Floral Visitor Diversity in the Sub-alpine Herb *Polemonium brandegeei*:
The Role of Secondary Visitors in Pollination

by

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Abstract

Many flowering plant species rely on animal-mediated pollination for sexual reproduction. I explored syndrome and non-syndrome floral visitor's contributions to pollen movement and seed set of *Polemonium brandegeei*, a species visited by diverse floral visitors. In 2015 and 2016, I excluded large pollinators (hummingbirds and hawkmoths) but allowed non-syndrome small visitors (mainly bees and flies) to interact with flowers. Small visitor pollination was compared with known pollinator efforts. Small visitors contributed to pollen deposition and seed set, especially in 2015. I conducted pollen supplementation experiments comparing seed set with open pollination. Seed set did not differ between open and hand pollinated plants suggesting that the *P. brandegeei* population is not pollen limited. However, 50% of floral tissue was lost to herbivory before seed set measurements were recorded. Results indicated a lack of strong selection imposed by visitors on floral shape and non-syndrome pollinators, which may occur frequently in natural systems.

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1.1 Pollination as a balanced antagonism

Approximately 85% of all flowering plant species rely on animal-mediated pollination for sexual reproduction (Ollerton *et al.* 2011; Dutton *et al.* 2016). Visitors are attracted to the flowers to forage on floral rewards such as nectar and pollen. The plants benefit by using the foragers as vectors for pollination (Proctor *et al.* 1996). However, there are costs associated with this relationship for both foragers and plants. Food rewards are mainly in the form of pollen and/or nectar, both of which cost the plant energy to produce. In addition, pollen consumption comes at an extra cost to the plant because it directly decreases the male gametes available for pollination. Foragers must also incur costs and expend energy to seek out floral rewards (Dukas 2001; Higginson & Houston 2015).

Given the potential costs, the term mutualism, where both organisms always benefit from the interaction, has proven too simplistic to describe all plant-pollinator relationships (Bronstein *et al.* 2009; Morris *et al.* 2010). The term "balanced antagonism" provides a more realistic description of plant-pollinator interactions as it accounts for the variability of natural pollination systems (Morris *et al.* 2010). Recognizing this spectrum accounts for both the relationships that are primarily beneficial (i.e. plant-pollinator mutualism) and those that are primarily detrimental to one partner (i.e. reward theft and deceptive flowers). It also allows for the presence of multiple plant and animal species and the interactions between them (Bronstein *et al.* 2009). Thus, a given plant species may have visitors ranging from pollinating nectar foragers to insects that consume pollen but contribute little to pollination (Hargreaves *et al.* 2012). In addition, the relative

contribution of different visitors to plant reproduction may depend on which species are present (Bronstein *et al.* 2009). The context-specific nature of plant-pollinator relationships should be reflected in pollination research. Establishing how different visitors affect reproductive fitness is a first step to understanding how they may affect floral evolution.

Pollinator performance, a general term to include all factors that influence pollen movement via vectors (Ne'Eman *et al.* 2010), varies between mutualistic, neutral and antagonistic visitors (Boyd *et al.* 2011; Sahli & Conner 2011; Bischoff *et al.* 2013). Research into pollinator performance has two main factors, 1. pollinator frequency, simply describing the rate of visits by a foraging species and 2. pollinator efficiency measures the quantity (number of pollen grains) and quality (amount of viable pollen grains) of pollen being out-crossed, and ideally includes a measure of male reproductive fitness (Ne'Eman *et al.* 2010; Bischoff *et al.* 2013). Studies have shown that not all visitors are pollinators, and among pollinators there is a varying degree of efficiency (Stebbins 1970; Aigner 2001; Hargreaves *et al.* 2012; Bischoff *et al.* 2013). Some visitors can be inefficient pollinators (relatively less effective at transferring pollen than the main pollinators). Efficiency can change depending on both the characteristics of the visitor and of the flowers.

1.2 Selection on floral traits

The morphology of a flower influences pollinator attraction and pollen movement (Darwin 1882; Stebbins 1970; Sahli & Conner 2011; Kulbaba & Worley 2012). The flower not only has to attract animal visitors but it also has to effectively 'fit' visitors so that they provide pollinator services by removing and depositing pollen (Galen & Cuba 2001; Karron & Mitchell

2012). Flower shape and floral sex organ shape / position evolve to promote effective pollen removal and deposition of outcrossed pollen (Galen & Stanton 1989). Floral morphology can also influence the amount of self-pollen deposition that occurs within the flower, in particular the position of the sex organs (Webb & Lloyd 1986; Medrano *et al*. 2012).

1.3 Sex organ position

Most flowering plants have hermaphroditic flowers that gain fitness through female and male function (Barrett 2002). Self-compatible plant species can produce progeny through self-fertilization, but these offspring may have reduced fitness due to inbreeding depression. In self-incompatible species, the deposition of self-pollen does not result in fertilization (Barrett 2002). However, there is still the potential for interference between female and male function, because the pollen physically occupies space on the stigma, thereby reducing the surface area for out-crossed pollen to adhere and germinate (Routley & Husband 2006). This sexual interference is especially likely when many flowers are open simultaneously. As a result, many species have evolved phenotypes to reduce transfer of self pollen (Barrett 2002; Medrano *et al.* 2012).

The physical proximity of the female and male sex organs affects the degree of sexual interference occurring in hermaphroditic flowering plants. Herkogamy is the term describing the spatial separation of the female and male sex organs within the flower. Approach herkogamy refers to a flower that has the stigma located above the anthers. Reverse herkogamy refers to anthers that are located above the stigma. It is most common for a plant species to display only one form of herkogamy, but occasionally species have a genetic polymorphisms for herkogamy, a condition referred to as heterostyly (Barrett 2010). For example, the distylous species *Primula*

vulgaris, displays two herkogamous phenotypes known as the approach (pin) and reverse (thrum) phenotypes (Li *et al.* 2011). Heterostyly reduces sexual interference and promotes pollen export (reviewed in Barrett 2010).

Continuous variation in herkogamy is a common, heritable, trait, and increased separation between sex organs can reduce contamination of the stigma with self-pollen (Holtsford & Ellstrand 1992; Dai & Galloway 2011; Medrano *et al.* 2012). Herkogamy can also influence pollen export and deposition of outcrossed pollen (Webb & Lloyd 1986; Forrest *et al.* 2011; Medrano *et al.* 2012; Kulbaba & Worley 2014). Relatively few studies have quantified continuous variation in herkogamy as a factor in pollinator efficiency. However, some studies have demonstrated that positioning of the sex organs determines how much contact visitors can have with the sex organs and thus, which visitors are effective pollinators (Sahli & Conner 2011; Medrano *et al.* 2012).

In recent years, continuous variation from approach to reverse herkogamy has been documented for a few species, including *Polemonium brandegeei* and *Mertensia fusiformis* (Kulbaba & Worley 2008, 2014; Forrest *et al.* 2011). When continuous variation occurs in sex organ position within a population, individual plants can display either of the two extreme phenotypes (approach or reverse) or any phenotype in between. The wide range of phenotypes may be maintained by the presence of multiple floral visitors (Forrest *et al.* 2011). However, field data supporting this possibility are limited and do not explain all variation in sex organ position (Kulbaba & Worley 2008, 2014; Forrest *et al.* 2011).

1.4 Specialized flowers & pollination syndromes

Selection on sex organ position, and other floral traits, depends on floral visitors. Flowering plants can be separated into functional groups based on their floral characteristics including food rewards, floral shape and scent (Proctor *et al.* 1996). The main pollinators associated with different flower types have complementary characteristics that allow them to feed on the floral rewards while providing pollination services. The association of both floral and pollinator traits is called a pollination syndrome (reviewed in Fenster *et al.* 2004).

A classic example of a pollination syndrome is hummingbird pollination. Red tubular flowers that offer nectar rewards are often associated with hummingbird pollination (Grant & Grant 1967; Campbell *et al.* 1991). Hummingbirds are attracted to red flowers. Furthermore, they have a beak shape that would allow them to reach down a long tubular flowers and feed on nectar (Lange *et al.* 2000; Temeles *et al.* 2013). Hummingbird pollinated flowers sometimes have exserted sex organs, i.e., sex organs that protrude beyond the corolla opening (Kulbaba & Worley 2008). This position ensures that the pollen contacts the bird's head when the beak is down the corolla tube (Temeles *et al.* 2013).

Flowering plants that are visited by obligate pollen consumers, including certain fly genera (Diptera) and beetles (Coleoptera), often have wide, shallow flowers with visible (often protruding) anthers. Insects that consume pollen are adapted with specialized morphological features, such as scraping and sponging mouthparts for pollen consumption (Marshall 2012). The wide flower shape allows the visitors to land and the shallow shape makes the pollen available for visitors with mouthparts that are modified for pollen consumption but not for reaching down long tubular flowers.

The above examples of pollinator syndromes are widely accepted and confirmed by many examples in nature (Lange *et al.* 2000; Rosas-Guerrero *et al.* 2014), but they generally involve pollination systems that have evolved under selection by only one primary visitor type. Pollination syndromes become less applicable as natural pollination assemblages become larger and the floral rewards collected more varied (Wilson *et al.* 2004; Waser *et al.* 2008).

Invoking pollinator syndromes based on floral traits can provide predictions about visitor types present in natural populations (Rosas-Guerrero *et al.* 2014). Although classifying pollination of species this way can be useful, it is not a failsafe method of identifying the correct pollinators present within a population (Ollerton 1996). It provides a coarse-scale estimate but does not address the common presence of multiple visitor types within a pollination assemblage (Mayer *et al.* 2011). It also does not account for within-group variations, nor does it account for seasonal or population-level changes in the main pollinator. There is also a certain degree of overlap between syndrome characteristics that allows other similar visitors to feed on flowers adapted to a different pollinator. For instance, hummingbird and bee-pollinated flowers are often similar enough that bees can visit hummingbird pollinated flowers and vice-versa (Lange *et al.* 2000).

Several recent studies of pollination in plant populations are discovering floral visitors that contradict pollination syndrome theory (Wilson et al. 2004; Bischoff et al. 2013). However, few, if any have studied the effectiveness of non-syndrome visitors in pollination and their potential for mutualistic or antagonistic relationships (Rosas-Guerrero *et al.* 2014). This thesis aims to investigate secondary (non-syndrome) visitors pollination performance and their role in selection on floral traits in the hummingbird-hawkmoth pollinated species *Polemonium brandegeei*.

1.5. Pollen limitation

Pollen limitation is the term given when the quantity and/or quality of the pollen available to a plant is inadequate to fulfill the total potential seed set (Ashman *et al.* 2004; Knight *et al.* 2005; Aizen & Harder 2007; Davila *et al.* 2012). Pollen receipt can be limited through many means, for example, low visitor abundance, pollen theft, low visitor efficiency, low pollen quality (i.e. self-pollen). Pollen limitation is expected to be less likely when pollinators are diverse (Červenková & Münzbergová 2014). In addition, it has also been suggested that self-incompatible species may be at higher risk of suffering from pollen limitation (Knight *et al.* 2005a). Plants therefore, must develop strategies to ensure the attraction of pollinators and the "fit" of flowers to the visitor.

Measuring pollen limitation in natural populations is an essential precursor to studying visitor-mediated selection on flower shape. Because visitors are the vectors for pollen movement, the efficiency of their pollination services could affect seed set if pollen is scarcely deposited. Visitor-mediated selection will have a strong influence on floral evolution when pollen is limited (Trunschke *et al.* 2017). Therefore, in years when reproduction is not pollen-limited then pollinator efficiency does not strongly affect floral evolution.

Research into the pollinator-mediated selection on flower shape, in particular sex organ orientation, has been conducted in experimental populations of *P. brandegeei* (Kulbaba & Worley 2014). Although, as of yet there has been no assessment of pollen limitation or direct measure of plant fitness in this population. In this thesis I aim to measure pollen limitation in a

natural *P. brandegeei* population to identify the factors affecting plant fitness and floral

evolution in more detail.

1.6 Study system and thesis objectives

My research explored how floral visitors contribute to pollen movement and seed set in

Polemonium brandegeei, a species visited by diverse floral visitors. *Polemonium brandegeei* is a

perennial sub-alpine herb found in the southern Rocky Mountains of the USA, in rocky scree

slopes and on moist cliff faces. The medium-sized, funnelform flowers are white to cream, and

are displayed in terminal cymes (Grant 1989 & Grant & Grant 1967). Their size and shape are

consistent with hummingbird pollination but their colour and strong sweet scent are more

consistent with moth pollination. Field observations (Kulbaba and Worley 2008, 2014) and

studies of experimental populations (Kulbaba & Worley 2012, 2013) have confirmed that white-

lined hawkmoths (*Hyles lineata*) and broad-tailed hummingbirds (*Selasphorus platycercus*) are

effective pollinators. However, numerous small insects including flower flies (Syrphidae) and

solitary bees (Halictidae) also visit flowers. The extent and nature of selection by these visitors

is unknown. The range of floral visitors makes *Polemonium brandegeei* an ideal system to

conduct a whole-assemblage study of the pollen movement, plant fitness and the selective

pressures imposed by floral visitors. During this study I explore the role the non-syndrome

visitors play in pollination of *Polemonium brandegeei*.

My research has four main objectives. First, I observe abundance and behaviour of all

floral visitors (known pollinators and small non-syndrome visitors). Second, I compare pollen

deposition by all floral visitors with non-syndrome visitors alone. My third aim quantified floral

visitor and non-syndrome visitor-contributions to seed production. Lastly, I assess the pollen available and seed set in a natural population to find out if *P. brandegeei* is pollen limited.

The above objectives were addressed through a field study investigating the previously unexplored secondary floral visitor contributions to pollen movement and seed set. The known primary pollinator's contributions to pollen movement and seed set were compared with that of the smaller secondary visitors through an exclusion experiment. The study was conducted over two consecutive years (2015 and 2016) to capture temporal variation in floral visitor abundance. The next two chapters describe my findings. Chapter 2 explores the pollen movement and seed set provided by different visitors in natural *P. brandegeei* populations. The abundance and behaviour of all visitors of *P. brandegeei* is also discussed. Chapter 3 investigates pollen limitation as a potential restriction to seed set. This chapter discusses the pollen supplementation experiment that was carried out in a natural population of *P. brandegeei*. Lastly, Chapter 4 comprises a final discussion of the overall thesis.

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Chapter Two: Pollen Movement in *Polemonium brandegeei*

2.0 Chapter Summary

Flowering plants are commonly visited by more than one visitor type. Some of these visitors may not match the pollination syndrome. Non-syndrome visitors are defined as secondary visitors and are either inefficient pollinators or pollen thieves. Little is known about the influence of secondary visitors on plant fitness and selection on floral shape. Therefore, an unanswered question in floral biology asks: what is the role of non-syndrome floral visitors in plant reproduction and ultimately the evolution of flower shape? This study examined the role of small visitors in a multi-visitor pollination system of the sub-alpine herb *Polemonium brandegeei*. I conducted a field exclusion experiment to separate the small visitor's pollination contribution from open pollination by larger pollinators. I compared the patterns of pollen movement under small visitor (non-syndrome visitor) pollination with previous studies that described larger hawkmoths and hummingbirds as pollen vectors. I documented that small visitors contribute significantly to pollen deposition and seed set. I concluded that the small visitors, or at least a subset of the small visitor group, are pollinators. I also witnessed no effect of floral shape on seed set. Seed set did not vary with variation in corolla dimensions or sex organ orientation. Pollination syndromes can be misleading and they do not account for secondary, non-syndrome pollinators, which may occur frequently in natural systems.

