it is unlikely that *P. brandegeei* seeds are spread far beyond the maternal plant.

Further, long-distance pollen transfer within and among populations of *P. brandegeei* is limited by the activity of its most effective pollinators, hawkmoths and hummingbirds. Previous work on *P. brandegeei* has shown that these large-bodied pollinators are highly effective and visit more flowers and plants per foraging bout than small-bodied bees and flies (Kulbaba & Worley 2008). For example, in a single foraging bout, hawkmoths and hummingbirds were observed visiting up to 30 and 48 flowers on multiple plants, respectively (Kulbaba & Worley

2008; Pond 2017). In comparison, small-bodied visitors typically visited one plant and often just a single flower per foraging bout (Kulbaba & Worley 2008; Pond 2017).

However, while large-bodied pollinators appear to be more effective, small visitors visit *P. brandegeei* much more frequently and reliably, suggesting that short-distance pollen transfer may be more prevalent in this system than previously thought (Kulbaba & Worley 2008, 2013; Pond 2017). For example, syrphid flies were common and reliable visitors in three populations of *P. brandegeei* across three years of observations while the number of hummingbird visits displayed large year-over-year variation (Kulbaba & Worley 2008; see also Brunet 2009 and Barbosa Leal et al. 2020 for further discussion on temporal variation in large-bodied pollinator abundance). Similarly, small flies and bees were the most observed visitors in 2015 and 2016, respectively, while visits by hummingbirds and hawkmoths were rare (Pond, 2017). Small-bodied pollinators, then, may be contributing more to pollination and seed set than expected. Indeed, excluding large-bodied pollinators from *P. brandegeei* plants in two populations reduced seed set in only one year out of two (Pond 2017). Thus, due to limited seed dispersal and seemingly variable long-distance pollen transfer, it is highly likely that genetic structuring exists in populations of *P. brandegeei*. This, combined with its patchy distribution, suggests that an optimal outcrossing distance may exist for this species. Further, a direct determination of the effects of long vs. short pollen transfer will confirm the increased importance of hawkmoths and hummingbirds to pollination in a generalist pollinated plant.

Finally, *P. brandegeei* occupies a variety of unique microhabitats throughout its populations. Microhabitats range from steep, exposed cliff faces with shallow soil, to gentle slopes with deep, organic soil, to windy, exposed sites with purely gravel substrates (pers. obs.). Thus, there is also potential for microsite variation to influence plant growth, pollinator behaviour, seed dispersal, and mating dynamics.

OBJECTIVES & HYPOTHESES

Objectives

Determine if physical distance between plants and environmental variation within populations both affect mating success in *P. brandegeei*.

Hypotheses + Predictions

Physical distance between plants and environmental variation both affect mating success in *P. brandegeei*.

- If inbreeding and outbreeding depression affect mating success, seed set and mass and seedling germination and survival in populations of *P. brandegeei* increases as the physical distance between mated *P. brandegeei* plants increases, until it plateaus at some intermediate distance.
- If both inbreeding and outbreeding depression affect mating success, seed set, and quality is higher for plants pollinated with pollen from different maternal families within the same population than it is for plants pollinated with pollen from the same maternal family or with pollen from different populations.
- If environmental variation exists within populations and among microsites, then:
 - Crosses between more similar microsites will be more successful than crosses between dissimilar microsites.
 - Some microsites provide better habitat and will be more conducive to successful crosses.

METHODS & MATERIALS

General Overview

To assess the effects of physical crossing distance on mating success, field crosses were performed within (0.05 – 3.5 m between plants) and between (6 - 390 m between plants) microsites at two populations of *P. brandegeei*: Deer Mountain, Colorado (DM) and Vedauwoo, Wyoming (VD). Environmental data from each microsite was collected to determine if environmental variables and/or environmental similarity between microsites affects crossing success. To supplement field data, I performed greenhouse crosses on plants with known parentage to directly assess whether genetic relatedness affects crossing success. I used plants from DM and from two additional populations at Sandia Crest, NM (SC) and Taylor Canyon, CO (TC) for greenhouse crosses.

Field Data Collection & Hand Crosses

Study Populations & Microsites

I selected two populations of *P. brandegeei* as primary field sites (Figure 3.1; Table 3.1). The DM population was located on a steep, loose slope and conditions were largely homogenous. The VD population was located on and around a large rock formation and plants



Figure 3.1: Distribution of *P. brandegeei* microsites at (a) Vedauwoo, WY and (b) Deer Mountain, CO. The primary feature of the Vedauwoo site is a large rock formation that runs through the centre of the population; the west side of the rock formation is more forested with more organic soil while the east side is less forested with gravelly soil. The Deer Mountain site is more homogenous and is located on a forested, SW facing slope.

were often found in gravel soil under rock overhangs other than on the west, more forested side of the rock formation (Figure 3.1a). Within each population, *P. brandegeei* was distributed in distinct patches (hereafter “microsites”) of 3-35+ plants (Figure 3.2). Following an extensive survey of the populations while plants were in bud in late May and early June 2022, I selected 8 microsites at Deer Mountain and 11 microsites at Vedauwoo for use in the crossing study based both on their distance from other microsites and on the availability of plants/flowers at the microsite. I performed crosses within microsites (0.05 – 3.5 m between plants) and between microsites (6 – 369 m between plants).

Microsite Data Collection

I georeferenced (Table 3.1; Figure 3.1), photographed (Figure 3.2) and recorded the general features of each microsite and included information on relative vegetation abundance, the plant community at the microsite, and soil type (broad categories).

Table 3.1: Location and description of the four populations of *P. brandegeei* used for field and/or greenhouse crosses in this study.

Population	Coordinates	Elevation	Description
Primary Populations (Used for Field + Greenhouse Crosses):			
Vedauwoo, Wyoming (VD)	41.15438 N, 105.37386 W	2519m	Plants found on/at the base of a large rock formation that divides the (accessible) population into distinct sides; plants growing in gravel, shallow soil, or in rock crevices, often sheltered by rock overhangs; high winds common; plants relatively exposed; habitat variable. Not used for greenhouse crosses due to lack of flowers
Deer Mountain, Colorado (DM)	40.38673 N, 105.59737 W	2880m	Plants found on steep, rocky, SW facing slope; plants growing in shallow soil or in rock crevices, sheltered by trees; plants less exposed; habitat largely homogenous
Secondary Populations (Used for Greenhouse Crosses Only):			
Taylor Canyon, Colorado (TC)	38.72829 N, 106.76393 W	2600m	Plants largely on or at the base of sheer, SW facing rock walls; soil very shallow; plants quite exposed or sheltered by trees; habitat largely homogenous
Sandia Crest, New Mexico (SC)	35.20793 N, 106.44771 W	3100m	Plants largely on or at the base of sheer, SW facing rock walls; soil shallow; plants sheltered by trees or quite exposed; gusty winds common; habitat variable

I also performed five soil depth measurements at each microsite by inserting a metal probe into the soil at random places at the site. Maximum depth was assumed to be reached when the probe could not be inserted further into the soil without excessive force. Also, as an index of vegetation cover, I counted the number of other (non-*P. brandegeei*) plants within a 30 cm radius of 5 haphazardly selected *P. brandegeei* plants per patch and recorded the number of *P. brandegeei* plants per site. Further, I counted the number of inflorescences (flowering stalks) and (approximate) number of open flowers for all *P. brandegeei* plants used in crosses.

In 2023, I returned to each microsite used for field crosses in 2022 and collected additional temperature, RH, shading, and site cover data. I took temperature and RH measurements every two hours over the course of one full day (09:00-15:30 at VD and 07:30-15:30 at DM) using hygrometers and/or a handheld weather meter. I collected a total of 4 replicate temperature and RH measurements at each VD microsite and a total of 5 replicate temperature and RH measurements at each DM microsite. I collected the temperature and RH measurements one day apart (June 17 at VD and June 18 at DM) on mostly clear days. Each time I took a temperature reading I also recorded if the microsite was shaded or not. As a measure of tree/rock cover, I assigned a qualitative % cover to each microsite based on how “open” a microsite was. I also recorded the direction of exposure of each microsite. In summary, I collected data on the following environmental variables at each microsite used in field crosses:

- *P. brandegeei* plants at the microsite
- # of non-*P. brandegeei* plants within a 30cm radius of a randomly chosen *P. brandegeei* plant (average of 5 counts)
- Soil depth (average of 5 measurements)
- Temperature (average of 4-5 temperature measurements over 1 day)
- Relative humidity (average of 4-5 RH measurements over one day)
- Cover % (measure of how exposed the microsite was)
- Shading (% of the time site was in shade over 1 day, average of 4-5 recordings e.g., shaded 4/5 times = 80% shaded)
- Soil type (organic, mixed, or gravel)



Figure 3.2: Typical microsites at a) VD and b) DM. *P. brandegeei* plants circled in white. Soil at VD has a much higher amount of gravel than at DM and encroachment by grasses is more common at VD.

Crossing Design

At DM, I determined which crosses were to be made in advance based on distances between microsites. I performed crosses within all microsites (i.e., near crosses), except at one microsite with a single large plant (DM 19). I then assigned crosses between microsites (i.e., intermediate crosses) to represent the full range of distances between (identified) microsites and therefore between plants at this population. In total, I performed 13 within microsite crosses (0.09 – 3.2 m between plants) and 46 between microsite crosses (6 – 218 m) at DM from June 16 – 22, 2022.

The crossing design at VD was more haphazard as flowers on many plants opened rapidly while I worked at DM, and I therefore applied crosses whenever flowers were available. I attempted to make crosses that represented the full range of distances between microsites and was largely successful. In total, I performed 15 within microsite crosses (0.05 – 3.5m between plants) and 33 between microsite crosses (21 – 369 m between plants) from June 9 – 21, 2022 at VD.

Hand Crossing Methods

I haphazardly selected two to four plants per microsite for use in crosses (except at two microsites, where only one plant was available). I generally selected larger plants with many available flowers and used plants as both pollen donors and recipients (with a few exceptions

due to flower availability or flower herbivory by a larval insect). To avoid self-pollinations, I ensured that plants used in within microsite crosses were not from the same "clump" of plants. I used 25 plants as pollen recipients and 26 plants as pollen donors at VD and 16 plants as pollen recipients and 15 plants as pollen donors at DM.

I removed any open flowers from selected plants. I then covered plants with fine mesh food covers (Figure 3.3) to prevent pollen deposition or removal by animal visitors. I was careful to avoid contact with plants when placing the covers and secured them with metal staples. I kept the covers on the plants until all crossing work was completed and stigmas had senesced.

In general, I used flowers as pollen donors as they opened. I removed anthers from flowers using fine forceps and placed them into Eppendorf tubes. Anthers from multiple flowers on the same plant were pooled, kept out of direct sunlight in a fanny pack when not in use and disposed of at the end of each day. I dipped forceps in 70% ethanol and wiped them down after anther collection from one plant was complete.

I used an average of 6 emasculated flowers (i.e., those with anthers removed as described above) as pollen recipients per cross. I used 4-6 replicate flowers per cross based on flower availability. I applied 1-5 crosses to each plant, again based on flower availability. I performed crosses simply by brushing anthers (held in forceps) onto receptive stigmas (Figure 3.3). Forceps were dipped in 70% ethanol and wiped down between crosses.

I considered stigmas to be receptive once they were fully opened and I confirmed pollen deposition on stigmas visually, as the bright yellow pollen is highly visible against the white stigma lobes. In general, I only applied pollen to stigmas once as stigmas senesce very quickly following pollination (within a day). If stigmas had not senesced the day following pollination, I applied pollen a second time. Following pollination, I applied a small amount of non-toxic acrylic paint to the calyx of pollinated flowers to keep track of pollen donors.

Once crosses on a given plant were complete, I removed exclusion tents and replaced them with cages made from ½ inch gauge hardware cloth to protect against seed predators (Figure 3.3). Cages were "custom made" to fit each plant, secured using metal staples, and removed following seed collection.



