FLORAL EVOLUTION IN POLEMONIUM BRANDEGEEI

(POLEMONIACEAE)

by

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Our treasure lies in the beehives of our knowledge. We are perpetually on our way thither, being by nature winged insects and honey gatherers of the mind. The only thing that lies close to our heart is the desire to bring something home to the hive.

Friedrich Nietzsche
The Genealogy of Morals (1887)

ABSTRACT

Floral traits are typically associated with specific groups of pollinators. Yet, many flowering plants are pollinated by more than one group of pollinators. To explore the influence of multiple effective pollinators on floral traits, I examined how the pollinator assemblage of *Polemonium brandegeei* affects pollen movement and selection on floral traits. I documented phenotypic and genetic variation in floral traits, and quantified the effectiveness of floral visitors. In natural populations, I quantified pollen removal and deposition over two consecutive flowering seasons. I then performed a pair of novel array experiments to estimate selection through female (seeds set) and male (seeds sired) function on floral traits by two important pollinators, hawkmoths and hummingbirds. My analysis of field populations demonstrated that height and relative positioning of sex organs were important for the removal and deposition of pollen. Individuals of *P. brandegeei* displayed a large degree of continuous and heritable variation, particularly in the relative positioning of sex organs. Plants in the same population displayed stigmas recessed below (reverse herkogamy), or exserted above anthers (approach herkogamy). My array experiments determined that variation in herkogamy is likely maintained through contrasting selection, because hawkmoths selected for recessed stigmas but hummingbirds selected for exserted stigmas. While my results were the first to detect selection for reverse herkogamy by hawkmoths, I also identified selection for traits that are typically associated with both pollinators. For example, hawkmoths selected for narrow corolla tubes, and hummingbirds selected for longer corolla tubes. The selection I detected on floral traits through female function was generally stronger than through male function, which runs counter to traditional theory of gender-biased selection. My findings indicate that floral traits can conform to intermediate dimensions between the optima of two pollinators (herkogamy), or appear specialized to one pollinator (tube length). Therefore, cumulative arrangements of floral traits (floral design) can effectively function under pollination by two pollinators that exert different selection pressures on traits.

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CHAPTER 1: INTRODUCTION AND GENERAL BACKGROUND

Flowers are the primary reproductive organs of flowering plants (angiosperms), and are displayed in a strikingly diverse range of shapes, sizes, and colours. Due to their sedentary nature, the majority (up to 87.5%) of flowering plants depend on animals as vectors of gamete dispersal (Ollerton et al. 2010; Barrett 2002a). The wide diversity in floral traits is largely viewed as a result of selection acting on the attraction and promotion of efficient pollen transfer by animal pollinators (Johnson 2006). Floral traits as they relate to pollinators have been formally studied since Darwin (1862), but the complex nature of plant-pollinator interactions leaves numerous unanswered questions. Examples include how selection operates through female versus male function, how floral traits evolve in species visited by diverse pollinators, and how conditions in natural populations affect pollen movement.

Flowers represent the ultimate interaction between plants and pollinators. The size, number, and arrangement of flowers (floral displays) have evolved to attract and promote pollinator visitation (Barrett 2002a). The characteristics of individual flowers (floral design) orient pollinators for the placement of pollen on the animal's body where it can be efficiently deposited during subsequent visitations. Therefore, floral designs influence the quantity and quality of pollen dispersed (Barrett 1998). However, some aspects of floral design may also influence pollinator behaviour (reviewed in Baker 1983; Kulbaba and Worley 2012). While the influence of selection by pollinators on floral

design has long been appreciated, the role of variable selection by multiple pollinators has remained ambiguous (Irwin 2006).

My doctoral research examined the interaction between flowers and animal pollinators, and how this interaction affects patterns of pollen transfer and selection on floral design. Below, I introduce some of the factors influencing selection on floral design. I also describe the roles of selection through female and male functions, and how gender functions and trait combinations contribute to the overall evolution of floral design. I then describe the quantitative genetic approach that I used to assess genetic variation in and selection on floral traits. I conclude with a description of my study species, and the experiments that comprised my thesis research.