2.1 Introduction

Many flowering plants are visited by more diverse floral visitors than predicted by traditional concepts of pollinator syndromes (Ollerton 1996; Waser *et al.* 1996). These visitors can range from having mutualistic interactions with the plant (i.e. pollinators) to antagonistic interactions (i.e. pollen and nectar thieves; Bronstein *et al.* 2009). We have a limited understanding of how diverse floral visitors affect floral evolution (Aigner & Scott 2004). To understand the role of floral visitors in floral evolution we must first quantify the contribution of different pollinators to plant fitness.

Floral visitors can often be split into groups based on pollinator performance and are referred to as primary and secondary pollinators. Secondary visitors often do not match expectations based on pollination syndromes and may be inefficient pollinators in comparison to the primary pollinators (Stebbins 1970; Wolf & Stiles 1989; Rosas-Guerrero *et al.* 2014). Interestingly, secondary pollinators tend to be associated with flowers of a particular syndrome (Rosas-Guerrero *et al.* 2014). For example, certain bee taxa, such as Halictidae, are often associated with bird and moth pollinated flowers (Rosas-Guerrero *et al.* 2014). It has been suggested that they may reflect ancestral pollinators and may also provide pollination (albeit less efficient) that bridges shifts between pollination syndromes through evolutionary time (Wolf & Stiles 1989; Aigner 2001; Mayer *et al.* 2011; Sahli & Conner 2011; Rosas-Guerrero *et al.* 2014). In contemporary populations, secondary visitors may develop a greater role in pollination and floral evolution due to climate change and recent habitat shifts (Miller-Struttmann *et al.* 2015). This chapter aims to investigate the role of "secondary visitors" in pollination within a multi-visitor assemblage. As the study plant, *Polemonium brandegeei* displays floral traits associated with bird and moth pollination, visiting bees and flies may be acting as secondary pollinators.

2.1.1 Role of solitary bees and flies in pollination

Solitary bees include leafcutter and mason bees (Megachilidae), carpenter bees (Apidae), mining bees (Andrenidae) and sweat bees (Halictidae), and are ubiquitous floral visitors world-wide (Marshall 2012). Solitary bees are sometimes the most abundant and frequent floral visitors (Beattie 1974; Boyd *et al.* 2011). They can be effective pollinators of species with small, pale open flowers, characteristics usually associated with bee- or generalist-pollinated plants. For example, several species belonging to the Halictidae family are the most common visitors of the shrubby reed-mustard (*Hesperidanthus suffrutescens* (Lewis & Schupp 2014)). Of these visitors, *Dialictus* species were one of the most important pollinators for this endangered Utah-native herb (Lewis & Schupp 2014). Similarly several Halictidae species were among the pollinators for the Costa Rican herbs, *Miconia brevitheca* and *M. tonduzii*. Kriebel & Zumbado (2014), both species with small, open, white flowers.

Solitary bees do not always feed on bee-associated or generalist-pollinated flowers and on occasion they have been observed feeding on 'non-syndrome" flowers, i.e. flowers associated with a different pollinator type (Wcislo *et al.* 1993; Hargreaves *et al.* 2009, 2012). Though solitary bees are identified as successful pollinators for some species they may also act as ineffective visitors when flower shape does not allow them to contact both female and male sex organs (Bischoff *et al.* 2013; Li *et al.* 2014).

The relationship between secondary visitors and flowers varies with floral morphology (Hargreaves *et al.* 2012). Many plant species pollinated by moths and birds have shifted from ancestral bee pollinators (Rosas-Guerrero *et al.* 2014). Some of these ancestral pollinators appear

in the current pollinator assemblage as secondary pollinators. For example, a Halictidae species was observed visiting several different *Aloe* (Alooideae) species (Hargreaves *et al.* 2012), which are normally pollinated by nectar feeding sunbirds (*Nectariniidae spp.*). Because of the dichogamous flowers of *Aloe*, and their deep-funneled flowers, the *Halictidae* bees were restricted from accessing the nectar and could only access pollen. Therefore, they visited only male-phase flowers. In this case the floral characteristics cause these visitors to act as resource thieves (Hargreaves *et al.* 2012).

Similarly, solitary bees were observed acting as pollen thieves in the high-altitude grasslands of Brazil (Jacobi & del Sarto 2007). Two *Dialictus* species act as pollen thieves of a shrub species (*Vellozia epidendroides* and *V. leptopetala)* pollinated by leafcutter bees (*Megachile spp.)* and hummingbirds. This alpine shrub species has large, widely open flowers that allowed the *Dialictus* bees to access and feed on the anthers without contacting the stigma (Jacobi & del Sarto 2007).

Flies have also been suggested to act as secondary pollinators or antagonists for some moth and bird pollinated species (Rosas-Guerrero *et al.* 2014). The Syrphidae family of flies is a vastly diverse family of flower visitors (Marshall 2012). They display a wide range of morphological adaptations to different flowers, such as long proboscises for funnel shape flowers (Ssymank *et al.* 2008). They can be effective pollinators but they are often thought to be ineffective or inefficient pollinators because they visit many plants where they may not match the pollination syndrome (Moroń *et al.* 2009; Asif & Saeed 2010; Bischoff *et al.* 2013).

In some cases plants are inaccessible to efficient pollinators, such as some arctic and high altitude plant species (Miller-Struttmann *et al.* 2015). In these habitats, highly efficient pollinators such as social bees are absent and less efficient species can then become the main

source of pollination services. For example, two alpine plant species in New Zealand, *Ourisia glandulosa* (Plantaginacea) and *Wahlenbergia albomarginata* (Campanulaceae), have white tubular flowers typical of moth pollination (Bischoff *et al.* 2013). However, syrphid flies (*Allograpta spp.)* and solitary bees (*Hylaeus matamoko*) were contributors to pollination, and ultimately seed set, in an area where hawkmoths and social bees, were absent (Bischoff *et al.* 2013). Syrphid fly efficiency as a pollinator was lower compared to solitary bees. Given that syrphid flies were the least efficient at depositing pollen after a single visit (Bischoff et al. 2013) they have the potential to act as antagonists and feed on the pollen without the reciprocal action of providing pollinator services (Jermy 1999).

2.1.2 Selection on floral traits

There has been some study of the selection by the primary pollinators on flower shape in multi-visitor assemblages (Beattie 1974; Sahli & Conner 2011; Kulbaba & Worley 2012, 2013). Less research has focused on selection by secondary pollinators (Sahli & Conner 2011).

Four beetle-pollinated plants (*Anemone coronaria, Papaver rheas, Ranunculus asiaticus and Tulipa agenensis)* belonging to the poppy guild were recorded to have a *Halictidae* bee species (*Lasioglossum marginatum*) as a secondary pollinator (Dafni *et al.* 1990). Although beetles preferentially foraged on flowers with black marking, the bees did not discriminate based on floral color, suggesting that these bees did not select on floral color. However, bees carried only an average of 110 pollen grains, which was one tenth that of the primary, beetle pollinators (Dafni *et al.* 1990). This difference was due to their relatively small size compared with the open

bowl-shaped flowers of the four plant species. This suggests that if bees were the only provider of pollination services they may select for smaller, less bowl-shaped flowers.

Sweat bees (particularly *Dialictus* bee species), although the least efficient, were one of the most frequent pollinators of wild radish, *Raphanus raphanistrum* (Sahli & Conner 2011). Wild radish was visited by many other pollinators including honey bees, bumble bees and syrphid flies. The study highlighted the selection on floral traits imposed upon the plant by each member of the pollinator assemblage (Sahli & Conner 2011). Within the sweat bee group, *Dialictus* species had a stabilizing effect on the anther exsertion and flower size. *Dialictus* bees were not the only pollinators that select for this particular floral design, suggesting that floral phenotypes were a result of additive selection from a sub-set of primary visitors. Perhaps visit frequency effects selection strength more than previously thought (Sahli & Conner 2011).

Sahli & Conner (2011) claimed to be the first study comparing selection by multiple visitor types. Since then there have been several studies that mention the presence of secondary pollinators (Zhao & Huang 2013; Kulbaba & Worley 2014; Gómez *et al.* 2015). However, very few, if any, studies have focused on the contribution of secondary pollinators to plant fitness and pollinator mediated selection. My research focuses on the secondary visitors in *Polemonium brandegeei*, a species with a high diversity of floral visitors (Kulbaba & Worley 2008, 2014).

2.1.3 Rationale

The multi-visitor pollination system of *Polemonium brandegeei* includes two known pollinators; hawkmoths and hummingbirds. These two pollinator taxa contact the sex organs and visit large numbers of plants and flowers when foraging (Kulbaba & Worley 2008). Previous

work on experimental populations shows that they are effective vectors of *P. brandegeei* pollen (Kulbaba & Worley, 2012 & 2013). However, so far seed set has not been explored in the natural populations. Seed set is a direct measure of female fitness in plants and is useful for assessing selection within a population.

Many small visitors were present in the *P. brandegeei* pollinator assemblage including syrphid flies and bees (Kulbaba & Worley 2014). My initial predictions assumed the small visitors were pollen thieves based on Kulbaba & Worley's description of their seemingly inconsequential foraging behaviour. Their non-syndrome morphology led me to question whether they would be contacting both sex organs and contributing to pollen movement while foraging. However, after the first field season, this expectation was revised to include their potential to act as secondary pollinators. As a group, syrphid flies and solitary bees are potentially inefficient pollinators of *P. brandegeei,* which provide inferior pollen movement services due to morphological-incompatibility, when compared to the known (hummingbird and hawkmoth) pollinators. Exploration of whole-assemblage pollination efforts is a relatively new area of study (Mayer *et al*. 2011) that can help us understand selection imposed on floral shape.

The quality of the pollen being deposited will also affect fitness (Knight *et al.* 2005). In self-incompatible species, such as *P. brandegeei,* good quality pollen would be viable out-crossed pollen, as opposed to self-pollen. The presence of self-pollen can inhibit out-crossed pollen germination by occupying space on the stigmatic surface (Galen & Stanton 1989). Small visitors are more likely to contribute to self-pollen deposition because of their foraging behaviour. Kulbaba & Worley (2008, 2014) observed that small visitors, such as syrphid flies and bees, would often forage only on one plant. Therefore, instead of cross-pollinating, small visitors may transfer self pollen within and between flowers of the same individual.

Selection on flower shape, especially sex organ arrangement, is closely influenced by the pollinator assemblage. Certain phenotypes will receive viable pollen more successfully under natural pollination conditions, compared to others. *Polemonium brandegeei* populations display a common phenotype that is not the optimum for pollen deposition under hawkmoth or hummingbird pollination. It is unknown whether this is a compromise between conflicting selective pressures, or if this intermediate phenotype is influenced by a third type of floral visitor (Kulbaba & Worley 2014). Perhaps the frequency of intermediate phenotypes is a result of stabilizing selection (Sahli & Conner 2011). Measuring reproductive success through pollen deposition and seed set can explain the importance of all floral visitors.

2.1.4 Research objectives and hypotheses

In this study, I explored the role of "non-syndrome" visitors to *Polemonium brandegeei* by testing the following hypotheses:

1. *Large visitor hypothesis:*

   If hummingbirds and hawkmoths are the primary pollinators then they will contribute to most pollen movement and seed set in natural populations of *P. brandegeei*.

2. *Small visitor hypothesis:*

   If small visitors are secondary pollinators then they will contribute to pollen movement but will contribute less to outcrossed seed set when compared to the large visitors.

3. **Sexual interference hypothesis:**

   If plants receive self-pollen, then seed set could be limited by the presence of incompatible self-pollen on the receptive stigmatic surface (Knight *et al.* 2005). I expect

that self pollen deposition will be high under small visitor pollination due to close proximity of the sex organs in many plants.

4. *Floral evolution hypothesis:*

Plants with anthers and stigmas close together (slight approach or slight reverse) will receive more pollen, and set more seeds under small visitors. This sex organ orientation is likely to increase the likelihood that small visitors will brush against the stigma and subsequently deposit pollen while feeding on the anthers.

2.2 Materials and Methods

2.2.1 Study Site(s) and Population

I conducted field experiments over two flowering seasons (2015 and 2016). This study was carried out at one site in the southern Rocky Mountain region in northern Colorado, 8.5 kilometres West of Estes Park. The site is on Deer Mountain (40°46′57″N, 105°53′01″W) in Rocky Mountain National Park, and has been previously used for pollination studies of *P. brandegeei* (Kulbaba & Worley 2008, 2012, 2013, 2014).

The Deer Mountain site was located at an elevation range of 2859m-2906m, on a steep scree slope. The slope was south-west-facing with several tree species present, including *Abies spp*. and *Pseudotsuga menziesii.* These two tree species identify the area as an altitudinal transitional zone, or ecotone, between the sub-alpine and the upper sub-alpine plant communities. There were also many snags and fallen trees in the site. Other plant species included tall shrubs (*Ribes cereum* and *Rubus deliciosus*) and several herbs, such as *Maianthemum racemosum, Penstemon virens, Geranium spp*. and *Frasera speciosa*. The total ground cover provided by the plants was low (<50%), due to large areas of scree, tree debris and exposed rock.

The *Polemonium brandegeei* population at the Deer Mountain site was recorded as having approximately 200 plants during the 2004-2006 flowering seasons (Kulbaba & Worley 2008).

2.2.2 Pollen Movement Experiment

I used three treatments to distinguish pollen movement caused by small versus large-bodied visitors, and by nectar-only versus pollen foragers. "Control" plants were uncovered and exposed to open pollination with their flowers freshly open and intact. "Tented" plants were covered with a coarse nylon mesh tent. The mesh gauge was 0.75 inch, a size that allowed all small visitors (bumble bee sized or smaller) to access the plant and excluded the large visitors, such as hawkmoths and hummingbirds (cf. Hargreaves *et al.,* 2012). A pilot study conducted with ten *Vicia americana* plants indicated that the mesh did not deter small visitors, such as the solitary bees and syrphid flies (Dawn Pond, unpubl. data), from visiting flowers. "Emasculated" plants had the anthers and any loose pollen removed from all open flowers and were not tented. This treatment removed pollen as a food reward and left only the nectar reward for floral visitors. In addition, pollen deposited on the stigma of emasculated flowers could only be out-crossed pollen.

The treatments were assigned randomly in blocks containing at least three plants, which whenever possible, were located in close proximity to one another. Prior to treatment all experimental plants were covered with a fine mesh tent (or 1-4 days to allow virgin flowers to open and remain unvisited until the treatments were applied. Treatments were applied to most of the open flowers (~20 flowers). I chose only fresh flowers that represent the most common flower shape on each plant, excluding old, damaged or deformed flowers. The flowers were available for visitors for at least two days. If moderate-heavy precipitation occurred during the two days post treatment, an extra day was added to the treatment time. Any flowers that opened before the full exclusion tent was applied were painted with a dot of yellow non-toxic acrylic

paint (on the calyx) and were not included in the experiment. The treated flowers were identified by red or blue dots. Flowers that opened after the experiment were not painted.