Figure 3.3: Images depicting methods used in Chapter 3. **A:** fine mesh food covers used to prevent pollen deposition before crossing; **B:** hand-crosses being performed; **C:** plants in Deepot racks and bins for bottom watering in the greenhouse; **D:** hardware cloth seed predator prevention cages.

Seed Collection

I collected seeds by placing all flowers/fruits from a given cross into a coin envelope (identified based on the calyx paint colour). I then counted and weighed the seeds from each cross and stored them in a 4°C fridge. Although most capsules appeared to be mature, the outer calyx of flowers from approximately 27% of crosses at DM and approximately 16% of crosses at VD was still somewhat green at time of collection. However, seeds appeared to have matured fully following collection.

Seed Germination (Greenhouse)

Physical scarification with fine-grained sandpaper followed by a four-week cold stratification treatment was the most successful germination method for VD and DM seeds (see Appendix 1) and was used for the germination study. I divided 20 seeds per cross into two batches of 10 seeds each. For crosses where fewer than 20 seeds were produced, I divided total seeds evenly into two batches or used a single batch only. I generally omitted crosses with fewer than 8-10 seeds from the germination study due to a lack of replication. I then sanded the seeds for about 1 minute, checked them under a dissecting scope to ensure some seed coat had been removed, placed them into 100mm x 15mm petri dishes lined with number 1 qualitative filter paper (Whatman), and cold stratified them for 4-weeks in a dark, 4°C fridge in two randomized batches. I kept Petri dishes in a closed container in the fridge to aid in moisture retention and checked them daily for germination. I replaced filter papers as needed if bacterial or fungal growth was noted.

I moved any seeds that germinated in the fridge to 72 cell plug trays (Deep-Root Seed Starters; Lee Valley) one or two days after germination was noted (radicle emergence) and transferred all remaining ungerminated seeds to soil at the end of the 4-week cold stratification period. Plants were grown in a soil mix consisting of 10g 15-9-12 slow-release fertilizer (Osmocote Plus; Scotts-Sierra Horticultural Products) per 1L growing medium (Pro-Mix High Porosity with Mycorrhizae; Premier Tech Horticulture) in the Crop Technology Centre greenhouse at the University of Manitoba. I transferred plants to 800-ml conical Deepots once they had reached an appropriate size (approximately 1 month after emergence). After about

one month of growth, I placed the Deepots (in racks) into 50 L plastic bins to facilitate bottom watering (Figure 3.3). I left plants in the bins until experiments ended, misted them regularly to increase RH, and spaced them as needed.

Once in the greenhouse, I observed seeds/seedlings closely over a period of four weeks. I recorded the number of emergent/surviving seedlings 1-week, 2-weeks, and 4-weeks after seeds were transferred to soil. I collected data on total percent germination (total germinated seeds / total seeds plated) and percent survival after 2 weeks in soil (# of surviving seedlings after 2 weeks / total seeds plated).

To enable greenhouse crosses between additional populations, I also germinated seeds from the Taylor Canyon, Colorado (TC) and Sandia Crest, New Mexico (SC) populations. Germination of seeds from these two populations was successful without the physical scarification treatment so (unsanded) seeds were simply cold stratified for two-weeks (as above) and placed into soil when they germinated or at the end of the two-week period. Once in soil, seeds were cared for and observed as described above.

Greenhouse Crosses

To supplement the field crosses, I performed additional crosses on greenhouse plants with known parentage as this allowed me to directly assess whether genetic relatedness affects mating success in *P. brandegeei*. I used plants from three populations (DM, TC, SC) and performed crosses using field techniques on a continuum from most related to least related (Table 3.2). Mature seeds were collected, counted, weighed, and stored in a 4°C fridge.

Table 3.2: Description and number of each type of greenhouse cross performed on *P. brandegeei* plants from three populations (SC, TC, and DM).

Cross Type (Description)	# of Crosses (SC)	# of Crosses (TC)	# of Crosses (DM)
Self	5	3	4
Sib (same maternal family)	3	4	4
Intra (same population, different maternal family)	4	4	5
Inter (different population)	8 (4 x TC, 4 x DM)	8 (4 x SC, 4 x DM)	8 (4 x SC, 4 x TC)

Statistical Analyses

All analyses were performed in R version 4.3.1 (R Core Team 2023).

Microsite Data

To determine if environmental variables and fine scale environmental variation affect mating success, I used multivariate ordination methods to quantify differences between microsites. I performed both a FAMD (factor analysis of mixed data) and a NMDS (non-metric dimensional scaling) with Bray-Curtis distances. Although both methods separated the microsites similarly, I chose to use coordinates from the NMDS to calculate my microsite dissimilarity metric as NMDS is a nonparametric, unconstrained ordination method that makes fewer assumptions about the data. I extracted the coordinates of each microsite on the NMDS plot and used these coordinates to calculate the Euclidean distance between each microsite in "environmental" (ordinal) space. The Euclidean distance between microsites serves as a microsite dissimilarity metric in my analyses.

I used student's t-tests to determine if there were any significant differences between populations for the seven quantitative variables listed above. t-Test assumptions of normality and homogeneity of variances were assessed for each variable.

Field Crosses

I analyzed the effects of the two main explanatory variables of interest (spatial distance and microsite environmental dissimilarity) on four measures of crossing success: seed set (seeds/flower), total seed mass, germination percent and percent survival to two weeks. I used general linear and linear mixed models to analyze these relationships and analyzed the effects of other fixed (NMDS axis one and two, # of inflorescences on maternal/paternal plants, population) and random (maternal/paternal ID, maternal/paternal microsite ID) effects as well.

A. Data Preparation

First, I removed crosses with noted issues (such as plant damage or uncertainty about parents) from the data set. I then removed all crosses where I had crossed or collected 3 or fewer flowers (mostly due to herbivory or other plant damage). In total, I removed 2 within-

patch and 6 between patch crosses from the DM dataset, and 4 within-patch and 13 between patch crosses from the VD dataset.

I explored all relationships graphically prior to analysis. I chose number of inflorescences as my metric of plant vigour for analysis, as larger and healthier plants were more likely to produce more inflorescences (pers. obs.). I chose number of inflorescences over number of open flowers as my vigour metric because 1) number of inflorescences was highly correlated with number of open flowers and 2) I directly counted inflorescences but approximated the number of open flowers.

There was a weak correlation between reciprocal crosses for seed set ($R_s = 0.37$, $P < 0.1$) so I chose not to average the reciprocal crosses for these analyses. Differences in seeds set between reciprocal crosses may be caused by differences in microsites or vigour of maternal plants, both of which were included in my analyses.

B. Seed Set

I standardized seed set by dividing total seeds collected per cross by total flowers collected (which ranged from 4-6 flowers). I chose total flowers collected over total fruits with seeds because empty fruits may reflect incompatible mating. After preliminary data analysis and examination of residual plots, I natural log transformed the (spatial) distance and maternal/paternal inflorescence number variables to stabilize the variance.

I performed separate analyses for the within and between microsite crosses because inbreeding effects are expected over small spatial distances only. Additionally, unlike the within microsite crosses, individual parents and microsites were used multiple times in the between microsite crosses which allowed for testing of random effects due to parent and microsite on the between microsite data.

Within Microsite Crosses

I included 24 microsites from both populations (12 VD, 12 DM) in the analysis of within-microsite crosses. I used general linear models to analyze the effects of (spatial) distance, NMDS axis one or two (microsite-environment effects), population, and/or maternal or

paternal vigour (inflorescence #) on seed set. I did not include random effects in any models as most plants and microsites were only used in crosses once and the random effects were therefore unreplicated. I included a maximum of two terms in each model. Highly correlated terms (e.g., NMDS axis 2 and population) were not used in the same model. I then compared model fit using AICc. Model assumptions of normality and homogeneity of variance were assessed graphically.

Between Microsite Crosses

I analysed the between microsite crossing data for each population separately. Although this did not allow for direct testing of a population effect, separating the two populations is justified given that VD appears less environmentally homogenous than DM which suggests that environmental effects and/or microsite dissimilarity may have a larger effect at VD than at DM. These effects may not be detected if the data were combined. Furthermore, separate analysis simplifies the analysis as it enabled me to keep a nested plant/population variable out of the already sample size-limited models.

First, I tested for relationships between seed set and the same explanatory variables as for within-microsite crosses (including Euclidean distance and removing population) using general linear models. I included a maximum of two explanatory variables in each model. As for within microsite crosses, highly correlated terms were not included in the same model (e.g., distance and Euclidean distance). I compared general linear model performance using the `aictab` function in R. Model assumptions of normality and homogeneity of residual variance were tested graphically.

I then used the “lmer” package in R to analyze the effects of both fixed and random effects on seed set. All fixed effects from within-microsite models were also used in between microsite mixed models, except population. I also included the random effects of maternal ID, paternal ID, maternal microsite ID, and paternal microsite ID in the mixed models. I added a maximum of one fixed and one random effect to each mixed model for the VD data and a maximum of two fixed and one random effect to each model for the DM data. For models with random effects, I removed crosses that were not replicated for that random effect from the

dataset prior to analysis (e.g., for models including maternal ID, I removed crosses with maternal plants that were only used in one cross). Mixed models were fit using reduced maximum likelihood (REML) and fixed effects were assessed using t-tests with Satterthwaite's method. I analyzed the performance of mixed models using the "model_performance" function in R, which extracts a conditional (full model) and marginal (fixed effects only) R^2 value for mixed models, as well as the intraclass correlation coefficient (ICC; variance explained by the random "grouping factor"). I also used the "ranova" function which compares models with and without the random effect using likelihood ratio tests.

For the overall analysis of the VD between microsite cross data, 26 between microsite crosses were used. When performing mixed models, 16 crosses remained when unreplicated fathers were removed, 21 crosses remained when unreplicated mothers were removed and 25 crosses remained when both unreplicated maternal and unreplicated paternal microsites were removed. A total of 41 between microsite crosses were used in the overall DM analyses and 39 between microsite crosses were used in mixed models (most random effects were sufficiently replicated).

C. Seed Mass

I included total seeds collected as a covariate in all models as this allowed me to assess whether increases in total seed mass are simply proportional to seed number or are also affected by other factors. Total seeds collected was natural log transformed prior to analysis as were all other explanatory variables (as above) other than Euclidean distance and NMDS axis one and two. Seed mass analyses was performed as for seed set unless indicated below.

I included total seeds collected and one additional explanatory variable in linear models of the within microsite cross data. As with seed set, 24 within microsite crosses were used in this analysis (12 VD, 12 DM).

For the between microsite crosses, I first used general linear models with total seeds collected as a covariate in every model along with one additional fixed effect at VD (due to sample size constraints) and two additional fixed effects at DM. To test for random effects, I then performed linear mixed models with total seeds collected and one of the random effects

for the VD data. For the DM data, mixed models included total seeds collected, a maximum of one additional fixed effect, and one random effect. Sample sizes were as for seed set.

D. Germination %

Out of the 91 crosses performed, 82 crosses had at least some seeds to perform a germination test. For analysis, I removed any crosses where less than 10 seeds had been used in the germination study which left a total of 59 crosses for the germination percent analysis. I then tested for any differences in germination percent between batch one and batch two using a paired t-test. Batch two had a significantly higher germination percent than batch one ($P < 0.001$; Appendix 1) and although most crosses germinated better in batch two than in batch one (47/62), there were exceptions with up to 80% higher germination in batch 1. Considering this, I chose to use the best germination percent across both batches in analysis.

I used general linear models and general linear mixed models to explore the relationship between germination percent and the same fixed and random effects as for seed set. I natural log transformed all variables other than Euclidean distance and NMDS axis one and two scores to stabilize the variance.