1.1 Selection on floral design

A major theme in pollination ecology is the association between specific floral designs and particular pollinators, or groups of pollinators. The floral designs involved have traditionally been known as pollination syndromes (Fenster et al. 2004). This traditional view invokes a "lock and key" relationship between floral traits and pollinators (cf. Grant and Grant 1965). For example, plants pollinated by hummingbirds often display flowers with brightly coloured corollas fused into a tube, and sex organs exserted beyond the opening of the corolla (Grant and Grant 1968). This floral design attracts hummingbirds and enhances pollen removal by ensuring that pollen is placed on a region of the bird where it can be deposited on the stigmas of subsequently visited flowers (see Campbell 1996). Although particular floral designs have been associated with specific pollinators, several studies have observed that many flowering plant species

are frequented by a variety of visitors with a range of morphologies and behaviours (e.g. Ollerton 1996; Waser et al. 1996; Aigner 2006; Waser 2006). The recognition that multiple floral visitors frequent the flowers of angiosperms has brought into question the relative effectiveness of visitors, the degree of trait specialization or generalization, and the effects of selection by multiple effective pollinators.

While ineffective floral visitors likely contribute little to selection on floral traits (Gómez and Zamora 2006), the fraction of effective pollinators may drive selection for reproductive success. Determining the relative effectiveness of floral visitors requires a detailed analysis of pollen transfer. For example, Sahli and Conner (1997) determined that a subset of the 15 genera of *Raphanus raphanistrum* floral visitors were more effective at transporting pollen, and exhibited a higher visitation frequency. This suggests that all floral visitors may not equally select on floral design for pollination efficiency. Direct estimates of female (seeds set) and male (seeds sired) fitness and pollen removal and deposition are ideal, but may be impractical for large community-wide studies (Alacórn 2010). Therefore, patterns of flower visitation may provide a surrogate measure. Calculating indices of relative pollinator importance is one such way to indirectly estimate pollinator effectiveness. The inclusion of the number of plants, number of flowers per plant visited, and whether or not visitors contact both sex organs are critical data for comparing the potential effectiveness of pollinators (Boyd 2004; Kulbaba and Worley 2008).

The observation that flowers are often visited by multiple groups of pollinators has also brought into question the relative degree of generalization or specialization of floral traits. Waser et al. (1996) and Ollerton (1996) began a critique of the traditional

view, and challenged the perceived frequency of generalized pollination systems. While specialized floral traits were traditionally viewed as a direct response to selection by the most effective pollinator (cf. Stebbins 1970), generalized systems were assumed to evolve when pollinators were infrequent (Johnson and Steiner 2000). Reconsidering the prevalence of generalized and specialized pollination systems has suggested a continuum of pollination systems. These systems range from highly specialized taxa (e.g. *Centropogon nigricans*; Muchhala and Thomson 2009) pollinated by a single pollinator, to generalist taxa pollinated by many animal species (*Erysimum mediohispanicum*; Gómez 2005). Overall, specialized pollination systems often involve large bodied pollinators on plants displaying flowers with bilateral symmetry and fused corollas. In contrast, generalized pollination systems are typically characterized by small-bodied pollinators (e.g. bees, flies) on plants displaying open flowers, with a large landing platform (Wilson et al 2004). The conditions promoting where a particular plant taxon resides on the continuum between generalization and specialization are still not fully understood. However, long-lived perennials and species capable of vegetative reproduction may be more likely to converge on specialized pollination systems. Conversely, short-lived annuals and species that employ a colonizing or weedy habit often display a generalized system as a form of reproductive assurance (Johnson and Steiner 2000).