Sample sizes varied between years, with 30 plants in each treatment in 2015 and 20, 20, and 18 plants in control, tented and emasculated treatments, respectively, in 2016. Fewer plants were used in 2016 (n=58) compared to 2015 (n=90) to allow more time to be spent on single visit trials, and because the population had a shorter flowering season compared with 2015. In 2015, an average of 19 experimental flowers were treated per plant. In 2015, some very small plants were also treated. In 2016, the small plants were excluded and the average experimental flowers per plant rose to 20. Larger plants were more likely to retain some seed for collection, given substantial herbivory at the site.

2.2.3 Floral Visitor Observations and Insect Capture

Two types of floral visitor observations were carried out. First, the experimental plants included in the pollen movement experiment were observed in both years. These treatment observations were carried out using microsites containing each treatment. The microsites were defined as natural patches of plants. Microsites were all located in the Deer Mountain population but were separated by various (small scale) topographical obstacles or enough distance that observations could not be carried out. Second, general observations were conducted in 2016 only, where patches of unmanipulated plants were observed. These were carried out in 30 minutes blocks and often included more plants than was used in the treatment observations. The purpose of the general observations were to: (1) determine whether the smaller visitors moved

between patches of plants; and (2) observe larger bodied visitors that might not be recorded during treatment observations because the target plants were close to an observer.

In 2015, 17 hours of treatment observations were conducted. These observations were conducted in 34 x 30-minute blocks and included a patch of plants (3-5) that contained at least one of each type of experimental plant (i.e. tented, emasculated and control). In 2016, 8.5 hours of treatment observations were conducted in 17 x 30-minute blocks and 19.5 hours of general observations were conducted in 39 x 30-minute blocks.

Plants were observed between 10:00 – 15:00 hours, which is the period of highest insect activity recorded in previous years (Kulbaba & Worley 2008). I observed the plant patches while keeping 1-3 meters away from the plants. I remained a minimum of 1 meter away to reduce my influence on the visitor behaviour and to avoid casting a shadow over the plants. I recorded tentative *visitor identification* (field assigned names were given based on morphology), *time on* and *time off* (time visitor first foraged within the observed patch of plants and the time the visitor stooped foraging) and *visitor pattern* (record of the number of flowers visited per plant, which plants were visited and in which order).

I collected specimens of the *P. brandegeei*-visiting insects for identification. Insect capture was achieved using insect aerial nets. Dropping the net over the entire plant while an insect foraged proved to be a successful method. A kill jar with ethyl acetate was used to euthanize the insects which were then stored in 70% ethyl alcohol. These insects were pinned and identified using Flies (Marshall 2012), the Canadian Journal of Arthropod Identification Key to Genera of Nearctic Syrphidae (Miranda *et al.* 2013) and the Peterson's Insect of Western North America Field guide book (Borror *at el.* 1998).

2.2.4 Single-Visit Pollen Deposition

To address the hypotheses that the pollen movement capabilities would differ among the *P. brandegeei* visitor types, I conducted a field pollinator efficiency trial. The trial was run within the flowering season of *P. brandegeei* in 2016 at the Deer Mountain Site, Colorado, simultaneously with the pollen movement experiment. Different plant individuals (n = 5) were used for this experiment from the pollen movement experiment. The plants chosen to be included in the efficiency trial were covered with a complete exclusion tent until at least ten virgin flowers opened. From each of the observed plants, five anthers (from the same flower) were harvested to measure the pollen production of the plant. This was done on the same day as the observations. The virgin flowers offered during the efficiency trial were observed for 30 minutes or until a visitor fed on a flower. The visitor type was noted along with the length of foraging bout. Immediately after the visitor left the flower, anthers and stigmas were harvested and the full exclusion tent was returned to cover the plant; this was to inhibit any further visits until the next observation period. The pollen on the stigma was counted to quantify deposition after a single floral visitor. On occasion, I used a plastic vial to capture visitors as they left the target flower. In these cases, the pollen load of the insect specimen was quantified. A total of 15 single visits were recorded within a total of 3 hours and 17 minutes of observation time.

2.2.5 Pollen Deposition in the Pollen Movement Experiment

Pollen deposited on the stigma was quantified after treated flowers in the pollen movement experiment had been open for visitation for 2-3 days. For control and tented plants, it

was assumed that stigmas were virgin when treatments were applied and therefore the visitors were responsible for all pollen deposition. This was verified by checking the stigmas with a hand lens before applying the treatment. One *after* stigma was harvested from each control and tented experimental plant. For emasculated plants, I destructively harvested an unvisited stigma to quantify any artificial self-pollination that occurred during the process of emasculation. The pollen on these *before* stigmas was subtracted from pollen found on *after* stigmas of the same plant to estimate outcrossed pollen deposition.

Stigma harvesting involved pinching the stigma off at the style and mounting it on a prepared fuschian jelly slide (Kearns & Inouye, 1993). Stigma harvesting was achieved using clean forceps to avoid contaminating samples with pollen from another flower. The coverslip was applied and then the slide was then held over a flame to melt the jelly. Once the jelly was melted, the coverslip was gently pressed to flatten the jelly onto the slide. Care was taken to mount the stigma, with all three lobes separated to facilitate the manual counting of pollen grains in the lab. Pollen counting was done using a dissection scope with an attached camera. The images from the camera were captured using computer image software QCapture Pro 7 by QImaging (https://www.qimaging.com/products/software/). The stained pollen grains were manually counted and tracked using ImageJ (https://imagej.nih.gov/ij/).

2.2.6 Seed Collection

Seeds were collected for every experimental plant from the Deer Mountain site. Flowers that had been open at the time of treatment were placed in coin envelopes. While collecting flowers, I recorded the total number of wilted flowers remaining on each plant, the total number

of "fruit" harvested (flowers with swollen ovaries) and number of missing inflorescences (most likely removed by herbivory). In the lab, any fruit with green calyces were air dried for a few days and then crushed to release the seeds for counting. The total number of seeds per fruit was recorded for each experimental plant, along with the number of undeveloped seeds per flower. The number of harvested flowers that did not set fruit was also recorded.

2.2.7 Floral and Plant-level Measurements

Floral measurements, based on measurements previously found to influence pollen movement in *P. brandegeei,* were taken prior to treating plants included in the experiment (Kulbaba & Worley 2012, 2013 & 2014; Figure 2.1). One flower was destructively harvested from each plant and the following measurements were taken to the nearest 0.1 mm using digital calipers: corolla tube length (mm), corolla basal diameter (mm), anther exertion (mm), stigma exertion (mm), anther-stigma separation (mm) and reproductive phase (male: anther dehisced or female: stigma matured but anthers not yet dehisced). The flower harvested for measurements represented the floral phenotype for the whole plant.

Plant size and floral display were also recorded for each plant in the pollen movement experiment. In 2015, these measurements were taken at the end of the flowering season for each plant. However in 2016, these measurements were taken at the time of treatment to record the size of the plant at the time of observation. Plant width (to the closest 0.5 cm) was measured from the leaf tip to leaf tip across the widest section of the plant from an aerial view. Plant height was measured from the base of the plant to the top of the tallest inflorescence. The number of open flowers and inflorescences was also recorded.

2.2.8 Statistical Analysis

Floral visitors

Total visits from floral visitors were analyzed using contingency table (Chi-square) analysis. Total observed visits were compared to the expectation of equal visits to each treatment (control, tented and emasculated). Similar analyses were applied for individual visitor taxa (morpho-species) with >15 total observed visits. To assess visitor group's variation in behaviour, a similar chi-square analyses was run to assess if one visitor type visited more or fewer flowers and plants per bout. Differences in bout duration per group were also tested using the same method. The bouts were analyzed in two categories (bouts lasting less than one second and bout lasting longer than one second).

Pollen deposition

Pollen deposition was analyzed using general linear models (Proc GLM) in SAS v 9.4 with separate analyses for each year of the study (2015 & 2016). Pollen deposited on stigmas was the dependent variable. Treatment type (control, tented and emasculated) was a categorical fixed effect, while microsite within the Deer Mountain population was a categorical random effect. Floral display characters (numbers of open flowers and treated flowers) were included in initial models as covariates. I also explored all two-way interactions among covariates, and categorical variables and covariates. A manual reverse-stepwise elimination process was employed to remove non-significant interaction terms, and covariates. To meet assumptions of normality and stabilize variances, data for the pollen deposited on stigmas and all continuous covariates were log transformed (x+1).

Seed set

Seed set was analyzed using general linear models (ProcGLM) in SAS v 9.4 and separate

analyses were conducted for each year of the study (2015 and 2016). Total seeds per plant

(plants with zero seed set were excluded to normalize data distribution) was analysed as the

dependant variable. Treatment (control, tented and emasculated) was a categorical fixed effect,

wass(number of open flowers) and number harvested flowers were added to the model as

covariates. Harvested flowers represent the amount of experimental flowers that were

recoverable and accounts for losses including loss of flowers to herbivory.

As with the analyses of pollen deposition, I explored all two-way interactions among

covariates, and categorical variables and covariates. A manual reverse-stepwise elimination

process was employed to remove non-significant interaction terms, and covariates (Sokal and

Rohlf, 1994). To meet assumptions of normality and stabilize variances, data for the total seeds

per plant and all continuous covariates were log transformed (x+1).

To test how the plants that set zero seed were distributed between treatments I used

contingency table (Chi-square) analysis. I expected that the tented treatment would have a larger

proportion of plants with zero seed set than the control or emasculated treatments.

Floral phenotype effects on seed set and pollen deposition

Measurements of floral phenotype were examined for correlations between them. This

was done by constructing a correlation matrix and resulted in a correlation coefficient for each

variable. I also examined the relationship between herkogamy and the two measures of female

fitness. These analyses included treatment, microsite as categorical effects, significant covariates from previous analyses (harvested flowers for seed number) as well as herkogamy and the square of this measure, to account for potential non-linear effects. Emasculated treatment was excluded because this treatment would result in an artificial floral shape due to the removal of the anthers. A manual reverse-stepwise elimination process was employed to remove non-significant interaction terms, and covariates (Sokal and Rohlf, 1994). To meet assumptions of normality and stabilize variances, data for the total seeds per plant and pollen deposition and all continuous covariates were log transformed (x+1).

2.3 Results

2.3.1 Visitors

A total of 136 visits to experimental plants were observed over 17 hours in 2015 whereas 59 visits were observed over 8.5 hours in 2016 (Table 2.1). In 2016, an additional 59 visits to *P. brandegeei* plants were recorded during 19.5 hours including treatment and general observations. The most frequent visitors in 2015 were the small brown flies (*Anthomyiidae*) and 2016 solitary bees (*Dialictus*). Hummingbirds, *Selasphorus platycercus* (n=5) and hawkmoths, *Hyles lineata* (n=2) were observed opportunistically throughout 2015 and 2016 at Deer Mountain and only one of the hummingbird was observed during formal observation periods. Hummingbirds visited between 1-5 plants per visit and between 3-48 flowers per bout. The hawkmoths I observed visited between 1-3 plants and 1-5 flowers per bout.

In 2015 there was no significant difference in total visits between treatments (total visits for control plants= 52; tented=40 and emasculated=44; $X^2 = 1.65; P=0.5$). Total visits varied between treatment in 2016 seasons (Table 2.1) with the emasculated plants receiving fewer total visits than the tented and control plants (total visits for control=28, tented=21 and emasculated=10; $X^2 = 8.37 P=0.01$).

Visits received by each treatment were also separated by visitor type. For both years, most of the visitor types did not discriminate between treatments and provided similar visit numbers to all treatments (Table 2.1). In both 2015 and 2016, the Diptera group (excludes syrphid species, which were analysed separately) visited control plants more than the tented and emasculated plants (2015: $X^2=7.26$ and $p<0.05$; 2016: $X^2=6.0$ and $P<0.05$). In 2016, *Dialictus* solitary bees also provided significantly different numbers of visits to each treatment. *Dialictus*

bees visited control and tented plants more than the emasculated plants (Table 2.1. $X^2 = 8.65$ and $P<0.01$).

2.3.2 Small visitor role in pollen movement

As a group, small visitors were the most frequent visitors to *P. brandegeei,* providing most of the visits over both years and throughout the entire flowering season of *P. brandegeei*. The most frequent of the small visitor types recorded were solitary bees (*Dialictus*), syrphid flies (multiple genera), small brown flies in 2015 (Anthomyiidae) and solitary bees (*Lasioglossum*) in 2016.

Almost all observed visits during general observations in 2016 were very brief with 94% of recorded visits were to a single plant. Approximately half of all visits involved a single flower (44%). A chi-square analysis showed there was no association between visitor groups (*Dialictus* bees, other bees, Syrphid flies and other flies) and the length of bout (seconds), number of flowers, number of plants visited per bout (measured as 2 categories: one plant/flower/second or greater than one flower/plant/second; Table 2.2). Hummingbirds (n=5) visited between 1-5 plants per visit and between 3-48 flowers per bout (Table 2.2). The hawkmoths I observed (n=2) visited between 1-3 plants and 1-5 flowers per bout. No statistical comparison was made with small visitors due to the low sample size.

Single visit trials included visits from single individuals of several species (Table 2.3) and 10 *Dialictus* visits (Table 2.3). Compared to the pollen deposition recorded after two days of open pollination (control plants) the pollen deposition by *Dialictus* was approximately 2 times greater in the single visit trials. The same trend is seen when the single visit deposition is compared to that of the tented plants after two days of small visitor pollination.

2.3.3 Pollen deposition on experimental flowers

Pollen deposition (the number of pollen grains per stigma) varied with treatment (2015: P <0.001 and 2016: P <0.05; Table 2.4; Figure 2.2). Pollen deposition was significantly lower for emasculated plants in both years. However, pollen deposition did not vary between tented and control treatments in either year (2015: average number of grains for control plants = 272; tented = 240 and emasculated = 114; 2016: control = 200; tented = 281 and emasculated = 69, Table 2.4, and Figure 2.2). The pollen deposition did not vary with floral display for either 2015 or 2016 (the number of open flowers; P>0.05). Microsite also did not influence pollen deposition in either year (P>0.05; Table 2.4).

2.3.4 Flower collection and herbivory

The number of harvested experimental flowers successfully recovered during seed collection indicated how many flowers escaped herbivory and other causes of tissue loss (Figure 2.4). On average, ten flowers (range of 0-37 flowers) were harvested from each study plant in 2015, and five flowers (range of 0-21) were harvested in 2016. These numbers corresponded to 54% and 26% of the flowers that had been treated in each year, indicating substantial loss of reproductive tissue between flowering and fruiting.

The number of harvested flowers did not differ between treatment for either year (P>0.05; Table 2.5). In addition, number of harvested flowers per plant did not differ across microsites in either year (P>0.05). The number of harvested flowers was both highly correlated with the floral display (number of open flowers) and with the number of treated flowers per plant

for both years (P<0.001). Only the number of treated flowers was included as a covariate in the final analysis and it had a significant positive effect on the number of harvested flowers per treatment for both years (P<0.001; Table 2.5).