For within microsite crosses, I included only one explanatory variable in models due to low sample size. A total of 12 within microsite crosses were used in this analysis (8 DM, 4 VD). Of those, only three crosses were performed between plants 1 m or less apart. For between microsite crosses at VD, I tested fixed effects individually and in combination using linear models. I included a maximum of two explanatory variables in each model. I did not perform mixed models on the VD data due to low sample size after removing terms that are unreplicated for each random effect. A total of 20 between microsite crosses were included in the VD analysis. For between microsite crosses at DM, I performed linear models on fixed effects individually and in combination and included a maximum of two explanatory variables per model. I then performed mixed models using a maximum of one random and one fixed effect. A total of 27 between microsite crosses were included in the analysis of the DM germination percent data.

E. Seedling Survival to Two Weeks

Normality and homogeneity of variance assumptions of linear models were not met for the survival data, likely due to the higher number of zeros in the survival data. I therefore used generalized linear models with a quasi-Poisson distribution (due to overdispersion) and log link. I used the number of seeds that survived to two weeks as my response variable and included an offset of the (log) total seeds germinated. I analyzed the effects of each fixed effect (as above) together with the offset. A maximum of one fixed effect was included in all analyses of survival data due to low sample sizes ($n = 12$ for within microsite crosses, $n = 20$ for between microsite crosses at VD, $n = 27$ for between microsite crosses at DM). No random effects were tested due to low sample sizes and a higher proportion of unreplicated random effects.

Greenhouse Crosses

I standardized seed set by dividing total seeds collected by total (crossed) flowers collected. I removed one cross (TC self) from which less than 3 flowers were collected from the dataset prior to analysis.

For analysis, I first combined all data and used a factorial type II ANOVA to test for differences in seed set (seeds/flower crossed) between the different cross types (self, “sib” = a cross with another plant from the same maternal line, “intra” = a cross with a plant from the same population but a different maternal line, and “inter” = a cross with a plant from a different population; Table 3.3). I then used a type II ANOVA to test for differences in seed set between the different interpopulation cross types. I used a (type II) ANCOVA to test for differences in seed mass between the different cross types with total seeds collected as a covariate. I used Tukey’s HSD test as a post-hoc test to test for all pairwise effects. Three to five self, three to four sib, four intra, and eight inter crosses were performed per population. ANOVA assumptions of normality and homogeneity of variance were assessed for each model.

RESULTS

Is there environmental/microsite variation within and among populations of *P. brandegeei*?

As predicted, there were environmental differences within and among populations of *P. brandegeei*. Populations separated along NMDS axis 2 primarily by relative humidity, vegetation cover, and gravel soil type (Figure 3.4). Intrapopulation variation was largely explained on NMDS axis 1, which was most influenced by percent cover and percent of day shaded (Figure 3.4). At VD, “peripheral” microsites from the forested side of the rock formation largely separated from those at the less forested “centre” of the population. Only one peripheral microsite at VD grouped with the central microsites, but this microsite is similar in soil type and exposure to the more central microsites. At DM, microsites are relatively more homogenous, and the lone “peripheral” microsite therefore grouped with the central microsites.

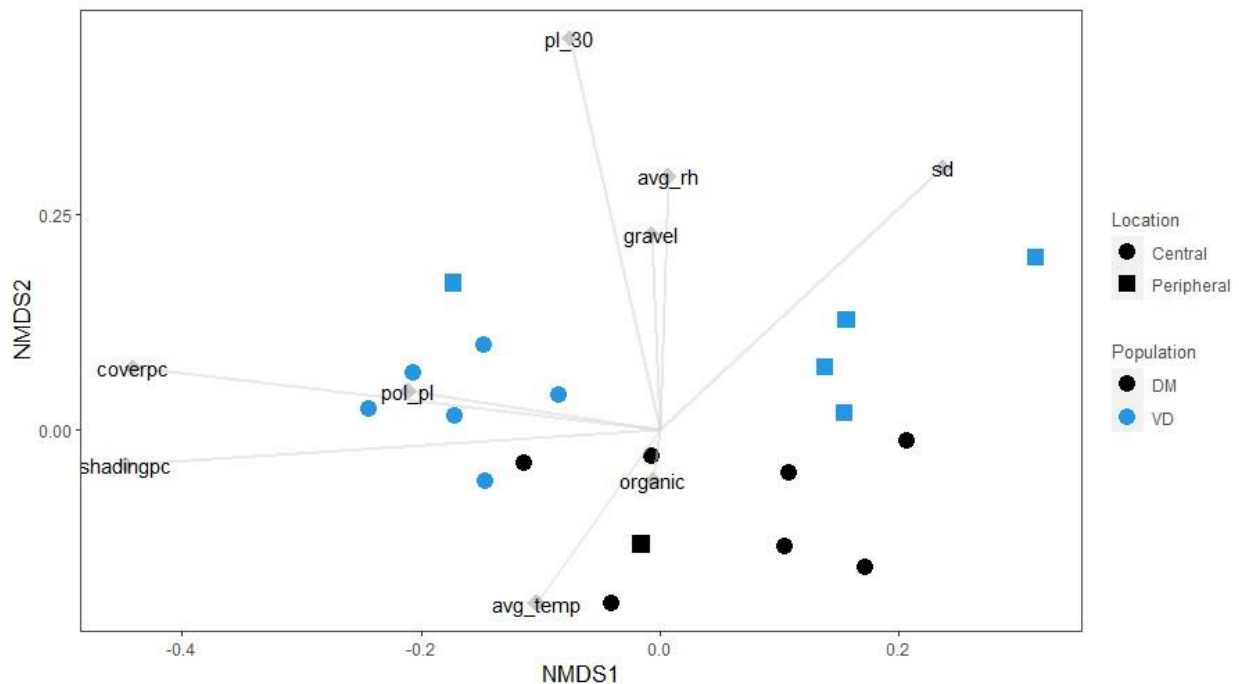


Figure 3.4: NMDS plot of microsite environmental data in two populations of *P. brandegeei*. Populations separate largely by vegetation cover and average relative humidity along NMDS axis 2. Variables included in ordination: pl_30 = plants within 30cm (index of vegetation cover), avg_rh = average relative humidity over one day, gravel/organic = soil type, avg_temp = average temperature over one day, coverpc = microsite “openness”, shadingpc = % of the time microsite was shaded over one day, pol_pl = number of *P. brandegeei* plants at the microsite, sd = soil depth (cm).

NMDS results were corroborated by comparisons of individual microsite traits and average relative humidity and vegetation cover separated the sites in both analyses (Figure 3.5). All values listed hereafter are in the form of mean $\pm$ SE. VD microsites included more than twice the number of non-*P. brandegeei* plants than did DM microsites (VD: 31.26 ± 2.09 ; DM: 13.43 ± 2.92 ; $t_{17} = -5.12$, $P < 0.001$, Figure 3.5a). Average relative humidity over one day was approximately 6% higher at VD than at DM (VD: $51.62\% \pm 1.08\%$; DM: $45.99\% \pm 1.64\%$; $t_{17} = -2.99$, $P < 0.01$, Figure 3.5b). Similarly, Additionally, 6/11 microsites at VD had gravelly soil compared to 1/8 at DM.

Populations did not differ in average temperature ($14.08\text{ }^{\circ}\text{C} \pm 0.31\text{ }^{\circ}\text{C}$), percent cover ($47.89\% \pm 4.25\%$), percent of day shaded ($66.58\% \pm 4.24\%$), soil depth ($15.39\text{ cm} \pm 0.92\text{ cm}$) or number of *P. brandegeei* plants at a microsite (11.68 ± 1.88). However, boxplots indicate intrapopulation variability in most of these variables, especially in percent cover, percent of day shaded, and the number of *P. brandegeei* plants, all of which contributed most to intrapopulation microsite separation along NMDS axis one (Figure 3.4, Figure 3.5). Overall, VD microsites were also more variable than those at DM for most traits (Figure 3.5).

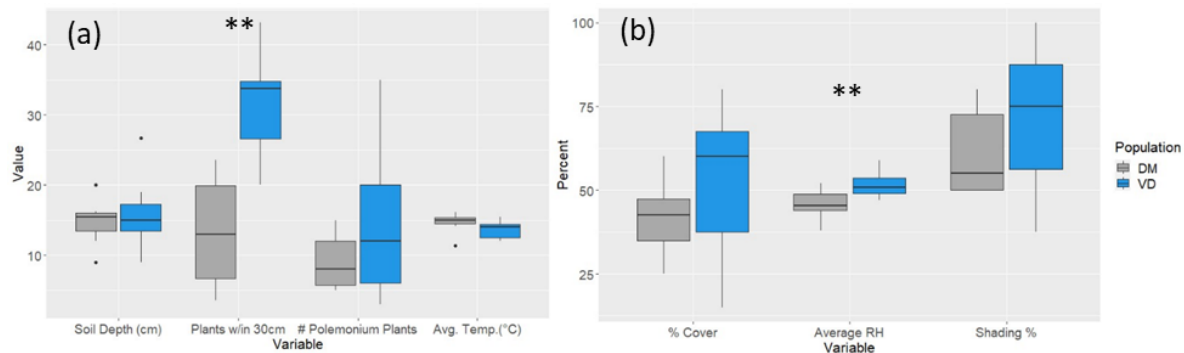


Figure 3.5: Boxplots of (a) microsite soil depth (cm), plants within 30cm (index of vegetation cover), # of *P. brandegeei* plants at each microsite, and average temperature ($^{\circ}\text{C}$) by population and (b) microsite % cover (microsite “openness”), average relative humidity (average RH), shading % (% of day microsite was shaded). Significant differences in average RH and plants within 30cm only. ** $P < 0.01$.

Does physical mating distance between plants and/or environmental variability affect mating success in *P. brandegeei*?

Within Microsite Crosses

As predicted, seed set increased as mating distance increased (slope $\pm$ SE = 0.45 ± 0.10 , $P < 0.001$) and distance explained 47% of the variation ($F_{1,22} = 21.29$, $P < 0.001$; Figure 3.6, Table 3.3). Additionally, within microsite crosses at VD set fewer seeds than those at DM (-1.44 ± 0.35 , $P < 0.05$) and population explained 14% of the variation in seed set ($F_{1,22} = 4.66$, $P < 0.05$) but this model was the 9th best by AICc (Δ AICc = 11.64). NMDS axis one and two score, Euclidean distance and maternal/paternal inflorescence number did not significantly affect seed set, contrary to expectations. On average, seed set for within microsite crosses was 3.45 ± 0.65 seeds/flower with a range of 0 – 9.43 seeds/flower across both populations.