Recent studies have just begun to examine selection on floral traits by more than one effective pollinator (see Aigner 2006; Conner et al. 2009; Sahli and Conner 2011). Earlier work has identified four potential responses to selection by two or more groups of effective pollinators (Aigner 2001; Sahli and Conner 2011). First the mean trait value

may conform to an "intermediate" phenotype between the optima of two pollinators, as a response to contrasting selection on the same trait. Secondly, the mean trait may specialize to both pollinator groups, if the pollinators select on the same floral trait in the same manner. Third, the mean value of a floral trait may specialize to one pollinator over another. This may occur when a particular trait is more critical for pollination under one pollinator, or the trait optimum for a second pollinator included the optimal dimension for the second pollinator. Thus, traits may directly respond to selection by less effective pollinators, providing this response does not interfere with pollination by more effective pollinators (Aigner 2006). Finally, selection on floral traits by each individual pollinator may not be independent, causing different targets or modes of selection when both pollinators occur simultaneously. These outcomes may result in a series of intermediate phenotypes, or floral designs comprised of a mosaic of traits apparently specialized to different pollinators (Aigner 2001). Thus, the overall response of floral design to selection by multiple pollinators may not be as simple as predicted by Stebbin's (1970) most effective pollinator principle. As a result, quantifying selection by all effective pollinators is crucial to understanding floral evolution. However, very few empirical studies have considered selection on floral traits by more than one effective pollinator (but see Sahli and Conner 2011).

Regardless of the number of effective pollinators, selection on floral traits occurs through both genders because most angiosperm species are hermaphroditic. Selection on floral traits through female and male function may be identical, in contrast, or asymmetrical and seemingly occurring through only one gender (Ashman and Morgan 2004). However, theoretical approaches imply that conflict among gender-specific

selection may be common; given sufficient variation is present at a stable trait, selection is predicted to be equal but opposite among genders (Morgan 1992; Ashman and Morgan 2004). Some evidence has been found for conflicting gender function on floral design (e.g. Campbell 1989), but examples are generally limited (reviewed in Delph and Ashman 2006), in part because few studies have directly measured male function.

Bateman's (1948) hypotheses as applied to flowering plants predicted that selection through male function should be stronger than through female function. This asymmetry between the magnitudes of gender specific selection was predicted because female fitness was traditionally assumed to be restrained by the availability of resources. In contrast, male fitness was thought to be limited by the number of siring opportunities (Burd 1994). Therefore, traits that enhance pollen export and siring success, such as traits that increased pollinator visitation, should be under strong selection (Wilson et al. 1994). However, empirical evidence for stronger selection through male function is limited (Ashman and Morgan 2004). Rather, the relative strengths of gender-specific selection may depend on the ecological context, where the strength of selection through female function varies with the degree of pollen limitation. When the number of seeds set is limited by the amount of pollen imported and not by available resources, selection through female function should favour any trait that enhances pollen import (reviewed in Ashman and Morgan 2004). Pollen limitation may be more prevalent than previously assumed, which urges a reconsideration of the assumption that floral traits evolved primarily to promote pollen export (Wilson et al. 1994).

The presence of both female and male sex organs in the same flower increases the opportunity for interference between pollen import and export, respectively. This sexual

interference can result in gamete wastage and reduced fitness (Barrett 2002b). Two common strategies have evolved to reduce the potential of sexual interference. First, many angiosperms display dichogamy, where one gender function matures before the other, resulting in a temporal separation of sex organs (Webb and Lloyd 1986a). Second, herkogamy is common trait of flowering plants where sex organs are separated spatially (Webb and Lloyd 1986b). Both strategies may also occur simultaneously in the same flower, and further limit the negative effects of sexual interference.

The potential for floral traits to respond to selection depends in part on the available genetic variation and covariation among floral traits. Aside from environmental influences, two intrinsic genetic interactions result in the genetic correlation between floral traits. First, pleiotropy occurs when the genotype at one locus affects more than one trait, resulting in a covariation of trait dimensions. Correlations between floral traits can either emphasize or constrain the response to selection (Conner 2006). For example, Campbell (1996) detected selection for increased corolla length, and time spent in "female" or pistillate phase. However, the negative genetic correlation between these two traits prevents a simultaneous response to selection in the same direction. Second, gametic-phase disequilibrium, or the non-random association of alleles at different loci that each affect a trait, can result in the "integration" of floral traits (Lynch and Walsh, 1998). For example, intermediate measures of anther exsertion beyond the corolla tube of *Raphanus raphanistrum* confer the highest relative fitness, resulting in stabilizing selection on this trait (Morgan and Conner 2001). However, the exsertion of anthers depends on the length of filaments (structures that elevate the anthers) and the length of the corolla tube in *R. raphanistrum*. Therefore, corolla and filament length are

functionally related, and selection on anther exsertion maintains a positive genetic correlation between them (Morgan and Conner 2001).