2.3.5 Seed set

Total seeds per plant (excluding plants that set zero seeds) were used to represent plant fitness. On average across all treatments, plants produced 10.4 seeds in 2015. There was no difference between total seeds for tented, emasculated and control flowers in 2015 (P<0.005; Figure 2.4 and Table 2.6). In 2016 seed set differed between treatments with an average of 3.7 seeds produced by tented and emasculated plants and an average of 11.1 seeds per control plants (P < 0.01, Figure 2.4 and Table 2.6). The number of harvested flowers was included as a covariate. The number of harvested flowers explained most variation in seed number between treatments in both years (P < 0.001). Microsite influenced seed set in 2015 (P <0.0001) but did not have a significant effect in 2016 (P >0.05; Table 2.6).

There were a number of plants that produced zero seeds for each treatment. In 2015 there were 14 plants that set no seed (4 control, 4 tented and 5 emasculated). In 2016 there were 23 plants that produced no seeds (7 control, 4 tented and 12 emasculated). In 2015, there was no significant difference between the number of plants that set no seed between treatments (X^2=0.21 and P=0.9). In 2016 there were significantly more plants that set no seed in the emasculated treatment (X^2= 10.71 and P<0.01).

2.3.6 Floral shape and female fitness

Most plants displayed an approach phenotype (69%; approach phenotype measured as stigma ≥ 1mm above anthers). An intermediate phenotype (where the stigma is within 1mm above or below the anthers) was displayed in 24% of experimental plants. Few plants were seen with a reverse phenotype (9%; reserve phenotype measured as stigma ≤ 1 mm below the anthers). All the herkogamy measurements from Deer Mountain in 2015 and 2016 fell within the range recorded by Kulbaba & Worley in 2004-2006 (approx. 8 mm to -10 mm; Figure 2.6). Some floral measurements were correlated with each other (2015: Table 2.7 and 2016: Table 2.8). The distance between the anthers and the stigma varied from -3.33 mm to +7.42 mm (Figure 2.6). Floral shape did not have an effect on seed set in either year, or pollen receipt in 2015 at the Deer Mountain *Polemonium brandegeei* population ($P > 0.05$). However, stigma-anther separation was negatively related to pollen deposition in 2016 (Table 2.9, $P < 0.001$). Thus, plants with reverse herkogamy received the most pollen in this year.

2.4 Discussion

Measuring visitor contributions to successful pollination and ultimately plant fitness is a complex process (Kessler & Baldwin 2011; Mayer *et al*. 2011). "Non-syndrome" floral visitors are present in many pollination systems (Stebbins 1970; Wolf & Stiles 1989; Rosas-Guerrero *et al.* 2014). However, few studies have assessed their role in pollination and floral evolution (Sahli & Conner 2011). "Non-syndrome" visitors are known to be present in the *Polemonium brandegeei* floral visitor assemblage (Kulbaba & Worley 2008) and their role in pollination until now was previously unexplored.

2.4.1 Large visitor's role in pollination

In contrast to my expectation, hummingbirds and hawkmoths were not the most frequent visitors to *P. brandegeei* during observation periods in either year (2015 and 2016). In my study the hawkmoth and hummingbird observations were collected opportunistically outside of formal observation periods, so I could not include them in the floral visitor analyses. However, compared with previous work conducted in the same *P. brandegeei* populations from 2004-2006 it appears that hawkmoths are usually rare visitors at the Deer Mountain site (0-5 visits recorded per year, 2004-2006; Kulbaba & Worley 2014). Hummingbirds on the other hand were very frequent visitors in certain years at this site. In 2004 around 25 visits were recorded and in 2005 more than 40 visits were observed. However, in 2006, 2015 and 2016 fewer than 5 hummingbird visits were seen per year at this site (Kulbaba & Worley 2014, this study).

The fluctuating abundance of hummingbirds within the *P. brandegeei* population on Deer Mountain suggests a long-term study would be necessary to more accurately assess the presence

of hummingbirds in this pollinator assemblage. In this study the hawkmoth and hummingbird observations were collected opportunistically so I could not include them in my floral visitor analyses. Numbers may have been low in some years due to fluctuations in the population size (Linhart, 1973; Newton 1998; Kulbaba & Worley 2008). Hummingbird populations have been known to fluctuate due to food-availability, and fluctuations on a local scale occur due to their territorial behaviour (Linhart, 1973). Territories are established in areas of high flower density (Linhart, 1973). Therefore, perhaps the low frequency of hummingbird visits in 2015 and 2016 to *P. brandegeei* flowers is due to a higher concentration of flowers on another species in the area. There was a large population of waxcurrant (*Ribes cereum*) in this site and it flowers over the same period as *P. brandegeei*. This could explain why there were many hummingbirds in the general area but few of them visiting *P. brandegeei.*

Differences in experimental design could also explain why there was a difference in observed large visitors between Kulbaba & Worley's work and this study. The proximity of the observer to the plants during observations could be a factor affecting observation of large visitors. Kulbaba & Worley (2008) situated the observers far enough away from the target plants that binoculars were required to identify floral visitors, whereas during this study observers were positioned 1- 1.5 meters from the plants. The possibility that hummingbirds were foraging less only when observers were around was assessed by using pollen movement and seed set as indicators of pollinator activity over the whole open pollination period.

Pollen deposition did not vary between tented and control treatment plants in either year. Seed set did not vary between treatment (control, tented or emasculated) in 2015 however, in 2016 there was significantly more seeds produced by control plants. The 2015 results disagree with my prediction that hummingbirds and hawkmoths are the primary contributors to pollen

movement between plants and seed set in natural populations of *P. brandegeei*. The results from 2015 also confirm that hummingbirds and hawkmoths were not simply being deterred from foraging on observed plants. The tented plants (available to only the small visitors) were receiving as much pollen as the open pollinated control plants, suggesting that the small visitors are providing most, if not all, of the pollination services during these years when primary pollinators are in short supply. The results from 2016 however, agree with the hypothesis that the control plants will set more seed due to the availability of these flowers to the hawkmoths and hummingbirds. I observed few hummingbird and hawkmoth visits to *P. brandegeei* but perhaps they were visiting when observers were in other areas of the site or absent.

In this study we found the pollen deposition over two days (average: 249 grains; 150 - 350 grains) did not differ between control and tented plants. Kulbaba & Worley (2014) recorded pollen deposition on day-pollinated plants at Deer Mountain ranging from approx. 0 - 1100 pollen grains (approx. average: 200 grains). In 2015 and 2016 maximum pollen deposition was one third that of the open pollinated plants in 2005 and 2006 (Kulbaba & Worley 2014). This suggests that there was a reduction in pollen deposition in years where foraging hummingbirds were in low abundance.

2.4.2 Small visitor's role in pollination

Dialictus and *Lasioglossum* solitary bee species were the most frequent small visitors. The next most frequent visitors were the syrphid flies. The syrphid fly group included multiple species; as a collective group they were relatively frequent visitors to *P. brandegeei*. Their frequency suggests that they may be contributing to pollen movement and therefore providing

some level of pollen services. Flies have been described as common visitors in a bee-associated *Polemonium* species (*P. viscosum)* in sub-alpine environments (Galen 1989). Syrphid flies are among the most frequent visitors in other systems (Boyd *et al.* 2011; Sahli & Conner 2011). They are frequent visitors of two common alpine herbs, *Ourisia glandulosa* (Plantaginaceae) and *Wahlenbergia albomarginata* (Campanulaceae) in New Zealand (Bischoff *et al.* 2013). Both of these plants are moth-associated. However, moths are not present in the alpine environment where the flies are the main visitors. Syrphid flies have also recorded as one of the most common visitors of wild radish (*Raphanus raphanistrum*, Brassicaceae). They are even common visitors to certain endangered species such as the 'three yellow-eyed grass' (*Xyris tennesseensis,* Xyridaceae). Syrphid flies were described as visiting *P. brandegeei* at "consistently high rates" between 2004 and 2006 (11 – 48 visits, Kulbaba & Worley 2008). In 2015 and 2016 the total visits to experimental plants from syrphid flies fell within that range (10-17 visits).

Bees in the Halictidae are also frequent flower visitors (Boyd *et al.* 2011; Sahli & Conner 2011). Solitary bees are often secondary pollinators of bird and moth syndrome flowers (Rosas-Guerrero *et al.* 2014). Some studies have found however, non-syndrome visitors can be the main pollinators in certain instances (Bischoff *et al.* 2013). *Dialictus* bees (Halictidae) have been recorded as the most frequent visitor in the multi-visitor pollination system of the herb, wild radish (*Raphanus raphanistrum*), accounting for 97% of observed visits and were described as one of the most important pollinators (Sahli & Conner 2011). A species of Halictidae bee is the most important pollinator, accounting for over half the total observed visits, for the endangered 'three yellow-eyed grass' (*Xyris tennesseensis* Kral, Xyridaceae).

Visitor behaviour affects pollinator efficiency along with frequency (Bischoff *et al*. 2013; Mayer *et al.* 2011). Many studies have used frequency as a measure of efficiency (Mayer *et al.*

2011) but more comprehensive studies include measures of foraging behaviour and pollen movement to determine visitors' roles in pollination (Sahli & Connor 2007).

Floral reward choices for small visitors were observed during my observations of *Polemonium brandegeei* and varied between taxa. The syrphid flies fed on only pollen, either while hovering above the flower, rarely touching the stigma whilst feeding, or landing on the flower to feed. Though I saw both behaviours (hovering and landing) in approximately equal amounts, Kulbaba & Worley (2008) noted that syrphid flies, during 2004-2006 observations, usually hovered while foraging. The *Dialictus* bees landed in the flower to feed on both nectar and pollen rewards. Due to their small size, to feed on the nectar they had to crawl down the corolla tube to reach the nectaries. As they fed on pollen they also loaded pollen into their corbiculae. These qualitative observations suggests that *Dialictus* were taking a relatively high amount of pollen when they visited. During their activity within the corolla tube they were likely to be contacting the stigma.

During general observations of open pollinated plants, small insects visited 1-2 plants per bout and 1-2 flowers per plant with the exception of the *Lasioglossum* bees that visited two plants seven flowers per bout on average. At the Deer Mountain population in 2004-2006 syrphid flies and bees only visited around two plants and never more than four flowers per bout (Kulbaba & Worley 2008). Pollinators that visit flowers on many individual plants in the population during a single foraging bout have more potential as out-crossed pollen vectors. In a self-incompatible species such as *P. brandegeei,* only out-crossed pollen will be viable so good quality pollen deposits are essential. In comparison, small visitors are likely to be less effective pollen vectors than hummingbirds, which visited 2.2 plants per bout and 18 flowers per bout (Kulbaba & Worley 2008).

Small visitors may be secondary pollinators over the lifetime of *P. brandegeei* plants or perhaps they are secondary pollinators of *P. brandegeei* when fluctuations in pollinator abundance are considered (Roubik 2001). The exclusion experiment cannot identify each taxa's role in the pollination of *P. brandegeei*. This could be tested by separating the groups to study pollen movement contributions for each species through single visit trials (Bischoff *et al.* 2013).

2.4.3. Single visit pollen deposition

Single-visit observations were recorded for *Dialictus,* the most common visitor. When comparing the single visit deposition observed in this study with the deposition recorded by Kulbaba & Worley in the same population in 2005 (Kulbaba & Worley 2014) the single visit *Dialictus* deposits (on average ~ 650 grains) are within the upper range of that seen in 2005 and 2006 (on average ~200 grains; range >0 - ~1100). The pollen deposition measurements on experimental plants were taken after two days of open pollination. This suggests that the *Dialictus* bees are depositing a large portion of the total received pollen in only one visit. By contrast, using the pollination syndrome to predict the pollinators of *P. brandegeei* would lead to the conclusion that small visits should be incidental members of the pollinator assemblage (Fenster *et al.* 2004).

Bee contributions to pollen deposition has been studied in other species (Bischoff *et al*. 2013; Boyd *et al.* 2011; Hargreaves *et al.* 2010). Solitary bee (*Hylaeus matamoko*) deposits an average of 10 germinating pollen grains per visit to *Ourisia glandulosa* and on average ~100 grains for *Wahlenbergia albomarginata* (Bischoff *et al*. 2013), in some cases depositing ten times the amount of pollen than that provided by a visiting syrphid species (*Allograpta sp*.).

Native bees deposit between 20-150 pollen grains under pollination of *Aloe sp*. this includes bees they categorize as small bees and *Apis sp*. (Hargreaves et al. 2010). Pollen deposition recorded during this study are higher when compared to those in the literature. The pollen deposition recorded in this study can be compared to the study by Kulbaba & Worley but it may not be so easily compared with others studies. Some pollen deposition studies have only counted viable germinated pollen grains (Bischoff *et al.* 2013), preventing a direct comparison.

Pollen quality could also be explored by measuring germinated pollen grain deposits (Bischoff *et al.* 2013). These results could also be used to compare with single visit measurements for other *P. brandegeei* visitors. The single visit trials did not distinguish between self and outcrossed pollen either so we cannot directly examine *Dialictus* bee's role in compatible pollen transfer. It is however a preliminary look into their capabilities of transferring pollen in *P. brandegeei.* Future studies could continue to use the single-visit method on emasculated plants to assess pollen movement and seeds set caused by solitary bees and other members of the *P. brandegeei* visitor assemblage in field conditions.

2.4.4 Quality of deposited pollen

The initial expectation that emasculated flowers would not be attractive to pollen feeding visitors was not met in 2015 but emasculation reduced visitation in 2016. In both years, the anthers, were removed to measure pollen deposition when self pollen was not present (Young & Stanton 1990).

My results suggest that small visitors are providing pollination services but the pollen is likely a mixture of self- and out-crossed pollen. In experimental flowers, where self-pollen was

absent, less pollen was deposited but equal seed set was achieved in 2015. Therefore the compatible pollen deposited was the same for all treatments. However, in 2016 the pollen appears to have been better quality in the control plants because seed set was higher compared to that of the tented even though pollen deposition did not significantly differ. The excess pollen present in intact flowers is likely to be, at least in part, self-pollen. Self-pollen deposition can be reduced by anti-selfing mechanisms such as herkogamy (Barrett 2002). I saw no difference in pollen deposition with total variation in sex organ position in flowers. However, this does not mean the ratio of self- out crossed pollen is not changing with floral phenotype.

Additional pollen deposited in control and tented flowers could also be excess pollen not necessary for optimal seed set. Identifying germinated versus non-germinated pollen grains on the stigmatic surface would lead to studies of the chemical mechanisms controlling seed set (Bischoff *et al.* 2013).

2.4.5 Herbivory

Loss of floral material was mainly attributed to herbivory due to the visual evidence and sightings. Small piles of broken up floral tissue, with whole seeds missing and often whole inflorescences were removed which suggests granivorous small mammal activity (Hulme 1994). Small mammal herbivores was frequently observed foraging in the *P. brandegeei* population on Deer Mountain. A significant number of flowers were lost before and during fruit maturation. This was measured by comparing the number of treated flowers per plant with the number of intact experimental flowers that matured on the plant. My results show that, although the loss of reproductive tissue was considerable, seed set and flower survival to fruiting stage did not vary

between treatments. However, flower loss may have reduced the power to detect selection by pollinators (which will be discussed further in chapter 3).