Total seed mass increased in proportion to the total number of seeds collected (slope $\pm$ SE = 0.97 ± 0.04 , $P < 0.001$) which explained 96% of the variation ($F_{1,22} = 497.7$, $P < 0.001$; Figure 3.7, Table 3.3). Distance and environmental variation metrics (Euclidean distance, NMDS axis one/two scores) as well as maternal/paternal inflorescence number and population had no

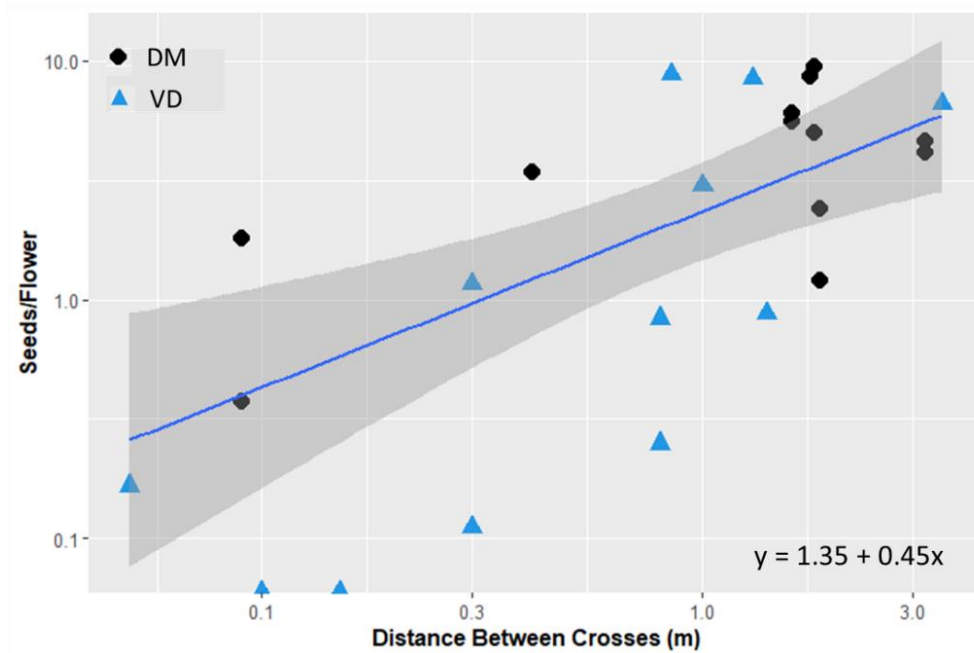


Figure 3.6: Scatterplot of seeds/flower (seed set) by (log transformed) physical distance between crosses for within microsite crosses in two populations of *P. brandegeei*. Physical distance between crosses explained 47% of the variation in seed set.

significant effect on total seed mass, although population and maternal inflorescence number were in the top three models (Table 3.3). Overall, average total seed mass for within microsite crosses was 19.14 ± 3.87 mg and ranged from 0 – 65.10 mg across both populations. Average seed mass per seed for within microsite crosses was 0.85 ± 0.09 mg and ranged from 0 – 1.83 mg across both populations.

Local environment (NMDS axis two score) explained 35% of the variation in germination percent (slope $\pm$ SE = -3.78 ± 1.44 , $P < 0.05$) ($F_{1,10} = 6.86$, $P < 0.05$; Figure 3.8, Table 3.3). However, the datapoint with the highest NMDS2 score, while not an outlier, has significant leverage over this result (Figure 3.8). No other variables, including distance, had a significant effect on germination percent. Germination percent for within microsite crosses across both populations was $71 \pm 7.50\%$ and ranged from 0 – 88.89%.

No variables had significant relationships with total seedling survival to two weeks. Average percent survival to two weeks for within microsite crosses was $41.10 \pm 9.28\%$ and ranged from 0 – 100%.

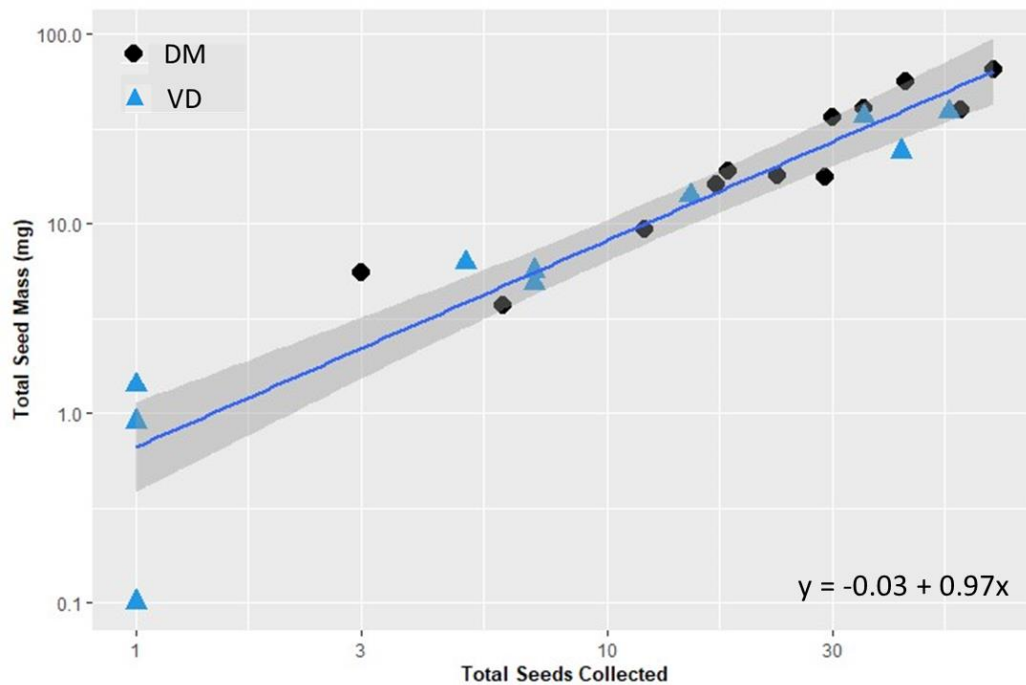


Figure 3.7: Scatterplot of (log transformed) total seed mass (mg) by (log transformed) total seeds collected for within microsite crosses in two populations of *P. brandegeei*. Total seeds collected explained 96% of the variation in total seed mass.

Table 3.3: Statistics for best linear models ($\Delta AIC_c < 2$) of within microsite crosses in two populations of *P. brandegeei* for seed set, total seed mass and germination %. Variables log transformed as per methods. Significant effects in bold. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Model	ΔAIC	R^2	F (DF effect, DF resid.)	Model p
<u>Seeds/Flower ~</u>				
Distance***	0.00	0.47	21.29 (1,22)	<0.001
Distance*** + Population	0.51	0.50	12.34 (2,21)	<0.001
<u>Seed Mass ~</u>				
Total seeds collected***	0.00	0.96	497.7 (1,22)	<0.001
Total seeds collected*** + Population	1.26	0.96	255.1 (2,21)	<0.001
Total seeds collected*** + maternal inflorescence #	1.94	0.96	247.7 (2,21)	<0.001
<u>Germination % ~</u>				
NMDS axis two score*	0.00	0.35	6.86 (1,10)	<0.05

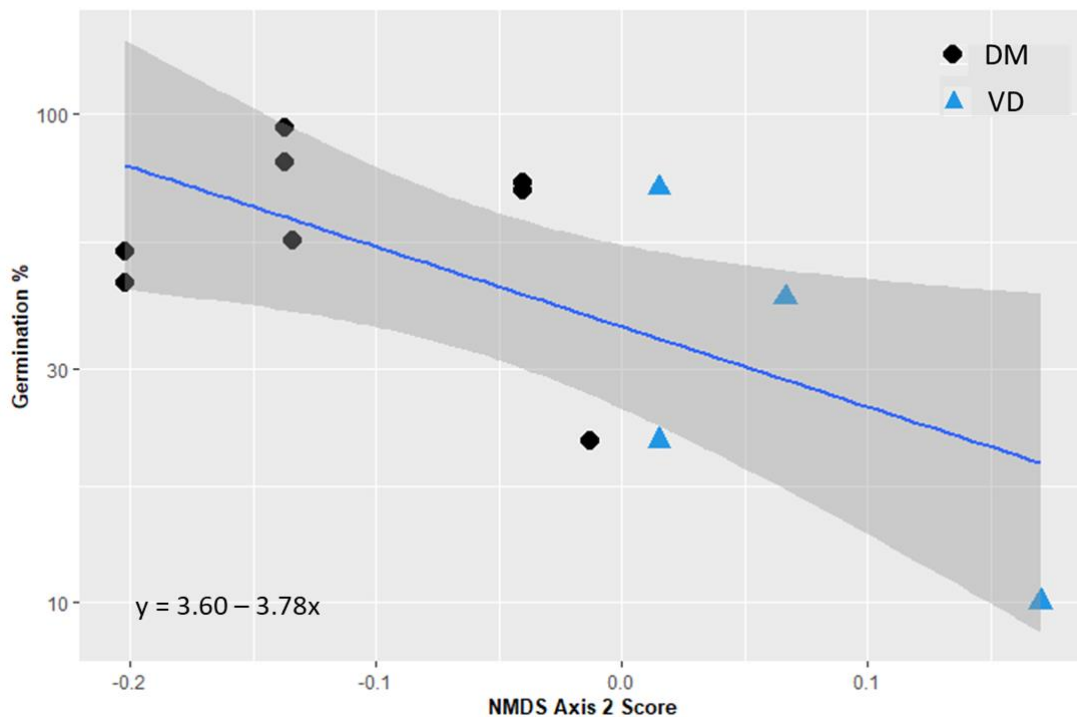


Figure 3.8: Scatterplot of (log transformed) germination percent by NMDS axis two score for within microsite crosses in two populations of *P. brandegeei*. NMDS axis two score explained 35% of the variation in germination percent.

Between Microsite Crosses

Contrary to predictions, there were no significant relationships between seed set and physical distance, Euclidean distance, NMDS axis one and two scores, or any other measured at VD or DM (all $F < 1.90$, all $P > 0.18$ in general linear models, all $t < 1.4$, all $P > 0.13$ in mixed models). In addition, none of the random effects tested in mixed models improved model fit; all $P > 0.11$ for tests of models including maternal ID or microsite and paternal ID or microsite versus models without these effects. On average, between microsite crosses at VD set 5.82 ± 0.56 seeds and seed set ranged from 1.75 – 15.50 seeds while between microsite crosses at DM set an average of 4.10 ± 0.55 seeds and seed set at DM ranged from 0 – 13 seeds.

Total seeds collected explained most of the variation in total seed mass at both VD and DM. At VD, total seed mass increased with total seeds collected (slope $\pm$ SE = 0.84 ± 0.12 , $P < 0.001$; Figure 3.9a, Table 3.4) and total seeds collected explained about 70% of the variation in total seed mass for between microsite crosses at VD. At DM, total seed mass also increased in proportion to total seeds collected (slope $\pm$ SE = 0.96 ± 0.03 , $P < 0.001$; Figure 3.9b, Table 3.4) and total seeds collected explained 97% of the variation in total seed mass for between microsite crosses at DM. Mixed models with maternal effects (maternal microsite, maternal ID) were significantly better than models without these random effects (all $P < 0.01$; Table 3.4) for both the VD and DM between microsite cross data, although random effects only increased R^2 slightly for DM mixed models.

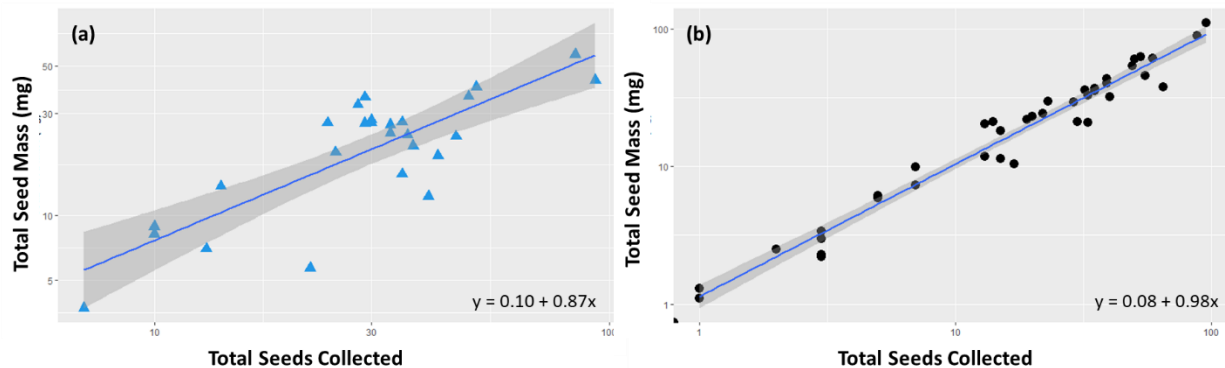


Figure 3.9: Scatterplots of (log transformed) total seed mass by (log transformed) total seeds collected for between microsite crosses in two populations of *P. brandegeei*: (a) VD; and (b) DM. Total seeds collected explained 65% of the variation in total seed mass at VD and 97% of the variation in seed mass at DM.