1.2 Heritability, genetic correlations, and measuring selection

The potential of floral traits to respond to selection is dependant on three criteria. First, floral traits must exhibit phenotypic variation within a population. Second, this phenotypic variation must result in corresponding variation in the number of seeds set and or seeds sired (fitness). Finally, the phenotypic variation in floral traits must have a heritable basis, so that the relatively more fit phenotypes produce more offspring (Freeman and Herron 2006). These are the same general conditions required for evolution to occur via natural selection in all organisms.

The majority of floral traits exhibit polygenic inheritance, and typically display normally distributed phenotypes (Barrett 2002a). Continuous variation in phenotypes results from the cumulative, additive effects of many individual loci, each of relatively small effect (Falconer and Mackay 1996). Quantitative genetics describes the inheritance of such traits, and has provided the statistical framework to quantify selection on polygenic traits.

The total phenotypic variance of a given trait is the result of both the genetic and environmental factors influencing that trait. The genetic influence is comprised of variation due to dominance (V_D), additive (V_a), and interactive (V_i, pleiotropic or epistatic) effects (Falconer and Mackay 1996; Lynch and Walsh 1998). The primary measure of genetic variation available for selection is the heritability (h^2) of a trait. The heritability of a given trait is the ratio of the additive genetic variance to the overall

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phenotypic variance $h^2 = \left(\dfrac{Va}{Vp} \right)$. This ratio is typically interpreted as the resemblance

between relatives on a per trait basis. This ratio ranges from 0 (no similarity between

parents and offspring) to 1 (complete resemblance of parents and offspring). However,

Houle (1992) has pointed out that low heritabilities may occur even when V_a is

substantial because heritability also depends on total variation (V_p), which is influenced

by environmental factors. He therefore advocated using the coefficient of additive

variation (or evolvability) of traits $CV_a = \left(100 \left(\dfrac{\sqrt{Va}}{\bar{x}} \right) \right)$. Although the heritability or

evolvability indicates the potential of an individual trait to respond to selection, traits that

are genetically correlated to other traits under selection may respond indirectly.

Therefore, the estimation of additive genetic correlations (r_a), while more difficult to

obtain (Lynch and Walsh 1998), is also important to understanding the response of traits

to selection.

Many studies estimate the heritable basis of quantitative traits and genetic

correlations under controlled, greenhouse conditions. Estimating h^2 and r_a relies on the

comparison of parent and offspring phenotypes, which can be difficult to accurately

assign in large natural populations. For example, pollen from a donor that was not

measured in the field may contribute to offspring phenotypes, limiting the ability to

accurately estimate the similarity between parents and offspring. Therefore, the constant

environment of the glasshouse provides a confined gene pool that is ideal for controlled

crosses. However, estimates from natural field populations can capture the environmental

component of phenotypic variation (e.g. Campbell 1996), and generally result in smaller

estimates of heritability.

Estimating selection on floral traits relies on the relationship between the variation in floral traits, and a measure of fitness (seeds set or seeds sired). A common measure of selection is the univariate selection gradient (β) as described by Lande and Arnold (1983). Univariate selection gradients are measured as the slope of the regression that describes the relationship between relative fitness and a trait. Measures of selection gradients allow convenient insights as to the direction and magnitude of selection on floral traits (Falconer and Mackay 1996). However, pollinators may exert selection on trait combinations. For example, during correlational selection for long and narrow corolla tubes in *Ipomopsis aggregata*, the effect of tube length on fitness will also depend on tube diameter (Campbell 2003). Therefore, estimating single univariate selection gradients may not yield the complete picture of selection. To accommodate correlated selection, Schluter and Nychka (1994) developed the technique of projected pursuit regression (PPR) to complement traditional estimates of univariate selection gradients. Without making *a priori* assumptions on the shape of the fitness function, PPR fits a spline curve to relative fitness data, and quantified the weighting of all floral traits along the fitness function simultaneously. The PPR approach allows the identification of selection among correlated characters, and the means to compare the degree of linear verses nonlinear selection when compared with univariate selection gradients.