2.4.6 Plant fitness and varying floral phenotypes

There was no significant variation in seed set caused by floral phenotype which disagrees with the hypothesis that intermediate herkogamy phenotypes with the stigma and anthers located close together would set more seed. This suggests that floral visitor assemblage is providing similar pollination services to all floral shapes. However, the natural distribution of phenotypes in the Deer Mountain population and my smaller sample size (compared to Kulbaba & Worley 2014) may have reduced my power to detect selection on herkogamy. The Deer Mountain population has a much larger number of plants displaying an intermediate phenotype opposed to the extreme phenotypes. Interestingly, pollen deposition was related to herkogamy in 2016. More pollen was deposited in flowers with reverse phenotypes. The reverse phenotype plants were distributed among the three treatments in 2016 (three emasculated, two tented and three control plants). Higher pollen deposition in reverse flowers of *P. brandegeei* has been previously associated with hawkmoth pollination (Kulbaba & Worley 2014). However, the increase in pollen deposition was recorded across all treatments, including the tented plants which could not be reached by hawkmoths. A possible explanation is that there is perhaps more self pollen deposited on reverse stigmas as the wind may knock pollen off the anthers down the corolla tube on to the stigma. To test this an extra treatment of fully excluded plant, with no access to visitors, could be included to test the amount of wind-transported pollen is deposited on the stigma.

From the studies by Kulbaba & Worley (2008, 2012 & 2013) we know that hummingbirds are, at least in some years, providing pollination services and are selecting for an approach herkogamy in *P. brandegeei* flowers. We also know from these studies that hawkmoths are also capable of providing pollination services and they are selecting for a reverse herkogamy. Hawkmoths were relatively infrequent visitors at this site so I would expect the net selection in this population would be towards the approach floral shapes.

Kulbaba & Worley discussed the conflicting selection imposed by two pollinators of *P. brandegeei* and suggested that the most common phenotype (intermediate or neutral herkogamy) was potentially a compromise between the selection pressures. The lack of variation in seed set across floral phenotypes and the successful pollination services provided by the small visitors suggests that they are not causing a directional selection of floral shape. Connor & Sahli (2011) showed that in certain plant systems with multiple visitors (small solitary bees) can have a stabilizing effect on floral phenotype. Connor & Sahli (2011) found several species of *Dialictus* bees selected for an intermediate phenotype when conflicting selection pressure was occurring due to other pollinators. These previous studies, coupled with my observations of high visit frequency and pollen deposition by *Dialictus* support a similar hypothesis for the high prevalence of the intermediate phenotype in the *P. brandegeei* population.

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2.6 Tables & Figures

Table 2.1. Number of visits by floral visitors to *Polemonium brandegeei* per treatment over two years (2015 & 2016) at Deer Mountain population, Colorado. Control treatment represents open pollination. Tented treatment excluded large visitors such as hawkmoths and hummingbirds while allowing small visitors to reach the flowers. Emasculated treatment removes self pollen deposition within the flowers as the plants had their anthers removed. The most common visitors for each year are separated from "other" members of the same order (Small brown fly/Anthomyiidae, syrphid flies/Syrphidae, *Lasioglossum* bees/Halictidae and *Dialictus* bees (sub-genus of *Halictidae*). Taxa with significant differences in the number of visits per treatment are in bold (all $P < 0.05$ in X^2 comparison).

	Floral visitor	2015			2016		
		Control	Tented	Emasculated	Control	Tented	Emasculated
Coleoptera	Coleoptera	0	0	2	1	1	1
Diptera	Anthomyiidae	17	11	8	0	0	0
	Other Diptera	16	8	8	3	1	0
	Syrphidae	7	3	7	5	3	3
Hymenoptera	Dialictus bee	9	11	13	13	8	2
	Lasioglossum bee	0	0	0	0	1	1
	Other Hymenoptera	3	7	6	0	2	4
	Total	52	40	44	24	16	11

Table 2.2. Floral visitor behaviour measurements observed on *Polemonium brandegeei* in 2016 at the Deer Mountain natural

population during general observations of untreated plants. Functional groups of visitors based on family or genus. ± 1 standard error.

* These observations were outside of the formal observation periods and over both years (2015 and 2016).

	Number of observations	Average bout length (seconds)	Average number of plants visited per bout	Average number of flowers visited per bout	Average number of seconds per flowers
Coleoptera	4	45.67 ± 23.54	1 ± 0	1 ± 0	45.67 ± 23.54
Other Diptera	10	39.7 ± 14.47	1.2 ± 0.14	2.8 ± 0.81	11.38 ± 3.27
Syrphidae	6	151.5 ± 131.26	1 ± 0	2.17 ± 0.6	44 ± 31.76
Dialictus bee	17	29.83 ± 8.17	1.12 ± 0.08	1.94 ± 0.3	14.08 ± 3.87
Lasioglossum bee	7	126.71 ± 70.25	2 ± 0.53	6.71 ± 3.21	14 ± 4.83
Other Hymenoptera	9	20.13 ± 9.94	1 ± 0	1.11 ± 0.2	13.43 ± 4.89
Hummingbird*	5	14.5 ± 8.54	2.2 ± 0.8	.18.25 ± 9.04	.
Hawkmoth*	2	.	3 ± 1.63	.	.

Table 2.3. Pollen deposits by floral visitors during single visit observations of *Polemonium brandegeei* in 2016 at the Deer Mountain population. Virgin flowers offered during single visit observations until a visitor fed on a flower. The visitor type was noted along with the length of foraging bout. Stigmas were harvested and the pollen grains on the stigma were counted to quantify deposition after a single floral visitor. Pollen deposited per single visit and the number of single visits observed are reported. Field identification for visitors used. * Average pollen deposition ± 1 standard error reported.

Floral visitor	Pollen grains deposited	Number of visits recorded
Dialictus bee *	650.3 ± 177.2	10
Lasioglossum bee	1085	1
Snake fly (Raphidioptera)	749	1
Medium thin beetle	328	1
Medium black beetle	95	1

Table 2.4. Analyses of pollen deposition on stigmas (log transformed) in relation to treatment (control treatment represents open pollination; tented treatment excluded large visitors such as hawkmoths and hummingbirds while allowing small visitors to reach the flowers; emasculated treatment removes self pollen deposition within the flowers as the plants had their anthers removed) microsite (naturally found patches of plants that were assigned numbers and treated as microsites) and number of open flowers (log transformed). Shown are general linear models for *Polemonium brandegeei*, during 2015 and 2016 field seasons at Deer Mountain site. Significant effects are in bold. Symbols: *P <0.05; **P<0.01; ***P<0.001.

Effect	2015	2016
Treatment	$\boldsymbol{F_{2,60} = 10.67}$***	$\boldsymbol{F_{1,42} = 4.24}$*
Microsite	$F_{10,60} = 0.53$	$F_{11,42} = 1.46$
Open flowers	$F_{1,60} = 0.40$	$F_{1,42} = 1.14$
R^2	0.34	0.44

Table 2.5 Analyses of number of flowers harvested in relation to treatment (control treatment represents open pollination; tented treatment excluded large visitors such as hawkmoths and hummingbirds while allowing small visitors to reach the flowers; emasculated treatment removes self pollen deposition within the flowers as the plants had their anthers removed) microsite (naturally found patches of plants that were assigned numbers and treated as microsites) and number of treated flowers. Shown are the general linear models for the treatment effects experimental flower harvest success in *Polemonium brandegeei*, during the 2015 and 2016 field seasons at Deer Mountain site. Significant effects are in bold. Symbols: $*P < 0.05$; $** P < 0.01$; $***P < 0.001$.

	2015	2016
Treatment	$F_{2,44} = 0.75$	$F_{2,42} = 1.26$
Micro site	$F_{11,44} = 1.09$	$F_{11,42} = 0.87$
Treated flowers	$\mathbf{F_{11,44} = 84.97***}$	$\mathbf{F_{1,42} = 27.64***}$
R^2	0.74	0.56

Table 2.6. Analyses of total seed number per plant (with plants that set zero seed removed to normalize data) in relation to treatment (control treatment represents open pollination; tented treatment excluded large visitors such as hawkmoths and hummingbirds while allowing small visitors to reach the flowers; emasculated treatment removes self pollen deposition within the flowers as the plants had their anthers removed), microsite (naturally found patches of plants that were assigned numbers and treated as microsites) and number of harvested flowers. Shown are the general linear models for the treatment effects on seed set in *Polemonium brandegeei*, during the 2015 and 2016 field seasons at Deer Mountain site. Significant effects are in bold. Symbols: *P <0.05; ** P<0.01; ***P<0.001.

Effect	2015	2016
Treatment	$F_{2,59} = 1.77$	$\mathbf{F_{2,22} = 6.95^{**}}$
Micro site	$\mathbf{F_{11,59} = 1.98^{*}}$	$F_{11,22} = 1.73$
Harvested flowers	$\mathbf{F_{1,59} = 21.41^{***}}$	$\mathbf{F_{1,22} = 6.8^{*}}$
R^2	0.46	0.62

Table 2.7. *Polemonium brandegeei* floral phenotype correlation matrix for measurements of the Deer Mountain population in 2015. Number plants included per interaction: Open flowers = 57, Corolla tube length = 58, Corolla tube diameter = 58, Anther –stigma separation = 52, Anther exsertion = 56, Stigma exsertion = 54, Plant size = 58. ** = $P <0.01$ *** = $P <0.001$. Anther-stigma separation was included in the analysis of seed set per treatment 1.control (represents open pollination) and 2.tented (excluded large visitors such as hawkmoths and hummingbirds while allowing small visitors to reach the flowers) and pollen deposition per treatment (control and tented). All variables are log (+1) transformed.

	Open flowers	Corolla tube length	Corolla tube diameter	Anther-stigma separation	Anther exsertion	Stigma exsertion	Plant size (height x width)
Open flowers	.	0.13	0.03	-0.01	-0.05	0.07	**0.52*****
Corolla tube length	.	.	-0.14	-0.13	-0.24	**-0.82*****	**0.37****
Corolla tube diameter	.	.	.	0.2	0.14	0.25	0.2
Anther-stigma separation	.	.	.	.	0.04	**0.43****	-0.17
Anther exsertion	.	.	.	.	.	**0.4****	0.13
Stigma exsertion	.	.	.	.	.	.	0.8

Table 2.8. *Polemonium brandegeei* floral phenotype correlation matrix for measurements of the Deer Mountain population in 2016. Number plants included per interaction: Corolla length = 42, Corolla diameter = 42, Anther-stigma separation = 41, Anther exsertion = 42, Stigma exsertion =41, Plant size 41. ** = *P* <0.01 *** = *P* <0.001. Anther-stigma separation was included in the analysis of seed set per treatment 1.control (represents open pollination) and 2.tented (excluded large visitors such as hawkmoths and hummingbirds while allowing small visitors to reach the flowers) and pollen deposition per treatment (control and tented). All variables are log (+1) transformed.

	Corolla tube length	Corolla tube diameter	Anther-stigma separation	Anther exsertion	Stigma exsertion	Plant size (height x width)
Corolla tube length	.	-0.14	-0.19	-0.23	-0.27	0.23
Corolla tube diameter	.	.	-0.03	-0.24	-0.14	-0.06
Anther-stigma separation	.	.	.	-0.01	**0.87***	0.0.1
Anther exsertion	.	.	.	.	**0.4**	-0.05
Stigma exsertion	.	.	.	.	.	-0.04

Table 2.9. Analyses of pollen deposition on stigmas (log transformed) in relation to treatment (control treatment represents open pollination; tented treatment excluded large visitors such as hawkmoths and hummingbirds while allowing small visitors to reach the flowers; emasculated treatment removes self pollen deposition within the flowers as the plants had their anthers removed) microsite (naturally found patches of plants that were assigned numbers and treated as microsites) and stigma-anther separation (log transformed; the distance in mm between the anthers and the stigma with a single flower). Shown are general linear models for *Polemonium brandegeei,* during 2016 field seasons at Deer Mountain site. Significant effects are in bold. Symbol: ***P<0.001.

Effect	2016
Treatment	$F_{1,27} = 3.3$
Micro site	$F_{11,27} = 2.11$
Stigma-anther separation	$F_{1,27} = \mathbf{21.78}$***
R^2	0.66