In the general linear models of the VD between microsite cross data, total seed mass also increased with Euclidean distance (slope $\pm$ SE = 1.14 ± 0.51 , $P < 0.05$), contrary to predictions, and Euclidean distance explained about 5% of the variation in total seeds collected. In partial support of predictions, total seed mass increased with Euclidean distance (slope $\pm$ SE = 1.04 ± 0.51 , $P < 0.05$), NMDS axis two score (slope $\pm$ SE = 1.30 ± 0.48 , $P < 0.01$) and maternal inflorescence number (slope $\pm$ SE = 0.14 ± 0.05 , $P < 0.01$) in the DM between microsite cross data, but these effects all explained less than 5% of the variation in total seeds collected. All other fixed and random effects were not significantly associated with total seed mass at VD or DM. At VD, the average total seed mass for between microsite crosses was 23.62 ± 2.47 mg and ranged from 3.70 to 56.60mg while the average seed mass per seed was 0.73 ± 0.05 mg and ranged from 0.26 to 1.18mg. At DM, the average total seed mass for between microsite crosses was 26.41 mg $\pm$ 3.86 and ranged from 0 to 110.40mg while the average seed mass per seed was 1.00 ± 0.05 mg and ranged from 0 to 1.57mg.

Table 3.4: Statistics of the best general linear mixed models of between microsite crosses in two populations of *P. brandegeei* for total seed mass. Best models selected by conditional R^2 . Conditional $R^2 = R^2$ of full model, marginal $R^2 = R^2$ of model with fixed effects only. ICC = intraclass correlation coefficient. P -value derived from the LRT (likelihood ratio test; “ranova” function) which compares models with and without random effects; a significant p value indicates that the model with the random effects fits the data best. Variables log transformed as per methods. Significant effects in bold. (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.)

Model		Conditional R^2	Marginal R^2	ICC	LRT	P
Fixed Effect	Random Effect					
Total Seed Mass (VD) ~						
Total seeds collected***	Maternal ID	0.921	0.737	0.701	9.89	< 0.01
Total seeds collected***	Maternal Microsite ID	0.854	0.698	0.516	7.65	< 0.01
Total Seed Mass (DM) ~						
Total seeds collected*** + maternal inflorescence #*	Maternal Microsite ID	0.983	0.966	0.491	8.77	< 0.01
Total seeds collected***	Maternal ID	0.988	0.965	0.651	14.95	< 0.001

There was a negative relationship between germination percent and paternal inflorescence number for between microsite crosses at VD (slope $\pm$ SE = -0.80 ± 0.30 , $P < 0.05$; Figure 3.10a). There was also a negative relationship between germination percent and Euclidean distance in general linear models of the VD between microsite cross data (slope $\pm$ SE = -3.33 ± 1.51 , $P < 0.05$; Figure 3.10b), as predicted. Together, paternal inflorescence number and Euclidean distance explained 28% of the variance in germination percent (Table 3.5). There were no other significant relationships. Average germination percent for the between microsite crosses at VD was $43.17 \pm 5.79\%$ and ranged from 0 to 88.89%.

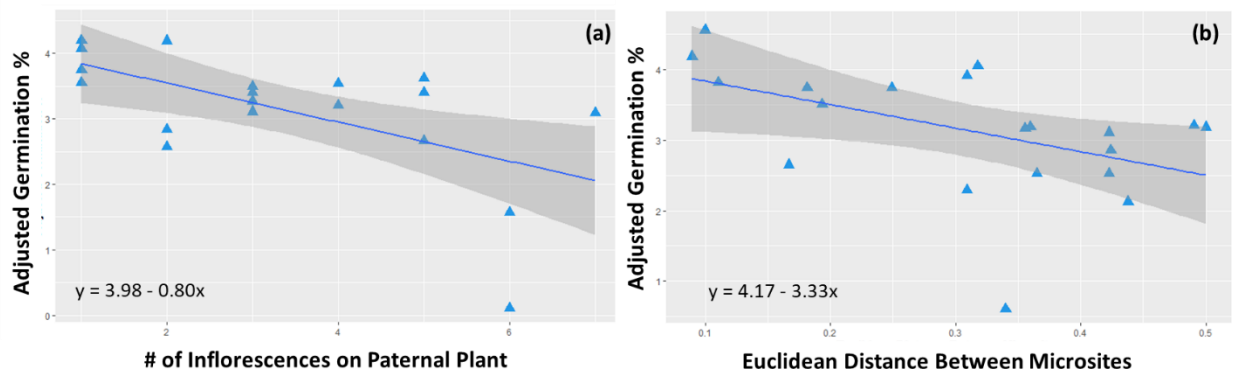


Figure 3.10: Scatterplots of (log transformed) germination percent (predicted values + residuals) by (a) # of inflorescences on the paternal plant and (b) by Euclidean distance between microsites for between microsite crosses in one population of *P. brandegeei* (VD). Values for paternal inflorescence # and Euclidean distance are adjusted (predicted values + residuals from the model). Together, paternal inflorescence # and Euclidean distance explained 28% of the variation in germination %.

For the DM between microsite cross data, there was a negative relationship between germination percent and paternal inflorescence # (slope $\pm$ SE = -0.36 ± 0.17 , $P < 0.05$) in general linear models of the between microsite crosses, but the model with physical distance and paternal inflorescence number explained only 11% of the variance in germination percent at DM (Table 3.5). No other relationships, including with distance, Euclidean distance, or NMDS axis one/two, were significant in general linear or mixed models of the DM germination percent data, contrary to predictions. In addition, none of the random effects tested in mixed models improved model fit; all $P > 0.35$ for tests of models including maternal ID or microsite and paternal ID or microsite versus models without these effects. Average germination percent for the DM between microsite data was $68.82 \pm 4.91\%$ and ranged from 6.25 – 84.21%.

Table 3.5: Statistics of the best general linear models (no random effects) of between microsite crosses in two populations of *P. brandegeei* (VD, DM) for germination %. Best models were selected based on ΔAICc scores. Significant effects in bold. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Model	ΔAICc	R^2 (adj.)	F (DF effect, DF residual)	p
<u>Germination % (VD) ~</u>				
Euclidean distance* + paternal inflorescence #*	0.00	0.28	4.73 (2,17)	< 0.05
<u>Germination % (DM) ~</u>				
Distance + paternal inflorescence #*	0.00	0.11	2.67 (2,24)	< 0.100

There were no significant relationships in the percent survival to two weeks data for either VD or DM, contrary to predictions. Average percent survival to two weeks for the between microsite crosses was $39.64 \pm 7.51\%$ and ranged from 0 – 100% at VD and at DM was $40.60 \pm 4.22\%$ and ranged from 0 – 100%.

Do greenhouse crosses support the presence of inbreeding and/or outbreeding depression in *P. brandegeei*?

As predicted, cross type had a significant effect on seed set ($F_{3,48} = 4.36$, $P < 0.01$). “Intra” (intrapopulation, different maternal family) crosses, which set an average of 2.50 ± 0.48 seeds and “inter” (interpopulation) crosses, which set an average of 2.82 ± 0.35 seeds, set significantly more seeds than self crosses ($P < 0.05$ and $P < 0.01$, respectively; Figure 3.11a). Self crosses set an average of 0.47 ± 0.51 seeds while sib crosses set an average of 2.08 ± 0.52 seeds. All means in this section are marginal means extracted from the model. Similarly, maternal population had a significant effect on seed set ($F_{2, 48} = 5.92$, $P < 0.01$). Specifically, crosses where a TC plant was used as the pollen recipient set significantly more seeds than crosses where an SC plant was used as the pollen recipient ($P < 0.05$; Figure 3.11b). There were no significant differences between the other populations. Crosses where TC plant was used as the pollen recipient set an average of 2.73 ± 0.42 seeds compared to 1.09 ± 0.41 seeds for crosses where an SC plant was used as the pollen recipient or 2.09 ± 0.39 seeds when a DM plant was used as the pollen recipient. The interaction between cross type and maternal population was also not significant ($F_{6,48} = 2.07$, $P = 0.07$).

Total seed mass increased as total seeds collected increased (slope $\pm$ SE = 0.92 ± 2.91 , $P < 0.001$). Cross type and maternal population, however, did not have a significant effect on seed mass, contrary to predictions ($F_{3,47} = 0.64$, $P = 0.60$; $F_{2,47} = 0.80$, $P = 0.46$, respectively), and the interaction between maternal population and cross type was also not significant ($F_{6,47} = 0.17$, $P = 0.98$). Average total seed mass for all greenhouse crosses was 11.76 ± 1.46 mg.

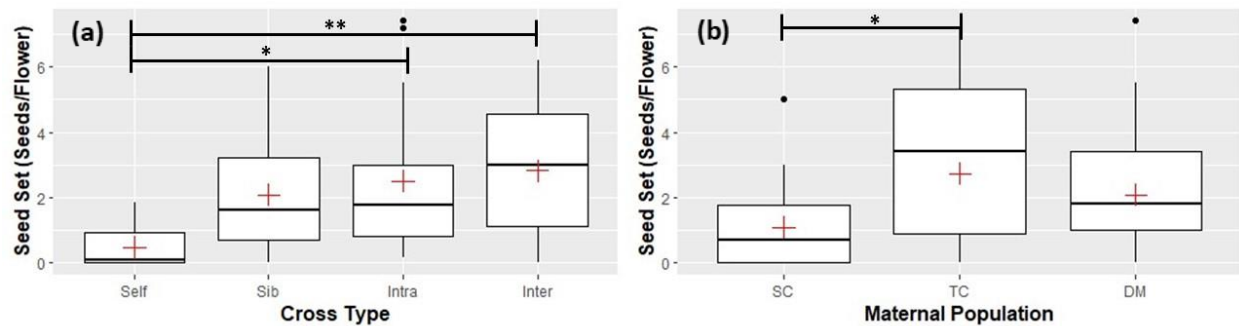


Figure 3.11 Boxplot of seed set (seeds/flower) of greenhouse crosses by (a) cross type and (b) maternal population. Intra (within population outcross) and inter (between population) crosses set significantly more seed than self crosses as did crosses where TC was used as the mother (* = $p < 0.05$, ** = $p < 0.01$). + = marginal means extracted from the model.

Interpopulation Crosses

In partial support of predictions, paternal population had a significant effect on seed set ($F_{2,18} = 5.67$, $P < 0.05$) but maternal population did not ($F_{2,18} = 1.87$, $P = 0.18$) and the interaction was not significant ($F_{2,18} = 0.03$, $P = 0.86$). Crosses where a SC plant was used as the pollen donor set an average of 4.5 ± 0.34 seeds which was significantly more seeds than crosses where a TC plant was used as the pollen donor ($P < 0.05$), which set an average of 1.03 ± 0.3 seeds. There were no significant differences in seed set between crosses where DM was used as the pollen donor, which set an average of 2.92 ± 0.74 seeds, and crosses where either TC or SC were used as the pollen donor. Contrary to predictions, maternal and paternal population had no significant effects on interpopulation cross seed mass (both $P > 0.23$). Total seeds collected was also not significant ($P = 0.25$).

DISCUSSION

The effects of physical distance, environmental variation, and other factors on mating success in *P. brandegeei* were assessed. Overall, seed set was affected by physical distance between crossed plants; crosses between closely related plants (greenhouse) and plants at distances of less than about one meter (field) set fewer seeds. Later-stage measures of mating success (seed mass, germination percent, and survival to two weeks) were not affected by physical distance between mated plants. Environmental variation within and among populations was found and microsite variables had some effect on mating success.

Physical distance between crosses affects mating success in of *P. brandegeei*.