1.3 Approaches to understanding floral evolution

My doctoral research explored the interaction between floral traits and pollinators, and how this interaction affects pollen movement and plant fitness. As such, my project

required a diverse series of field and laboratory-based experiments. This section describes the general approaches used in my thesis.

Examining how multiple pollinators interact with floral traits and influence pollen movement and ultimately plant fitness, requires detailed observations in both natural and controlled conditions. The first step is to describe how floral traits vary within a species by measuring floral traits from natural populations. A multi-population approach, over consecutive years, is often necessary to fully appreciate the geographic and temporal variation inherent to floral traits (Herrera et al. 2006). Secondly, examining visitation frequencies in natural pollinator assemblages can aid in discriminating among effective pollinators and floral visitors. Finally, estimates of selection on floral traits by confirmed pollinators can yield insights into the patterns of selection under each pollinator.

Since most flowering plants are visited by more than one functional group of pollinators, examining selection by each pollinator in isolation is required to explore selection on floral design. Field studies of pollen removal and deposition can be difficult to interpret due to the presence of multiple visitors, not all of which are effective pollinators. Direct estimates of female and male fitness in field populations are complicated by the input of gametes from outside the study population that unexpectedly import novel genes (Conner 2006). Therefore, Campbell (2009) suggested a program of isolating (in natural or controlled conditions) a subset of the pollinators. Under controlled conditions, this approach has the advantage of limiting the gene pool to only known pollen donors, and allows the examination of selection on specific phenotypes. Further, manipulating the phenotypic distribution of one or a few traits independently of the remaining naturally varying traits allows the testing of trait-specific evolutionary

hypotheses. When consecutive experiments or "arrays" of plants are pollinated and evaluated under all effective pollinators, a more complete picture of the cumulative selection on floral design emerges.

1.4 Study system and objectives

My doctoral research sought to determine how pollinators and floral traits interact to effect pollen movement, fitness, and the evolution of floral design. The family Polemoniaceae contains many instances of closely related species that display strikingly different pollination systems (Grant and Grant 1965). *Polemonium brandegeei* in particular provides a unique opportunity to study the interaction of multiple effective pollinators and variable floral traits. My research focuses on the pollinators, pollen movement, and selection on floral traits of *Polemonium brandegeei*.

Polemonium brandegeei is a subalpine perennial herb, distributed along the Rocky Mountains from New Mexico through to Wyoming and southern Montana. The flowers of *P. brandegeei* are relatively long (19-32 mm) and narrow (3.0-6.0 mm), and overall resemble taxa pollinated by hummingbirds. However, the corolla is a cream-white colour and the flowers emit a strong sweet odour more reflective of taxa pollinated by hawkmoths. In addition, *P. brandegeei* displays a continuous range of approach to reverse herkogamy, which is congruent with these major pollinators.

This thesis describes a series of studies exploring the factors influencing the evolution of floral design in *Polemonium brandegeei*. The first two studies are descriptive because no previous work on reproduction in *P. brandegeei* exists. Chapter 2 first describes the floral visitors and morphological variation of *P. brandegeei* flowers in

three populations near the centre of its range. The study was conducted over three consecutive years (2004 to 2006) to capture temporal variation in both floral design and floral visitors. I also measured heritability of and genetic correlations among *P. brandegeei* floral traits, in a greenhouse crossing experiment. Chapter 2 appears largely as published in the *International Journal of Plant Sciences* (Kubaba and Worley, 2008). Chapter 3 describes pollen removal and deposition in the same three populations studied in Chapter 2. These patterns of pollen movement are considered in the context of variable floral visitors and the abiotic conditions spanning two flowering periods.