Figure 2.1. Floral measurements taken for each *Polemonium brandegeei* experimental plant. (1) Distance between the centre of the anthers and centre of the stigma (mm). (2) Distance between the point where the petal fuse and the centre of the stigma (mm). (3) Distance between the point where the petals fuse and the centre of the anthers (mm). (4) Distance between point that the petals fuse and the point where the flower joins the pedicle (mm). (5) The diameter of the corolla tube at the height where the sepals fuse (mm).
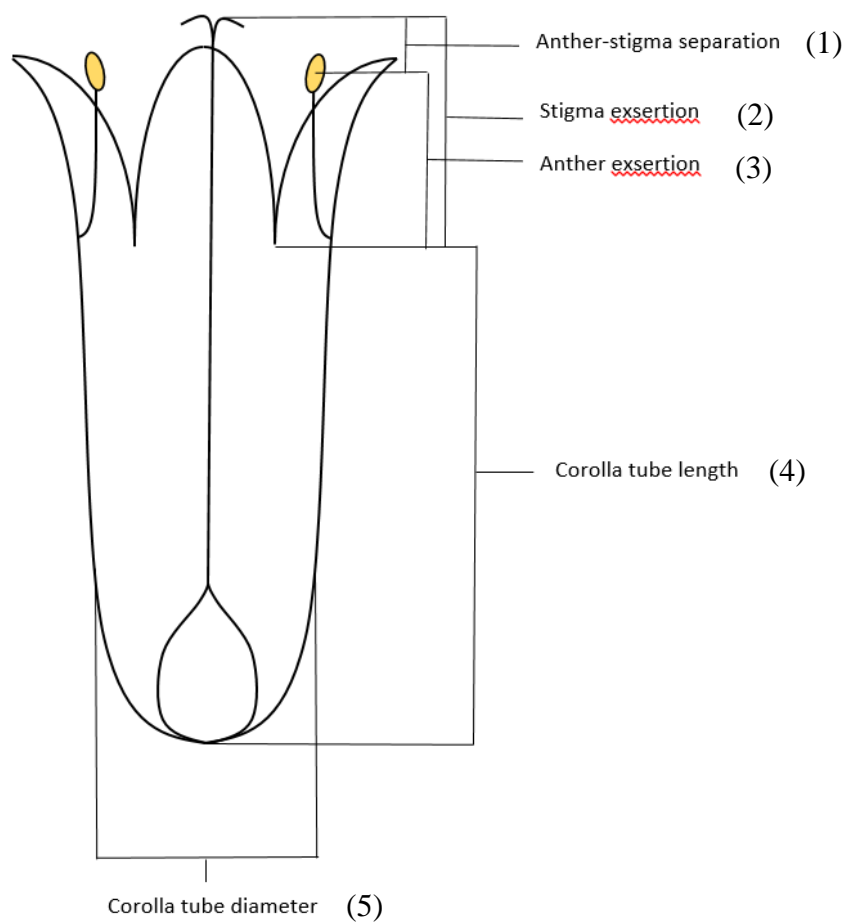
Figure 2.2. Mean pollen deposition on stigmatic surface in *Polemonium brandegeei* flowers per treatment; control, tented and emasculated (control treatment represents open pollination; tented treatment excluded large visitors such as hawkmoths and hummingbirds while allowing small visitors to reach the flowers; emasculated treatment removes self pollen deposition within the flowers as the plants had their anthers removed). Experimental flowers were available for floral visitors for two days at Deer Mountain field population across two flowering seasons (2015 and 2016). Least squared means were back-transformed from the analyses presented in Table 2.4. Error bars show -/+ 1 standard error.
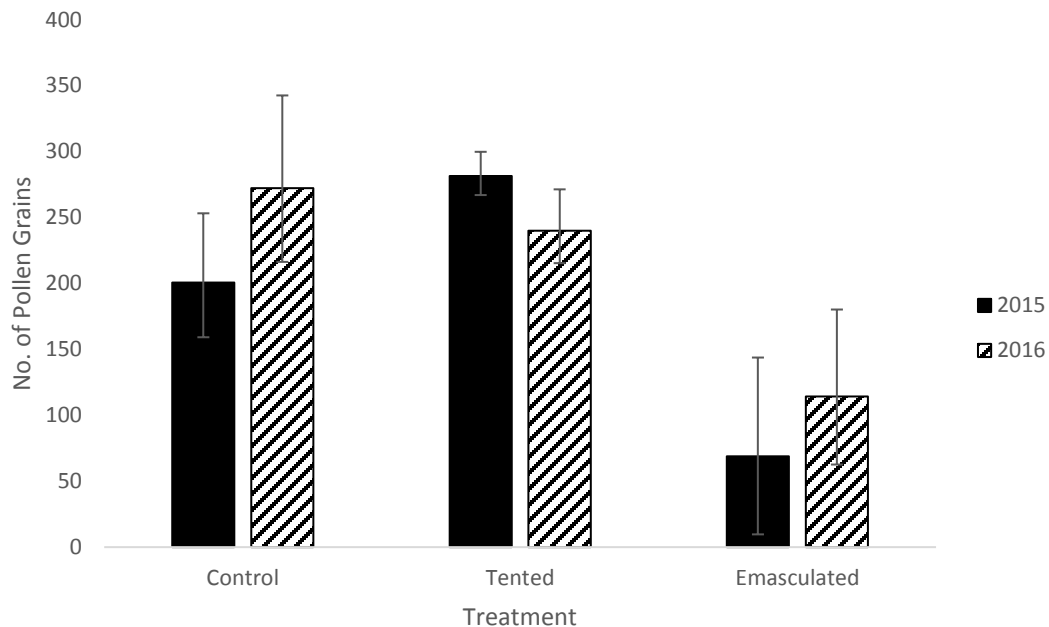
Figure 2.3. Number of open flowers, treated flowers, harvested flowers and flowers that set seed for each treatment at Deer Mountain field population. This shows loss of floral tissue through the proportion of treated experimental flowers that set seed. Measured for both 2015 (a) and 2016 (b) flowering seasons. Error bars show -/+ 1 standard error.
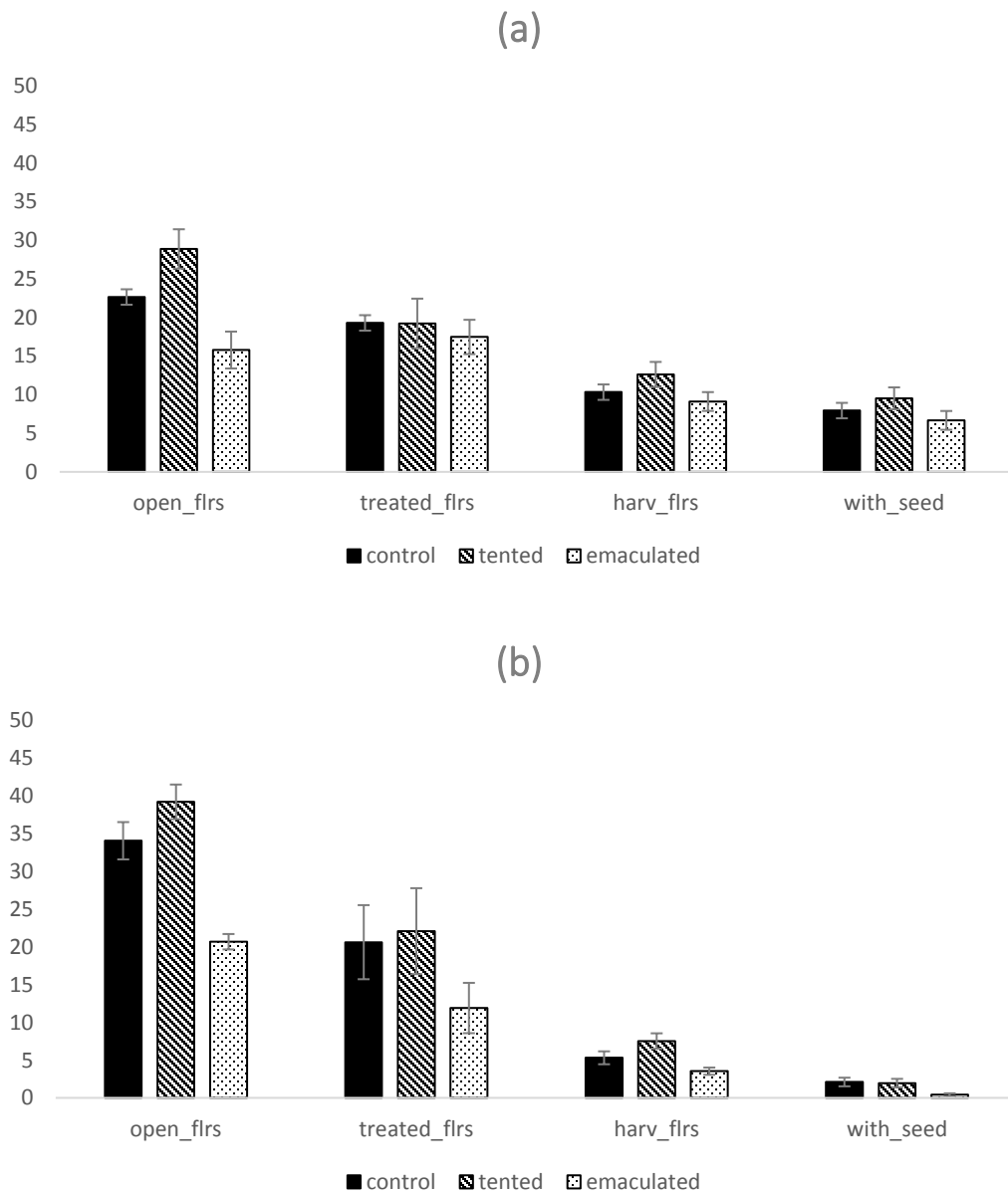
Figure 2.4. Mean seed set (total seed harvested per plant) for treated *Polemonium brandegeei* flowers per treatment; control, tented and emasculated (control treatment represents open pollination; tented treatment excluded large visitors such as hawkmoths and hummingbirds while allowing small visitors to reach the flowers; emasculated treatment removes self pollen deposition within the flowers as the plants had their anthers removed). Experiment conducted at the Deer Mountain field population across two flowering seasons (2015 and 2016). Data from the analyses presented in Table 2.6, which accounted for variation in the number of harvested flowers. Error bars show -/+ 1 standard error.
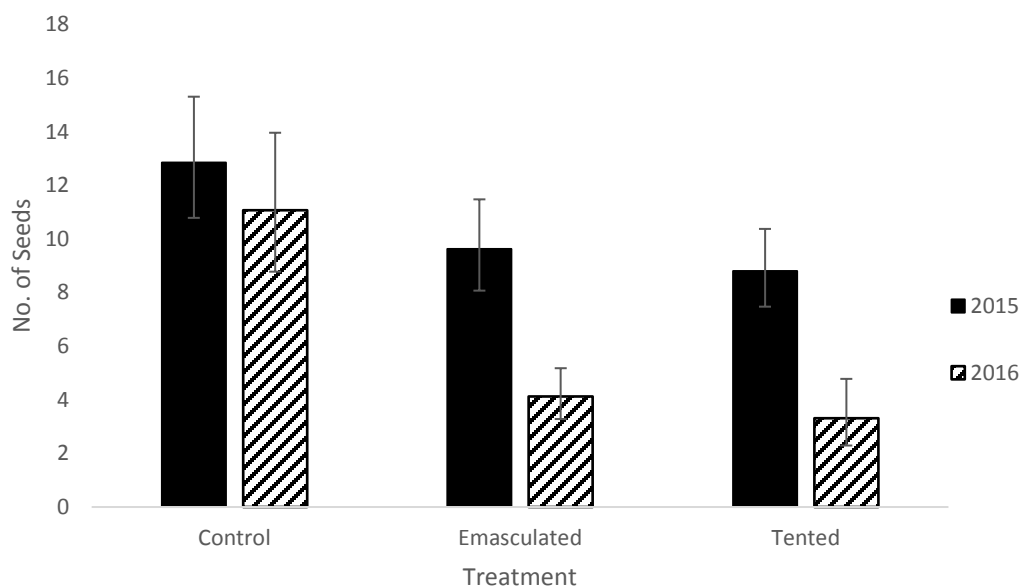
Figure 2.5. Ranked stigma-anther separation of Deer Mountain population

Polemonium brandegeei in 2015 (a) and 2016 (b). Stigma-anther separation (distance from

center of the anthers to the center of the stigma) is ranked from highest to lowest distances. On

the horizontal axis the highest ranking individuals are on the left and the lowest rankings are on

the right. Data markers above horizontal line are from approach herkogamous flowers, while

those below the line are reverse herkogamous.
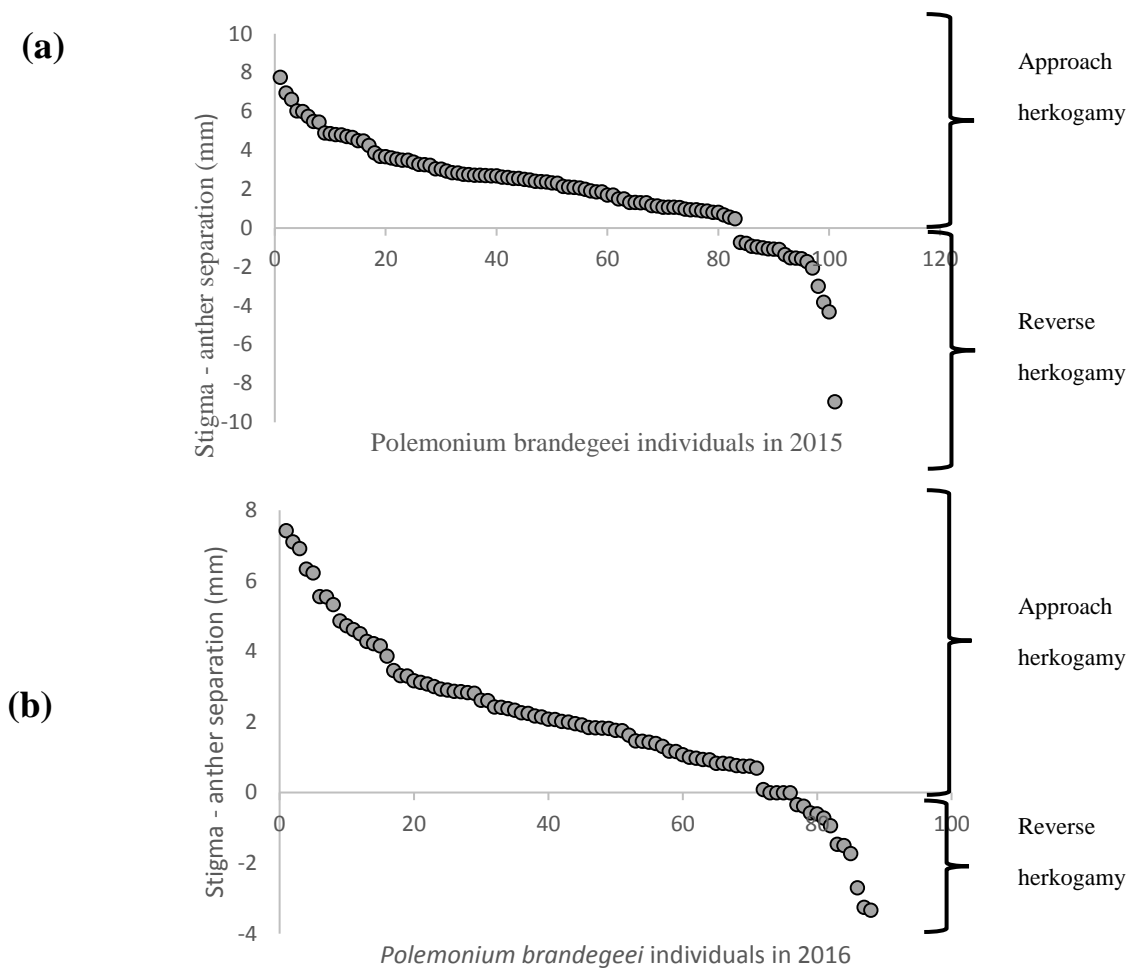
Chapter Three: Pollen Limitation & Herbivory

3.0 Chapter Summary

Plants depend on pollen vectors to reproduce due to their sessile form. Pollen limitation describes the situation where the quantity and quality of pollen received limits seed production. The severity of pollen limitation in turn affects the opportunity for pollinator mediated selection. Thus to study selection on floral shape and the pollination services from floral visitors, knowing if the plant species is naturally pollen limited is vital. In this study I measured pollen limitation in a natural *Polemonium brandegeei* population. I conducted a pollen supplementation experiment by hand pollinating plants and collecting seeds to compare to seed set under natural open pollination. The results of this experiment showed that this *P. brandegeei* population was not pollen limited because seed set did not differ between open pollinated and hand pollinated plants. I also measured loss of reproductive tissue between treatment and seed harvest. Much of this loss was due to herbivory although some may be lost through dispersal. The loss of flowers was considerable across all treatments (over 50% of the experimental flowers). This had an overall effect on fitness but tissue loss did not differ between treatments, and so should not have confounded my results. The lack of pollen limitation in this system suggests that pollinators are not providing a strong selection pressure on floral shape and that there are likely other factors affecting female fitness more strongly.

3.1 Introduction

3.1.1 Pollinator-mediated selection and pollen limitation

Plant reproductive fitness through female function is dependent on pollen receipt and resource availability (Burd 1994; Knight et al. 2005; Johnston *et al.* 2017). Pollen limitation is the term given when the quantity and/or quality of the pollen available to a plant is inadequate to fulfill the total potential seed set (Aizen & Harder 2007; Davila *et al.* 2012). Pollen can be limited through many means, including low visitor abundance, pollen theft, low visitor inefficiency, and low pollen quality (i.e. self-pollen). When present, pollen limitation can intensify selection pressure on floral phenotypes to optimize pollen receipt. In animal-pollinated species, floral visitors influence the quality and quantity of pollen received (Harder & Johnson 2009). Therefore visitor-mediated selection on floral phenotype exists when pollen is limited (Trunschke *et al*. 2017). The exact selection pressure parameters will change in response to the relationship between pollen receipt and relative fitness (Johnston *et al.* 2017).

Pollen limitation has been well studied (Knight *et al.* 2005, 2006; Rosenheim *et al.* 2016). However, less is known about the relationship between floral visitors and pollen limitation (Lázaro *et al.* 2015). Natural populations are often pollen limited to some extent (Knight *et al.* 2005c). Some argue that this conclusion is drawn from a publication bias in the literature caused by only studies with pollen limited species being published (Rosenheim *et al.* 2016). However, multi-species surveys of the occurrence of pollen limitation in natural communities have shown that the number of species that benefited from the receipt of supplementary pollen varied between studies (Lázaro *et al.* 2015; Rosenheim *et al.* 2016).