Seed set increased as physical distance between plants increased, but only for the within microsite crosses. Based on this and the lack of a relationship between seed set and physical distance for the between microsite crosses, mating in *P. brandegeei* is optimized provided crosses are made between plants that are at least 1 to 3.5 metres apart. Crosses at distances of less than about 1m are likely to be crosses with closely related individuals (due to limited seed dispersal) which may result in biparental inbreeding depression. All crosses beyond 3.5m had similar seed set which suggests that outbreeding depression does not occur within populations of *P. brandegeei*. Plants at different microsites throughout the populations have apparently not sufficiently adapted to local environmental conditions for genetic incompatibilities to develop, although the effects of incompatibilities may only be observed in later life stages and subsequent generations (Villegas et al. 2020).

Similar mating success at any physical distance greater than about one meter in *P. brandegeei* contrasts previous work on other members of the Polemoniaceae. For example, Waser and Price (1989) detected both inbreeding and outbreeding depression in their work with *Ipomopsis aggregata* and crosses at 1-10m distances were more successful than self crosses or crosses between plants 100m apart. However, significant outbreeding depression was only detected in some cohorts of *I. aggregate* in a follow up study (Waser et al. 2000).

The lack of outbreeding depression in two populations of *P. brandegeei* is, however, consistent with results for some other perennials. Studies on numerous other species have also

found that matings within populations are often similarly successful at a range of distances between plants, provided crosses are not made on the same plant or with immediate neighbours. For example, Villegas et al. (2020) found that seed set and progeny fitness in *Pinguicula moranensis* (Lentibulariaceae) is similarly optimized at 1, 4, 460 (intrapopulation), and 9900 metres (interpopulation) compared to self crosses. A similar study on two *Trillium* (Liliaceae) species also found that self crosses reduced fitness but outcrosses at various distances (1, 10, 100, 1500 m) were all equally successful (Irwin 2001). These results are therefore consistent with my finding that seed set in *P. brandegeei* increases as physical distance increases for within microsite crosses only.

Increased seed set for crosses between plants greater than one meter apart also supports previous work on mating and pollination in *P. brandegeei*. These results help inform why seed set decreased when more effective large-bodied pollinators (hawkmoths, hummingbirds) were excluded from *P. brandegeei* plants in one year of two years of study (Pond 2017). Large-bodied pollinators have larger foraging ranges and are more likely to visit multiple plants throughout a population, thereby increasing the likelihood of outcrossing (Kulbaba & Worley 2008; Rhodes et al. 2017). In contrast, small-bodied pollinators (bees, flies) typically have shorter foraging ranges and are more likely to transfer pollen between flowers on the same plant or to near neighbours (Kulbaba & Worley 2008; Rhodes et al. 2017; Diller et al. 2022). This short distance pollen transfer would, as this study found, cause reduced seed set through self-incompatibility mechanisms or inbreeding depression due to selfing or due to mating with a closely related individual and suggests that pollen transfer by large-bodied pollinators is indeed more effective. Thus, changes to the relative abundance of large-bodied pollinators may affect seed set in this sub(alpine) species.

The lack of relationship between physical distance and total seed mass, germination percent, and percent survival to two weeks runs contrary to predictions, however. First, although some studies have found that both seed set and mass increase as crossing distance increases (Forrest et al. 2011), others have found that even though seed set is positively influenced by crossing distance, seed mass is not related to (Oostermeijer et al. 1995) or is negatively related to crossing distance (Ayre et al. 2019). Second, the lack of a relationship

between physical distance and total seed mass may be explained by the extremely high correlation between total seed mass and total seeds collected in this data. This strong relationship suggests that seed size within populations of *P. brandegeei* is relatively constant. Finally, seed mass may be more influenced by other factors than by physical distance, such as by nutrient availability (De Malach et al. 2019).

Finally, while other studies have also found positive relationships between physical crossing distance and both germination percent and survival percent (Ayre et al. 2019; Forrest et al. 2011), the apparent lack of relationships in the *P. brandegeei* crossing data may be explained by several factors. First, inbreeding depression may only affect seed set in *P. brandegeei* and may have limited effects on progeny fitness, as was found by Trame et al. (1995) in a similar study on *Agave schottii* (Asparagaceae). Further, a lack of seeds produced by near neighbour crosses (i.e., crosses between plants less than 1m apart) excluded most near crosses from the germination study (Figure 3.12). Given that the only relationship with physical distance in the seed set data involved within microsite crosses, the decrease in sample size for germination percent may have reduced the power to detect any effects of short crossing distances on germination percent.

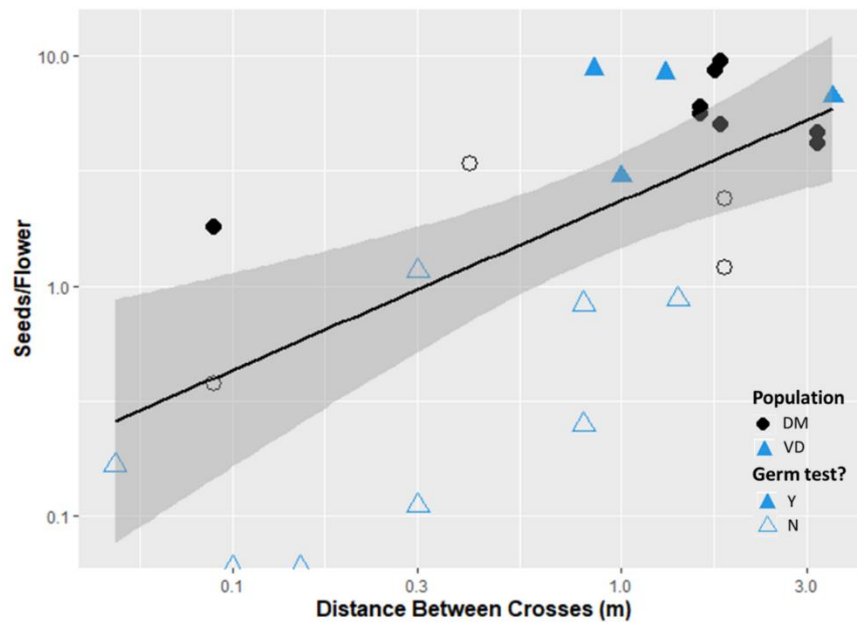


Figure 3.12: Scatterplot of seeds/flower by physical distance between crosses in two populations of *P. brandegeei*. Points coloured by population and whether a germination test was performed for that cross.

Greenhouse crosses support the presence of inbreeding but not outbreeding depression in *P. brandegeei*.

Across three populations grown in the greenhouse, crosses between closely related individuals (self crosses and crosses within maternal families) were less optimal compared to crosses with less closely related individuals (crosses outside maternal families and interpopulation crosses), both of which set significantly more seeds than self crosses. Although greenhouse crosses set fewer seeds overall compared to field crosses, this supports my interpretation of the field crossing data, as near neighbour crosses in the field at distances of less than one metre are also likely to be crosses with closely related individuals due to limited seed dispersal mechanisms.

The lack of an outbreeding depression effect in the greenhouse also matches with the field data and offers even more evidence that outbreeding depression is unlikely in *P. brandegeei*. This result is unsurprising given that members of the genus *Polemonium*, including *P. brandegeei*, are known to readily hybridize (Grant 1989; Rose 2021; Rose et al. 2021) and are therefore unlikely to suffer from early acting outbreeding depression within a species or population. Further, outbreeding depression is often not detected in optimal mating studies and most studies report that interpopulation crosses at large distances are often as successful as intrapopulation crosses (e.g., Ayre et al. 2019; Villegas et al. 2020) although there are certainly exceptions (e.g., Ruane et al. 2015; and see introduction).

Environmental variation within and among populations of *P. brandegeei* has some effect on mating success.

Although there was much overlap in environmental variables between populations, microsites at VD and DM differed in average relative humidity over one day, in vegetation cover, and in soil type and the two populations were separated in the NMDS ordination. Differences between populations in relative humidity over one day could simply be a result of sampling date variation, as similar trends were not detected in datalogger data from 2022 (Appendix 5). Differences in vegetation cover may be due to differences in substrate between

the populations as the loose scree slope at DM may prevent the establishment of some fast-growing annual plants and grasses (Giupponi et al. 2023).

Intrapopulation differences were driven primarily by differences in percent cover, the percent of the day that microsites were shaded, and by the number of *P. brandegeei* plants within each microsite. Overall, microsites at VD were more variable than were microsites at DM and microsites from the more forested west side of the rock formation at VD appeared distinct in the NMDS plot. These "peripheral" microsites at VD typically had deeper, more organic soil and *P. brandegeei* plants were less abundant overall. Further, microsites on the west side were less likely to be covered by rock overhangs and were less likely to be located alongside boulders than microsites on the east side of the rock formation. Thus, intrapopulation habitat and environmental variation is quite common at VD, and considerably more common than at DM.

Environmental variation had some effect on various measures of mating success in field crosses. First, total seed mass was positively related to Euclidean distance for between microsite crosses at both VD and DM and positively related to NMDS2 score for DM between microsite crosses. With the caveat that these effects explained much less of the variation compared to total seeds collected, these results suggest that microsite variability does affect seed mass in *P. brandegeei*. Although the positive relationship with Euclidean distance runs contrary to predictions related to local adaptation, as increasing Euclidean distance suggests increasing microsite dissimilarity, it is consistent with the lack of an apparent outbreeding depression effect and supports the interpretation that microsites within populations of *P. brandegeei* are not distinct enough for fine-scale local adaptation to occur. I have found no other optimal mating studies that consider microsite variation. However, habitat heterogeneity can affect plant health and reproductive success (Rodriguez-Rodriguez et al. 2015). Crosses between more productive microsites might, therefore, be more successful irrespective of apparent microsite dissimilarity. Indeed, maternal microsite and maternal ID both explained some variation in total seed mass at DM and VD which further suggests that the characteristics of the microsites and of the maternal plants themselves likely contribute more to seed mass than does similarity or dissimilarity between microsites (Pittman & Levin 1989).

Furthermore, pollinator behaviour is influenced by fine-scale habitat variation. For example, within population variability in con- and heterospecific flowering plant density (Janovsky et al. 2013) and amount of direct sunlight received (Herrera 1995) can influence pollinator composition and behaviour. Thus, even though microsites may be dissimilar by the metrics measured for this study, other unmeasured environmental factors could be more similar and may affect pollinator behaviour and crossing success in open pollinated plants.

There was also a negative relationship between germination percent and both NMDS2 score for the within microsite crosses and Euclidean distance for the VD between microsite cross data. This also suggests that germination percent may be influenced by habitat variability within populations. However, the interpretability of the germination test data is limited for two reasons. First, the small samples sizes for the germination data may under or overestimate significant relationships. Second, germination and survival studies were only standardized for the four-week cold stratification period and conditions such as soil moisture levels, temperature, light levels, and humidity were variable in the greenhouse. This may have influenced germination and survival in the greenhouse, as *P. brandegeei* seedlings are very susceptible to damping off due to overwatering in the early stages of growth (pers. obs.).

CONCLUSIONS & FUTURE DIRECTIONS

Seed set in *P. brandegeei* is optimized when crosses are made between plants at a minimum distance of 1 – 3.5m from each other. Meeting this distance threshold apparently reduces inbreeding depression. There was no evidence of outbreeding depression within populations of *P. brandegeei* as crosses between plants at distances greater than 1 – 3.5m showed no differences in seed set, seed mass, germination percent, or seedling survival. Environmental variation within and among populations had some effect on seed mass and germination percent. Together, these results indicate that pollination by pollinators with larger foraging ranges is likely preferred in this (sub)alpine perennial, and that changes to the abundance of such pollinators may affect plant fitness. Also, environmental variation can affect crossing success and should be considered when performing crosses in natural populations.

Future work could include a higher proportion of near crosses to fully test the relationship between germination percent and physical crossing distance and to determine the specific range of distances over which inbreeding depression influences crossing success. Genetic analysis of populations of *P. brandegeei* would confirm spatial structuring within populations and microsites and aid in the interpretability of these data. Interpopulation crosses in the field would also provide confirmation on the lack of outbreeding depression in this species. As discussed above, these data will also be used to inform previous studies on mating and pollination in *P. brandegeei* as they offer further support for the increased effectiveness of large-bodied pollinators with larger foraging ranges (as per Kulbaba & Worley 2008; Pond 2017).

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Chapter 4: Thesis Conclusions

Many plants have generalist pollination systems and are pollinated by a diverse array of pollinators (Brosi 2016). Within generalist systems, pollinator effectiveness often varies, and certain pollinators transfer larger amounts of higher quality pollen than others (Mayfield et al. 2001). However, the relative role different pollinators play in generalist systems has historically been understudied (Jaeger et al. 2022). *Polemonium brandegeei* receives pollination services from hawkmoths, hummingbirds, and various small bees and flies, of which hawkmoths and hummingbirds are the most effective and most likely to promote outcrossing between plants (Kulbaba & Worley 2008; Pond 2017). However, excluding hawkmoths and hummingbirds from the system only decreased seed set in one year out of two which suggests that small-bodied pollinators also contribute to seed set (Pond 2017). In this research, I used hand crosses between plants at varying distances from each other to determine if longer distance pollen transfer, such as that consistent with pollination by hawkmoths and hummingbirds, results in increased reproductive output compared to shorter distance pollen transfer consistent with pollination by small bees and flies (Chapter 3). This research increases our understanding of pollination in a generalist system by explaining the relative contribution of more effective, longer-distance pollinators to reproductive output (Jaeger et al. 2022) and offers insights into the potential effects on generalist plants of changes to pollinator assemblages, such as a loss of more effective pollinators, due to ongoing pollinator declines (Rhodes 2018).

Further, determining the role different pollinators play in generalist systems can offer insights into past and current selection on floral traits. For example, more effective pollinators often exert stronger selective pressures on floral traits due to their outsized importance on reproductive success (Stebbins 1970). Temporal and spatial variation in the abundance of highly effective pollinators (and in selective pressures) may lead to local adaptation and population genetic and phenotypic differentiation (e.g., Chapurlat et al. 2018). However, plastic responses to environmental variation can also cause interpopulation phenotypic variation (Palacio-Lopez et al. 2015). Due to their importance to the attraction and orientation of pollinators, variation

in floral traits such as morphology and scent can affect pollinator behaviour and plant fitness and it is therefore important to understand this variation and its causes (Eisen et al. 2022).

In Chapter 2, I characterized among population variation in *P. brandegeei* floral morphology and scent and used a common environment study to determine if among population variation has a genetic component or is caused primarily by phenotypic plasticity. Common garden studies can explain among population trait variation and plant responses to environmental change, but they have not often been used with (sub)alpine plant species or to understand plasticity in floral scent emissions (Majetic et al. 2009; Berend et al. 2019). The clear among population variation in floral traits (Kulbaba & Worley 2008; pers. obs.), the lack of data on floral scent chemistry, and the addition of a study population in New Mexico, made *P. brandegeei* an excellent study system for an analysis of the scale and potential causes of interpopulation variation in floral morphology and scent.

In this final chapter, I summarize and discuss the main findings of this thesis and identify areas of further study.

Populations of *P. brandegeei* vary in floral morphology and scent.

All populations included in this study differed from each other in both morphology and floral scent although the scale of the differences among populations was variable. The southernmost population (Sandia Crest, New Mexico) was the most distinct in all analyses; flowers from this population were larger, had highly recessed stigmas (reverse herkogamy), and differed significantly in the relative proportion of both abundant and less abundant floral VOCs. These differences may reflect more intense selection by hawkmoths at SC or to genetic admixture/hybridization with other species of *Polemonium* (Kulbaba & Worley 2012; Rose et al. 2021) although further research is required to confirm this. The other populations (Taylor Canyon, Colorado, Deer Mountain, Colorado) were less distinct from each other, as was reported by Kulbaba & Worley (2008). However, flowers from the most northern population (Vedauwoo, Wyoming), had wider corollas and differed in some floral VOC compounds compared to flowers from the two Colorado populations. Also, flowers from the Taylor Canyon, Colorado population were considerably smaller than flowers from all other populations and

displayed subtle differences in floral scent composition. These differences may reflect past and current selection on floral traits across the substantial elevational and latitudinal distribution of this (sub)alpine species (Szenteczki et al. 2021).

As the first full characterization of the floral scent profile of *P. brandegeei*, this study is just the beginning of potential work on this topic (see also chapter 2). Further characterization of the floral scent profile of this species is necessary to confirm patterns identified in this study and to increase the sample size, particularly for the VD population. Further research on diel (day vs. night) patterns of floral scent emission (e.g., Chapurlat et al. 2018), within-flower patterns of emission (Garcia et al. 2021), and the role different trichomes play in floral VOC synthesis and emission (e.g., Guo et al. 2020) would also complement this baseline research, as would comparisons to other species of *Polemonium*. Beyond floral scent related research, increased pollinator observation hours, particularly at SC would improve understanding of pollinator mediated selection in the different populations. Genetic analysis, especially of the SC population, would confirm the species relatedness of all populations. Finally, including additional populations in future studies would further illuminate the latitudinal gradient in floral traits.

Patterns of population differentiation were maintained in the greenhouse.

As a first step to determine if variation among populations has a genetic component or is the result of phenotypic plasticity, I compared morphological and chemical traits between field and greenhouse grown plants from the same three populations. All major patterns (as presented above) were preserved in the greenhouse which indicates that differences among populations have a genetic component and are not caused strictly by plastic responses to environmental variation (de Villemereuil et al. 2020). However, greenhouse grown flowers were overall larger and produced substantially more VOCs than field grown flowers which suggests that most *P. brandegeei* floral traits are plastic to some degree (Majetic et al. 2009). This indicates that among population variation *P. brandegeei* has a strong genetic underpinning and that this (sub)alpine plant can respond to rapid changes in environmental conditions such as temperature through phenotypic plasticity, as has been reported for other alpine species (e.g., Dai et al. 2017). This also adds to the limited body of knowledge on both the plasticity of

floral scent emissions (Majetic et al. 2009) and on the heritability of among population differences in floral scent (Eisen et al. 2022) . Future work to characterize the genetic variability among populations of *P. brandegeei* would confirm that interpopulation variation has a genetic component and reciprocal crosses in field populations would further determine responses of an alpine plant species to variable environmental conditions (Dai et al. 2017; Berend et al. 2019).

Mating is optimized when crosses are made between plants >1m from each other.

Crosses between near neighbours (about 1m between plants) set less seed than crosses between more distant plants. This indicates that populations of *P. brandegeei* have some spatial genetic structure, and that inbreeding depression acts at relatively short distances in this species, as has been reported for other hummingbird pollinated species (e.g., Ayre et al. 2019). Thus, longer distance pollen transfer, such as that facilitated by hawkmoths and hummingbirds, is likely to be preferred in *P. brandegeei*. This provides an explanation for the reduced seed set in one year when large-bodied pollinators were excluded from the system (Pond 2017). Further, this finding offers insights into the potential effects of ongoing pollinator declines in a generalist system (Rhodes 2018). For example, loss or decreased abundance of pollinators with longer foraging bouts due to climate or habitat changes could significantly reduce seed set in *P. brandegeei* and other species with similar generalist systems (Pond 2017).

There were no significant differences in seed set, seed mass, germination, or survival among between microsite crosses and within population outcrosses and crosses with different populations set statistically similar amounts of seed in the greenhouse. This suggests that outbreeding depression does not occur within populations of *P. brandegeei*. Environmental variation had some limited effects on crossing success, especially regarding maternal microsite conditions, and environmental factors should be considered in optimal mating studies.

An increased number of crosses between near plants and crosses in more populations would confirm the presence of inbreeding depression over small spatial scales in *P. brandegeei*. Further, the inclusion of between-population and between-species crosses in the field would confirm the lack of outbreeding depression found in this study. Finally, a genetic analysis of populations would confirm spatial genetic structuring in populations of *P. brandegeei*.

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Appendix 1 – Germination Experiments

This is a summary of various methods used to stimulate germination in *P. brandegeei*.

Method 1 – Cold Stratification Only

As per previous studies and preliminary work in Fall 2021, I randomly selected seeds from some crosses and cold stratified them for two weeks. Following cold stratification, I transferred petri dishes to an incubator (I-36 Series Controlled Environment Chamber; Percival Scientific, Inc.) set at 21°C during the day and 15°C at night, with a day length of 15 hours. Humidity in the incubator was variable but averaged around 40% RH. I kept all filter papers moist and replaced them if fungal/bacterial growth was noted. Percent germination with this treatment was minimal (Table A.1). After being moved to soil, all seedlings failed to survive past one week. After 8-10 weeks in the incubator with no germination, I moved all remaining seeds to soil where no further germination occurred.

Table A.1: Total number of crosses, total seeds used, and % germination under two-week cold stratification treatment on seeds from two populations of *P. brandegeei*.

Population	# of Crosses Used	Germination %
Deer Mountain	14 (114 seeds total)	5/114 = 4.4%
Vedauwoo	18 (161 seeds total)	5/161 = 3.1%

Method 2 – Acid Scarification + Cold Stratification

I applied three acid and two cold stratification treatments (in combination) to open-pollinated seeds collected at DM and VD to stimulate germination through acid scarification. Acid treatments included a control (no acid), a 20hr soak in 0.5M hydrogen peroxide, a 20hr soak in 6% hydrogen peroxide, and a 7-minute soak in 95% sulfuric acid. I rinsed seeds with DI water following acid treatment and plated them. Percent germination increased only marginally (Table A.2). I moved all seeds to soil after about 4 weeks in the incubator, but very little further germination occurred.

Table A.2: Total number of crosses, total seeds used, and % germination under various stratification and scarification treatments performed on seeds from two populations of *P. brandegeei*.

Treatment	Population	# of Maternal Families	% Germination
4 Week Cold Stratification (CS) (Control)	DM	4 (50 seeds total)	25%
	VD	4 (36 seeds total)	5%
0.5M H ₂ O ₂ + 4 Week CS (e.g., Msanga & Maghembe, 1989)	DM	4 (29 seeds total)	0%
	VD	4 (29 seeds total)	0%
6% H ₂ O ₂ + 4 Week CS (e.g., Msanga & Maghembe, 1989)	DM	4 (30 seeds total)	3%
	VD	3 (21 seeds total)	0%
95% H ₂ SO ₄ + 4 Week CS (e.g., McDonough & Laycock, 1975)	DM	4 (41 seeds total)	19%
	VD	4 (40 seeds total)	2.5%
8 Week CS (Control)	DM	4 (28 seeds total)	12.5%
	VD	4 (37 seeds total)	0%
0.5M H ₂ O ₂ + 8 Week CS (e.g., Msanga & Maghembe, 1989)	DM	4 (29 seeds total)	0%
	VD	4 (27 seeds total)	0%
6% H ₂ O ₂ + 8 Week CS (e.g., Msanga & Maghembe, 1989)	DM	4 (29 seeds total)	0%
	VD	4 (19 seeds total)	0%

Method 3 – Physical Scarification + Cold Stratification

Finally, I used sandpaper (fine grain silicon carbide contour sanding sponge; Mastercraft Canada) to physically scarify a random selection of open- and hand-pollinated seeds from both populations (Table A.3). Seeds were placed between two sanding sponges and sanded for a total of one minute. I checked seeds with a dissecting scope to ensure some seed coat had been removed, placed them in petri dishes on moistened filter paper, and placed them in the fridge for four weeks. This method proved most successful and was used to germinate the remaining seeds used for these thesis projects.

Table A.3: Total number of crosses, number of seeds used, and % germination under physical scarification + four-week cold stratification treatment for seeds from two populations of *P. brandegeei*.