3.1.2 Resource reallocation and pollen limitation

Resource availability, like pollen limitation, regulates female fitness and can limit the maturation of seeds and fruit (Burd 1994; Knight *et al*. 2006; Worley & Harder 1999; Rosenheim *et al.* 2014). Resource reallocation reflects the plants ability to distribute resources between different tissue types (vegetative and reproductive) and within tissue types (Caswell 2017).

When a portion of flowers are supplemented to test for pollen limitation, resource allocation must be considered because flowers receiving supplemental pollen may receive an increased proportion of the resources available for seed maturation (Knight *et al.* 2006). Up to a 6-fold difference in fraction of ovules maturing to seed is seen when measuring supplemented flowers only versus all flowers on a plant (Burd 1994; Rosenheim *et al.* 2016). If resource reallocation is not accounted for in supplementation experiments, it can lead to misinterpreted results. Exploring the difference between flowers on the same plant identifies the possibility of resource allocation as a result of supplementation experiments (Saikkonen *et al*. 1998).

3.1.3 Herbivory & loss of reproductive tissue

Resource allocation is used as a strategy to maximize resource efficiency (Hawkes & Sullivan 2001; Johnson *et al.* 2015) and will also shape the plant response to herbivory (Hawkes *et al.* 2001). Most plants suffer some degree of loss of reproductive tissue. Some of this loss is due to herbivory in natural populations (Juenger & Bergelson 1997). When herbivory is prevalent in a population, plant fitness can be severely reduced (Hawkes *et al.* 2001; Turcotte *et al.* 2014). The ability to recover after herbivory requires resources to be available, and resource availability can vary among natural plant populations (Hawkes *et al.* 2001). Resource response to

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herbivory depends on the length of the growing season. Limited time to react to the herbivory event (for example when the herbivory effort occurs at the end or after flowering season) may not allow for the plant to produce more flowers to improve reproductive success (Rosenheim *et al.* 2014).

Very little literature on the relationship between herbivory defence and plant reproduction exists (Johnson *et al.* 2015). However herbivory is an important factor to consider when studying plant fitness and the evolution of plant traits (Turcotte *et al.* 2014). Herbivory was not a factor I initially set out to explore however, the high rates of tissue loss seen in 2015 when collecting seed led to a measure of herbivory being considered essential in the analysis.

3.1.4 Rationale

The multi-visitor pollination system of *Polemonium brandegeei* includes two known pollinators; hawkmoths and hummingbirds that select for two conflicting phenotypes (Kulbaba & Worley, 2012 & 2013). However, selection of floral phenotype has not been measured in the natural populations. Any study of visitor-mediated selection on floral shape must investigate pollen limitation to determine the potential strength of pollinator mediated selection. We would assume that if there is visitor-mediated selection in the natural population that pollen would be limited (Trunschke et al. 2017).

Therefore, I predict that: 1) if pollen supplementation increases natural seed set then pollen is limiting female fitness; and 2) if seed set is not different between plants, but it differs between flowers on the same plant, then resource reallocation is occurring and preventing the seed set difference between control and supplemented plants.

In this chapter I assessed *P. brandegeei* populations for pollen limitation by hand pollinating a subset of flowers per plant and to test pollen limitation as a key factor contributing to the intensity of visitor-mediated selection

3.2 Methods and Materials

3.2.1 Study sites and population

I conducted field experiments over two flowering seasons (2015 and 2016). The study was carried out at Deer Mountain in Colorado (described in chapter 2).

3.2.2 Pollen limitation experiment

Pollen limitation was assessed by supplementing natural pollen receipt to measure the seed set response to abundant pollen. Pollen was supplemented by hand-pollinating plants with viable, out-crossed pollen from donor plants within the same population. The experimental plants were assigned one of two treatments; *supplemented* or *control. Control* plants were naturally pollinated and not manipulated in any way. *Supplemented* plants were hand pollinated using out-crossed pollen from two donor plants from the natural population that were located a minimum of 7 meters away from the *supplemented* plant. The experiment was run over two years and included 35 control and 31 supplemented plants in 2015, as well as 20 control and 18 supplemented plants in 2016.

The supplementation treatment provided a threshold for maximum seed set to compare with the seed set from the *controls* and the two treatments from the pollen movement experiment (see chapter 2). The experiment was run simultaneously with the pollen movement experiment (during flowering season, early-late June) and the treatments were assigned to plants within the population randomly.

Within the supplementation treatment, I included two internal control treatments to account for resource re-allocation within the plant, between flowers. Control flowers on the same inflorescence (*inter-inflorescence control*) as the supplemented flowers and control flowers on separate inflorescence (*intra-inflorescence control*) to the supplemented flowers. The proportion of flowers treated was allocated based on how many inflorescences were in flower. Approximately 2/3 of the inflorescences would be assigned as 'supplemented' and would include approximately 2/3 supplemented and 1/3 inter-inflorescence control flowers. The other 1/3 of the inflorescences would be treated as the intra-inflorescence flowers. Treated and control flowers were marked using non-toxic acrylic paint on the calyx of experimental flowers (Figure 3.1 and 3.2). A different colour of paint was assigned to each treatment.

3.2.3 Seed collection

Seeds were collected for every experimental plant from the Deer Mountain site over both years in late July-early August. Seeds from supplemented, intra and inter inflorescence control and external control flowers were counted and collected separately. Fruit was harvested from the flowers that had been in flower at the time of treatment and placed in coin envelopes. The total number of seeds per fruit was recorded for each experimental plant, along with the number of undeveloped seeds per flower. The number of harvested flowers that did not set fruit was also recorded (seed collection conducted using the same method described in section 2.2.7).

3.2.4 Statistical analysis

To assess pollen limitation at the whole-plant level, I compared total seed number in control and supplemented plants. Total seed production in supplemented plants included counts from all flowers with seeds (supplemented + both controls). A general linear model was used to analyze seed set as a dependant variable, and included treatment (control or supplemented plant) as a categorical fixed effect and micro-site as a random effect. The numbers of treated flowers and the harvested experimental flowers were included as covariates to account for variation in the number of treated flowers on each plant and extensive floral herbivory (see Results). Seed numbers were log transformed ($log+1$) to meet assumptions of a normal distribution and equal variances. Separate analyses were conducted for each year of the study (2015 and 2016).

Resource allocation

To assess whether supplementation caused resource re-allocation, I compared seed numbers in control flowers and supplemented flowers on the same plant through paired *T*-tests. Within stem and between stem controls were combined because grazing meant that few plants from each category remained at the time of harvest. I analyzed differences in seeds per flower, rather than total seeds, to account for variable numbers of flowers collected from each treatment and plant. Separate analyses were conducted for each year of the study (2015 and 2016).

Herbivory

To assess the loss of reproductive tissue due to herbivory, I compared the total number of flowers harvested from control and supplemented plants. Total flowers harvested in supplemented plants included counts from all flowers (supplemented + both controls). A general linear model was used to analyze number of flowers harvested as a dependant variable. Explanatory variables included treatment (control or supplemented plant) as a categorical fixed effect, micro-site as a random effect, and number of treated flowers as a covariate.

3.3 Results

3.3.1 Numbers and recovery of treated flowers

On average, nine flowers (range of 0-31 flowers) were harvested from each study plant in 2015, and four flowers (range of 0-13) were harvested in 2016. These numbers corresponded to 60% and 22% of the flowers that had been treated in each year, indicating substantial loss of reproductive tissue between flowering and fruiting.   However, flower recovery in 2015 was not influenced by treatment, micro-site, the number of treated flowers, or the number of open flowers at the time of treatment (Table 3.1 & Figure 3.3). In 2016 number of treated flowers affected the number of harvested flowers consistently indicating that the two treatments had comparable levels of herbivory, and that the number of flowers escaping herbivory increased with higher numbers of treated flowers ($P = 0.01$, Table 3.1, Figure 3.3).

3.3.2 Plant level seed set

Seed set from experimental plants in both 2015 and 2016 was analyzed. There were no significant differences in total seed number between control and supplemented plants in either year ($P > 0.05$, Table 3.2). In 2015, supplemented plants produced an average of 9.0 seeds and control plants produced 10.2 seeds. In 2016, supplemented plants produced 14.4 seeds and control plants produced 9.8 seeds. In addition seed number did differ in 2015 between microsites ($P = 0.01$) and varied positively with the number of harvested flowers ($P < 0.001$, Table 3.2). In 2016 neither microsite nor number of harvested flowers affected seed set ($P > 0.05$).

3.3.2 Within-plant resource allocation

There was no significant difference between seed set of the supplemented and the control flowers for 2015 (mean seeds per fruit $\pm$ 1 standard error: control flowers = 1.49 $\pm$ 0.17, min/max= 0.7-4; supplemented flowers =1.34 $\pm$ 0.2, min/max = 0-5, paired T_{d18} = -0.65, $P >$ 0.05) or 2016 (mean seeds per fruit: control flowers = 2.88 $\pm$ 0.54; min/max = 1-6; supplemented flowers = 5.46 $\pm$ 0.96, min/max = 0.5-10, paired T_{d6} 1.89; $P > 0.05$).

3.4 Discussion

3.4.1 Seed set and selection on floral phenotype

Seed set in the Deer Mountain population of *P. brandegeei* was not limited by pollen receipt. Pollen limitation was undetected in this population suggesting that the visitor abundance was sufficient for optimal seed set for the plants that set seed, even though there were some plants that did not set fruit. Pollen limitation often decreases with greater visitor frequency (Lazaro *et al.* 2015). These are particularly interesting findings because the large pollinators were in low abundance in 2015 (Chapter 2) suggesting that the abundant small visitors were providing sufficient pollination services to the plants that set seed.

Plant fitness was not affected by variation in corolla tube dimensions or sex organ orientation. Opportunity for pollinator-limited selection may have been limited in 2015 and 2016. Fitness in this system appears to be weakly associated with visitor-mediated selection based on the lack of pollen limitation (Trunschke *et al.* 2017). Previous experimental investigations into hawkmoth and hummingbird-mediated selection in *P. brandegeei* suggested that disruptive selection on floral traits might be imposed. However, the distribution of phenotype frequencies in the natural population suggested that perhaps there was a stabilizing force affecting selection. The lack of pollen limitation weakens visitor-mediated selection for floral phenotype in a natural population.

3.4.2 Resource reallocation

Accounting for resource reallocation is an essential component of any pollen supplementation experiment (Knight *et al.* 2006). The results of paired *T*-tests comparing

supplemented flowers to the control flowers within a plant showed no signs of resource reallocation in any direction. The low overall seed set and the lack of resource reallocation suggests that seed production in this population of *P. brandegeei* in 2015 and 2016 may be resource limited rather than pollen limited (Juenger & Bergelson 1997; Knight *et al.* 2005c). 'Resources' encompasses a suite of factors including water, nutrients and sunlight. Nutrient availability has been identified as a major factor influencing seed set in addition to herbivores and pollinators (Juenger & Bergelson 1997).

Future work could explore nutrient availability throughout the flowering season. Pre- and post- pollination resource reallocation in *P. brandegeei* could be investigated by incorporating an early and late season nutrient availability and plant growth factor in the analysis (Rosenheim *et al* 2014). The plants that receive supplemental pollen early in the season may have a different tissue growth/seed set reactions than those supplemented late in the flowering season. I treated plants continuously throughout the mid-late flowering season without treating throughout the entire flowering season or grouping them into 'early' and 'late' treated plants. Accounting for temporal changes in plant reaction to hand pollination could identify within-season variation in optimal seed set. Future work on *P. brandegeei* could more closely investigate the balance between pollen limitation and resource limitation.

3.4.3 Seed production

Seed and fruit set was slightly low (0-40 seeds per plant) compared to seed set of experimental *P. brandegeei* population (2-50 seeds per plant). The previous study, conducted under greenhouse conditions, recorded that all the plants set at least some seed (Kulbaba &

Worley 2013). Perhaps water or nutrient availability limits seed set of natural populations more than does pollen receipt (Verhoeven *et al.*1996).

Pollen limitation is dependent on ecological factors and the balance may change in these populations over time (Knight *et al.* 2005). Pollen limitation may be occurring intermittently in environments where conditions, and therefore floral visitors, are variable (Fulkerson *et al.* 2012). Pollen not being a limiting factor for sub-alpine *P. brandegeei* species in 2015 and 2016, suggests that frequent small visitors may be providing stable pollination services year to year (at least in 2015 and 2016). Given the perennial life history of *P. brandegeei* (Grant 1989) perhaps two years is not long enough to see the effects of pollen limitation on total, life-long fitness.

3.4.4 Herbivory

The observed loss of reproductive tissue was likely due to herbivore activity. The loss did not vary across treatment types (control and supplemented) which suggests that herbivores are not preferentially targeting plants based on the applied treatments. Small piles of broken up pieces of the fruit and flowers were observed in piles close to the base of the plants, which is an indication of grazing activity by small mammal granivores (Hulme 1994). Herbivory can reduce fitness by reducing seed set (Maron & Vila 2001). Dispersal of floral tissue and seeds was also observed and is another explanation for some of the tissue loss recorded. *Polemonium brandegeei* flowers desiccate and break off of the plant when seeds are mature and ready to disperse. Although loss of tissue reduced my sample size, it did not affect the trends recorded as there was no discrimination in loss between treatments.

High degrees of loss to herbivory can select for dominant pre-pollination resource allocation. In other words, plants can combat high levels of reproductive tissue loss by reproducing more reproductive tissue pre-emptively. Allocating resources to the production of more flowers acts as a balance between surviving to set seed and resource efficiency (Rosenheim *et al.* 2014). To explore resource reallocation responses to herbivory in *P. brandegeei* future studies could measure the mass of vegetative and reproductive tissue when herbivory is present and when herbivory is excluded (Galen & Cuba 2001).

Herbivory weakens the visitor-mediated selection intensity on floral shape due to loss of reproductive tissue. Herbivory reduces the variation in relative fitness therefore, reduces selection that occurs due to changes, such as floral phenotype (Juenger & Bergelson 1997; Galen & Cuba 2001). We would also expect variation in fitness to be unrelated to floral phenotype in a system where pollen is not a limiting factor which may lead to *P. brandegeei* being falsely identified as not pollen limited. Therefore my ability to detect pollen limitation may be obscured.

The results of this study support the idea that pollen limitation appears to be less common than previously suggested (Knight *et al.* 2005). By assessing pollen limitation in natural populations we can continue to build on our knowledge of the relationship between pollen receipt quantity and quality and plant fitness.

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3.6 Tables and figures

Table 3.1 Relationship between the total number of flowers harvested per plant, treatment (control and supplemented), microsite, and number of treated flowers per plant. Shown are general linear models for *Polemonium brandegeei,* during the 2015 and 2016 field seasons at *Deer Mountain* site. All continuous variables were log (+1) transformed. There was a significant difference in harvested flowers explained by the number of treated flowers in 2016. Significant effects in bold. * $P < 0.05$.