Population	# of Maternal Families	% Germination
Deer Mountain	4 (59 seeds total)	41%
Vedauwoo	4 (56 seeds total)	23%

Germination Study – Batch Effects

I used method 3 for my germination study. I performed germination studies on seeds from the same cross in two batches. I took the best germination percent for analysis although batches did differ in overall germination % (Table A.4).

Table A.4: Germination % by batch for hand crosses performed in two field populations of *P. brandegeei* (Vedauwoo, Wyoming and Deer Mountain, Colorado).

	Batch 1	Batch 2
Mean Germination %	36.56	56.85
Median Germination %	31.67	61.25
SEM	3.26	3.78
SD	25.68	29.80

Appendix 2 – Leaf Volatiles

Leaf samples were filtered as described in Chapter 2. Compounds were included in this final list if they passed these filters and if they were found in at least two (of the 11) leaf samples. Compounds not found in an authoritative database (pherobase.com; Knudsen et al. 2006) are marked with an asterisk.

Table A.5: List of 38 VOCs found in leaf samples of *P. brandegeei*. CAS# = CAS registry number; # of samples = number of leaf samples compound was found in (out of 11). Compounds with an asterisk (*) were not found in an authoritative database.

Compound Name	Other Name	CAS #	# of Samples?	In floral samples?
(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	(1R)- α -Pinene	7785-70-8	11/11	Y
(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene	Humulene Epoxide	19888-34-7	3/11	N
(E)-4,8-Dimethylnona-1,3,7-triene	-	19945-61-0	3/11	N
B-Myrcene	-	123-35-3	10/11	Y
B-Pinene	-	127-91-3	4/11	Y
1-Octanol	-	111-87-5	6/11	N
1-Octen-3-ol	-	3391-86-4	4/11	N
*1,4-Butanediol, 2,3-bis(methylene)-	-	93578-48-4	3/11	N
1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, (E)-	Nerolidol	7212-44-4	3/11	N
1,7-Octadien-3-one, 2-methyl-6-methylene-	-	41702-60-7	3/11	N
*2-Cyclopenten-1-one, 2,3-dimethyl-	-	1121-05-7	9/11	N
*2-Cyclopenten-1-one, 3-ethyl-2-hydroxy-	-	21835-01-8	4/11	N
*2-Ethyl-1-dodecanol	-	19780-33-7	3/11	N
2-Furanmethanol, 5-ethenyltetrahydro- $\alpha,\alpha,5$ -trimethyl-, cis-	Linalool Oxide	5989-33-3	3/11	N
2-Hexenal	-	6728-26-3	9/11	N
*2-Isopropyl-5-oxohexanal	-	15303-46-5	3/11	N
2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	Cosmene	460-01-5	7/11	N
*2H-Pyran-2,6(3H)-dione	-	5926-95-4	5/11	N
3-Hexen-1-ol	-	928-96-1	4/11	Y
3-Hexen-1-ol, acetate, (Z)-	Hexenyl Acetate	3681-71-8	9/11	Y

3,7-Octadiene-2,6-diol, 2,6-dimethyl-	-	13741-21-4	3/11	N
6-Octenal, 3,7-dimethyl-, (R)-	Citronellal	2385-77-5	3/11	N
*6,8-Nonadien-2-one, 8-methyl-5-(1-methylethyl)-, (E)-	-	54868-48-3	3/11	N
Acetic acid, hexyl ester	Hexyl Acetate	142-92-7	7/11	Y
Benzeneacetic acid, methyl ester	-	101-41-7	3/11	N
*Benzenemethanol, α ,4-dimethyl-	-	536-50-5	5/11	N
*Benzofuran, 2,3-dihydro-	-	496-16-2	6/11	N
Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	Sabinene	3387-41-5	9/11	Y
Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1 α ,3 α ,5 α)]-	Pinocarveol	547-61-5	4/11	N
Caryophyllene	-	87-44-5	3/11	Y
cis- β -Farnesene	-	28973-97-9	9/11	Y
*Cyclohexanol, 2-methyl-3-(1-methylethenyl)-, acetate, (1. α .,2. α .,3. α .)-	-	54244-81-4	3/11	N
*Cyclopentanol, 1,2-dimethyl-3-(1-methylethenyl)-, [1R-(1 α ,2 α ,3 α)]-	-	4028-60-8	4/11	N
*Isopulegol acetate	-	57576-09-7	5/11	N
Pentadecanal	-	2765-11-9	4/11	N
Tetradecanoic acid	-	544-63-8	4/11	Y
trans- β -Ocimene	-	3779-61-1	3/11	N
trans-Linalool oxide (furanoid)	-	34995-77-2	8/11	Y

Appendix 3 – Floral VOC Sampling Environmental and Experimental Data NMDS

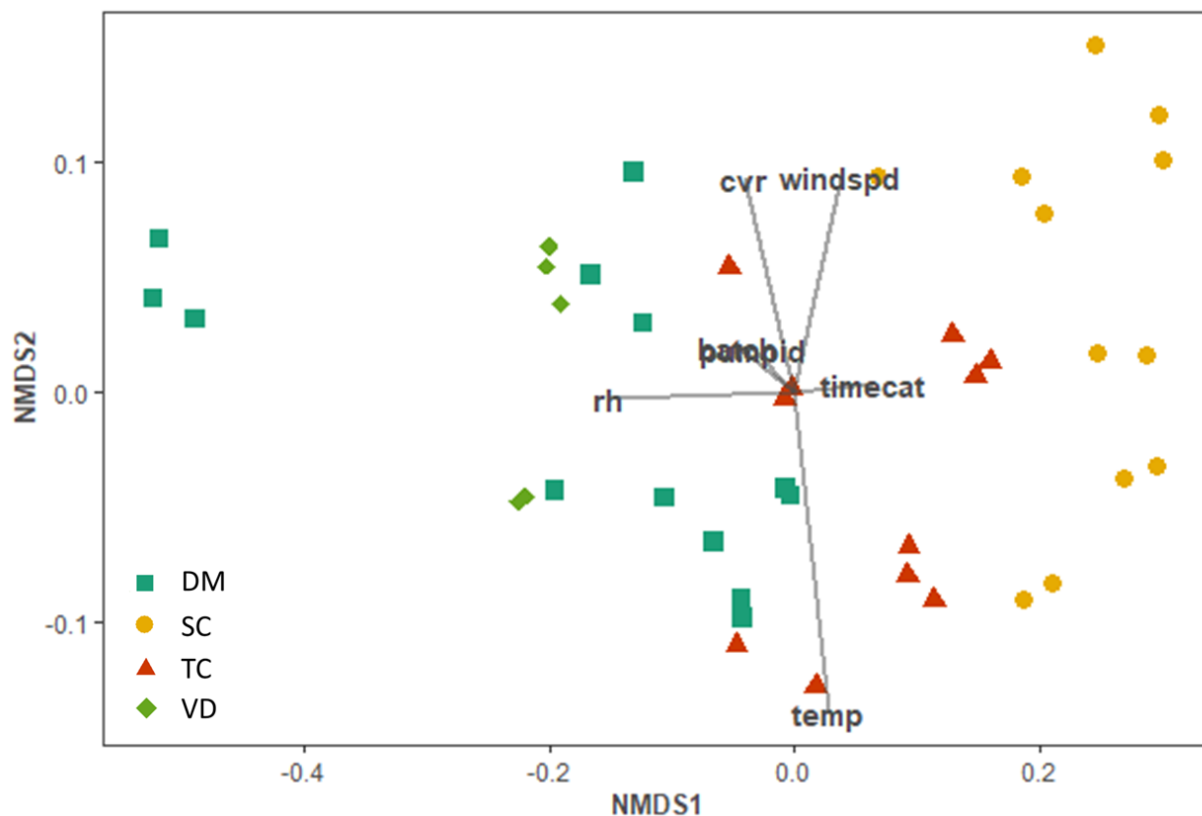


Figure A.1: NMDS plot of environmental and experimental variables collected during field floral scent sampling. "cyr" = cloud cover (categorical), "windspd" = windspeed, "rh" = relative humidity, "timecat" = time of day (categorical), "batch" = analysis batch, "pumpid" = ID of the pump used for sample collection, "temp" = temperature.

Appendix 4 – Mean Male and Female Flower Measurements and Correlations

Table A.6: Mean + SE, and min/max for 11 floral measurements taken on male and female flowers from four field populations (field) and three populations in the greenhouse. $P < 0.05$ = significant difference between male and female flowers for that floral trait (paired t-test). r_s = Spearman's correlation between male and female flowers for that floral trait. All measurements in mm except dry mass, which is in mg.

Field	Mean + SE Female, Field)	Mean + SE (Male, Field)	Min/Max (Field)	P	r_s
Flower Length	28.02, 0.55	28.58, 0.55	21.62/43.02	0.07	0.87
Corolla Width	16.22, 0.46	15.13, 0.42	5.78/20.37	< 0.01	0.72
Tube Length	23.32, 0.50	22.87, 0.49	15.69/39.18	0.09	0.83
Apical Diameter	4.52, 0.15	4.49, 0.13	2.17/6.73	0.75	0.67
Basal Diameter	2.41, 0.09	2.33, 0.08	1.53/4.02	0.15	0.75
Style Length	25.00, 0.48	25.30, 0.44	17.62/32.35	0.29	0.82
Anther Length	24.28, 0.49	24.02, 0.47	17.12/37.46	0.30	0.83
Stigma Exsertion	-3.03, 0.41	-3.27, 0.36	-12.47/0.64	0.51	0.54
Anther Exsertion	-3.74, 0.31	-4.55, 0.28	-7.63/-0.87	< 0.05	0.61
Stigma/Anther Separation	0.71, 0.39	1.28, 0.31	-7.39/5.70	0.12	0.57
Dry Mass	8.54, 0.40	8.59, 0.32	4.5/15.6	0.80	0.79
Greenhouse	Mean + SE (Female, GH)	Mean + SE (Male, GH)	Min/Max (GH)	P	r_s
Flower Length	30.86, 0.56	31.68, 0.55	22.17/41.5	< 0.001	0.90
Corolla Width	14.70, 0.32	14.61, 0.30	9.79/18.09	0.80	0.49
Tube Length	27.53, 0.58	27.09, 0.59	17.09/37.10	< 0.01	0.96
Apical Diameter	4.56, 0.07	4.55, 0.07	3.34/5.60	0.93	0.60
Basal Diameter	2.37, 0.03	2.30, 0.03	1.96/2.90	< 0.05	0.52
Style Length	28.18, 0.49	27.46, 0.45	20.07/37.92	< 0.001	0.93
Anther Length	28.10, 0.55	27.56, 0.55	19.12/37.86	< 0.01	0.95
Stigma Exsertion	-2.70, 0.39	-4.24, 0.45	-10.24/4.70	< 0.001	0.77
Anther Exsertion	-2.72, 0.26	-4.10, 0.30	-8.18/2.96	< 0.001	0.59
Stigma/Anther Separation	-0.03, 0.34	-0.22, 0.39	-8.16/5.31	0.35	0.87
Dry Mass	12.34, 0.31	11.28, 0.28	7.90/18.30	< 0.001	0.78

Appendix 5 – 2022 Temperature and RH Data

Table A.7: Average temperature and RH during the night and day in two populations of *P. brandegeei* collected by dataloggers during field work in June 2022.

	DM (June 4-6 & 16-24, 2022)	VD (June 6-15, 2022)
Average Temperature (°C)	Day: 18.76 Night: 11.85	Day: 19.62 Night: 12.11
Min/Max Temp (°C)	Min: 5.50 Max: 36.50 (48.50*)	Min: 3.00 Max: 35.00
Average RH (%)	Day: 39.29 Night: 50.83	Day: 30.25 Night: 48.29
Min/Max RH (%)	Min: 10 Max: 100	Min: 9.5 Max: 92