	2015	2016
Treatment	$F_{1,41} = 0.83$	$F_{1,31} = 2.35$
Micro site	$F_{13,41} = 1.12$	$F_{12,31} = 0.99$
Treated flowers	$F_{1,41} = 1.70$	**$F_{1,31} = 8.20$***
R Square	0.43	0.70

Table 3.2 Relationship between seed set (total seeds harvested per plant) and treatment (*Supplemented* and *control* plants) microsite, number of flowers harvested per plant and number of flowers treated per plant. All continuous variables were log (+1) transformed. Shown are general linear models for *Polemonium brandegeei*, during the 2015 and 2016 field seasons at Deer Mountain site. Significant effects are shown in bold. ** P < 0.05, ** P < 0.001, *** P <0.0001.*

	2015	2016
Treatment	$F_{1,46} = 1.36$	$F_{1,15} = 1.36$
Micro site	$\mathbf{F_{14,46} = 2.91**}$	$F_{11,15} = 0.31$
Harvested flowers	$\mathbf{F_{1,46} = 58.75***}$	$F_{1,15} = 2.25$
R Square	0.76	0.53

Figure 3.1: Pollen limitation experiment set-up diagram of supplemented (left) and control plants

(right). Supplemented flowers shown as grey and control flowers shown as white triangles.

Internal and external controls on the same plant were used to account for any potential resource

re-allocation that may affect intra and inter inflorescence seed set.
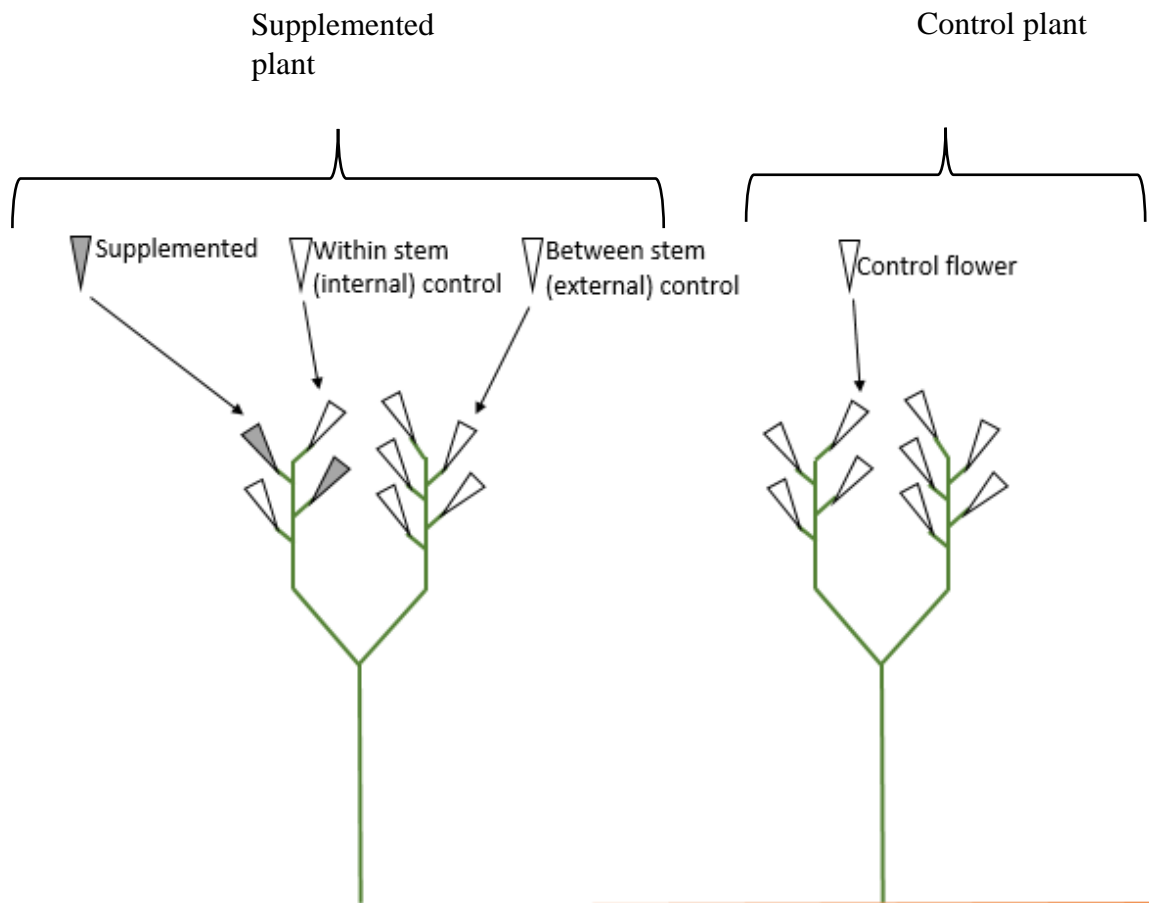
Figure 3.2. Method of hand pollinating the supplemented flowers on the supplemented experimental *Polemonium brandegeei* plants used at the Deer Mountain site (photo credit: Dylan Pond).

Figure 3.3 Experimental flowers treated and flowers recovered for seed set. Number of open flowers, treated flowers, harvested flowers and fruit set (number of flowers harvested that set seed) averages from the Deer Mountain population of *Polemonium brandegeei* in 2015 (a) and 2016 (b). All experimental flowers counted for the control plants and the supplemented values includes both the supplemented flowers and the internal control flowers.
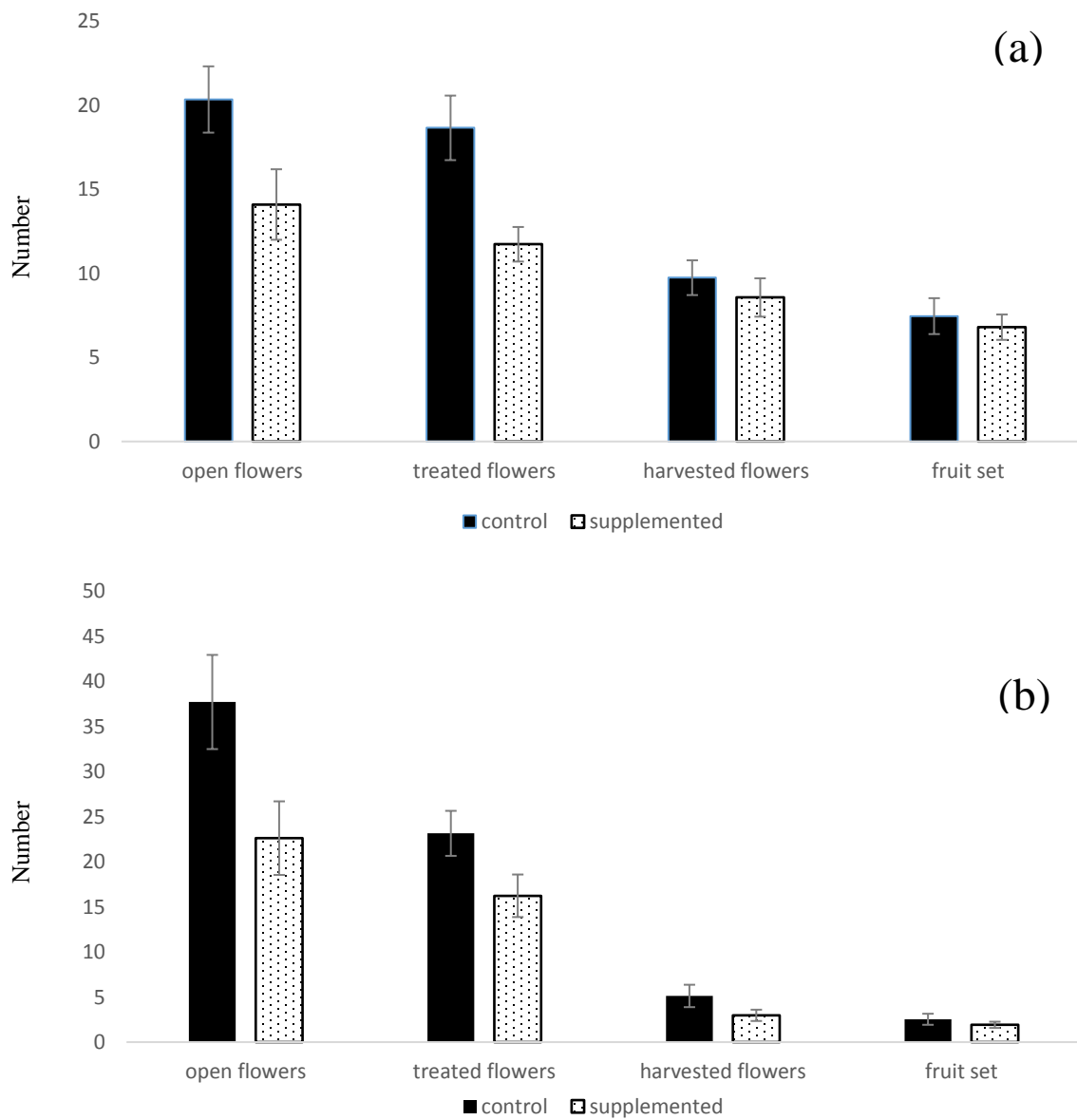
Chapter Four: Thesis Conclusions

To understand the effects of all floral visitor's roles in pollen movement it was important to explore previously overlooked visitor types. Several visitors had unidentified roles in the pollination of *P. brandegeei*. Previous work (Kulbaba & Worley 2012, 2013, 2014) that identified hummingbirds and hawkmoths as pollinators led to continued examination of pollination of *P. brandegeei* and the evolution of floral design in this thesis. I continued to document floral visitors in natural populations over two consecutive flowering seasons and I measured pollen deposition over the same flowering seasons (Chapter 2). To measure limitations to plant fitness in *P. brandegeei* populations a field experiment was conducted to assess *P. brandegeei* seed set when pollen was supplemented (Chapter 3). This final chapter summarizes the main findings from my thesis, and interprets them based on the current understanding of pollination biology. I also identify future areas of research based on what we have learnt about this study system.

4.1 Floral visitors

Small visitors, such as syrphid flies and solitary bees are often ignored, or their roles under-estimated, in pollination studies (Byers & Schiestl 2015). This is even more common when the visitors do not match the pollination syndrome associated with the plant (Rosas-Guerrero *et al.* 2014). Previously thought to be inconsequential visitors to *P. brandegeei*, I have found that small visitors play a significant role in pollination. Previous work on *P. brandegeei* surveyed the floral visitors observing their foraging behaviour and pollen movement efforts (Kulbaba & Worley 2014). They concluded that small visitors, when compared with the foraging behaviour of the

large visitors (hawkmoths and hummingbirds), were visiting fewer plants and flowers per bout than the large visitor. Therefore they suggested that the small visitors were less efficient. Kulbaba and Worley (2014) go on to conclude that this observation excludes small visitors from the pollinator category. My findings from 2015 disagree with this claim as I observed small visitors providing as much, possibly more, seed set through their role as a pollen vector in the small visitor only treatment compared to the large pollinators contribution to open pollinated plants. The next question to be asked is why might this disparity between studies exist?

Direct measures of fitness, such as seed set, determine ultimate pollination effect on fitness. Seed set was not measured by Kulbaba and Worley (2014), which left them to rely on foraging behaviour to assign pollinator/visitor statuses. I measured seed set and, in 2015, saw no significant difference between the tented (small visitor only) plants and the open pollinated plants. I conclude that small visitors were pollinators, at least in the two years I observed *P. brandegeei.* Small visitors appeared to be the main visitors because they were the most frequent visitors and provided pollination services. Small visitors may be secondary pollination (defined as less efficient non-syndrome visitors by Rosas-Guerrero *et al.* 2014) over a larger time scale to account for potential annual fluctuations in pollinator populations.

Large visitor abundance was low in the years I recorded visits to *P. brandegeei.* Visits to *P. brandegeei* by hummingbirds in 2006-2008 were roughly 10-fold more common compared to my observations in 2015 (n=4) and 2016 (n=1). This perhaps has been a result of fluctuations in the hummingbird population (Linhart, 1973) or a shift of main food source to another, more densely flowering, species. The hummingbirds were observed feeding mainly of *Ribes cereum* (wax currant) bushes that were abundant in the Deer Mountain site. *R. cereum* flowering period overlapped with that of *P. brandegeei* so hummingbirds always had *R. cereum* flowers available

during the *P. brandegeei* flowering season. Perhaps there are shifts associated with local climatic changes or perhaps there is a cyclical pattern in hummingbird populations when studied on a larger scale. Transitional ecotones like the sub-alpine environment are more sensitive to environmental fluctuations and are worth studying long term (Bischoff *et al.* 2013; Miller-struttmann *et al.* 2015).

Although it is true that not all floral visitors are pollinators, more research including all floral visitors in an assemblage and pollen movement provided by each visitor would lead to more concrete conclusions that includes antagonistic visitors and inefficient pollinators (Mayer *et al.* 2011). Their role appears to be context specific with some species of small visitors having different relationships with different flower shapes (Hargreaves *et al.* 2012). Perhaps the pollination syndrome hypothesis has led to mis-guided assumptions that hummingbirds and hawkmoths are the main pollinators in this plant system (Ollerton *et al.* 2009). Small visitor's role in pollination has been underestimated, perhaps due to the moth and bird pollination-associated traits of the *P. brandegeei* flowers.

4.2 Variation in herkogamy

My field experiment included the natural variation in floral visitors from which I can conclude that floral shape did not influence seed set under natural or small visitor pollination. My findings disagree with the observations of visitor-mediated selection on floral shape recorded by Kulbaba and Worley's (2012 and 2013). They isolated the pollination efforts of the two large bodied pollinators and represented a known range of floral phenotypes through experimental populations. My study was conducted in the natural population with many floral visitor types

present and floral phenotypes varied and plants were not chosen to be an experimental plant based on phenotype. The distribution of floral phenotype (specifically anther-stigma separation) followed a similar trend as observed previously at the same site (Kulbaba & Worley 2008).

4.3 Pollen limitation

To understand selective pressure on floral phenotypes we first must know if pollen receipt is limiting fitness (Lázaro *et al.* 2015). If pollen is limiting seed set then I would expect to see directional selection towards a floral phenotype to maximize pollen receipt through an attractive flower that morphologically fits the visitor and allows for successful pollen removal and receipt.

If pollen was limited in this population of *P. brandegeei,* small visitors may have been stabilizing the divergent evolution of flower shape in this species and could explain the presence of a variety of floral shapes in a natural population. It could also answer the question why is there such a high frequency of intermediate phenotypes when the two large pollinators select for the two extreme phenotypes? Measuring visitor contributions to successful pollination and ultimately plant fitness is a complex question (Kessler & Baldwin 2011; Mayer et al. 2011).

Pollen receipt was not limiting reproduction, which indicated a lack of strong selection imposed by visitors on floral shape (Trunschke *et al.* 2017). In this species, other factors influencing reproductive success and survival are likely having a stronger effect on seed production.

4.4 Herbivory

Herbivory is prevalent in many plant systems and the effect of herbivory should be considered when conducting field pollination experiments (Hawkes & Sullivan 2001; Turcotte *et al.* 2014). Herbivores can be excluded from pollinator field studies by caging the plants (Galen & Cuba 2001). Exclusion is a way to remove the effects completely, which would reduce loss of clarity when studying many factors of pollination and floral phenotype evolution. Perhaps for future work with *P. brandegeei* an exclusion technique could be employed to remove the herbivory effects on fitness and see the effects on evolution of floral phenotype more clearly.

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