Based on the finding of the previously described chapter, I conducted two experiments to estimate selection by the two major pollinators of *P. brandegeei*. Chapter 4 describes the development of microsatellite markers specific to *P. brandegeei*, and used to estimate male selection gradients. I demonstrate the variability of 6 polymorphic loci, describing 39 alleles. Further, I demonstrate that these 6 loci are suitable for *P. brandegeei*'s sister taxon, *Polemonium viscosum* and the hybrids formed between these two species. This chapter appears largely as it was published in the *American Journal of Botany* (Kulbaba and Worley, 2011), with additional results on hybrid individuals. These microsatellites were used in the following two chapters to estimate selection through male function (seeds sired).

The next two chapters describe my experiments measuring selection by the two major pollinators of *P. brandegeei*. Chapter 5 explores selection on *P. brandegeei* floral traits by hawkmoth pollinators. These results are from a series of controlled pollination arrays, where I estimate selection through both female and male function on *P. brandegeei* floral traits, under visitation by the hawkmoth *Hyles gallii*. Chapter 5

appears mostly as when it was published in the journal *Evolution* (Kulbaba and Worley

2012). Chapter 6 takes a similar approach to measuring selection by pollinators on

P. brandegeei floral traits, but with the hummingbird *Archilochus colubris*. The patterns

of selection between hawkmoths and hummingbirds are also contrasted in this chapter, to

describe important selective factors shaping *P. brandegeei* floral design. Finally, Chapter

7 presents a general discussion of the overall thesis.

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CHAPTER 2. FLORAL DESIGN IN POLEMONIUM BRANDEGEEI (POLEMONIACEAE): GENETIC AND PHENOTYPIC VARIATION UNDER HAWKMOTH AND HUMMINGBIRD POLLINATION

2.0 CHAPTER SUMMARY

Many flowering plants reduce sexual interference between male and female functions through herkogamy, the spatial separation of anthers and stigmas. Many species are monomorphic and present stigmas either above or below the anthers, "approach" or "reverse" herkogamy, respectively. Although numerous studies have examined species that are polymorphic for approach and reverse herkogamy, species with continuous variation in sex-organ position have received little attention. I examined continuous variation in anther position, style length, herkogamy, and flower shape in the self-sterile, perennial herb, *Polemonium brandegeei*. I observed pollinators, measured flower shape and sex-organ position in the field, and estimated heritabilities and genetic correlations among floral traits in the greenhouse. The two major pollinators were hummingbirds, *Selasphorus platycercus,* and hawkmoths, *Hyles lineata* and *H. gallii*, which are believed to select for exserted and recessed sex-organs, respectively. Herkogamy was not polymorphic, but rather ranged continuously from reverse to approach, independently of corolla shape, size and age. Corolla-tube length and width, sex-organ heights and herkogamy were all heritable. Genetic variation for the spatial separation of stigmas and anthers was particularly high ($h^2 = 0.851$; $CV_a = 36.88$). Temporal fluctuations in pollinator frequency likely maintain the variation in herkogamy by imposing heterogeneous selection on floral traits.

2.1 INTRODUCTION

The flowers of animal-pollinated plants attract pollinators and promote efficient pollen dispersal. Some of the most common floral designs involve herkogamy, the spatial separation of anthers and stigmas in hermaphroditic flowers (Webb and Lloyd 1986). Two major forms of herkogamy occur. Approach herkogamy refers to the presentation of the stigma above or beyond the anthers so that pollinators contact the stigma before the anthers. In reverse herkogamy, the anthers are presented at the mouth of the corolla tube and the stigma is recessed below them (Webb and Lloyd 1986); this arrangement causes pollinators to contact the anthers before the stigma. Both forms of herkogamy reduce deposition of self pollen, and therefore reduce wastage of gametes in species with self-incompatibility or inbreeding depression (Barrett 2002). Although approach herkogamy is common, and associated with many types of floral visitors, reverse herkogamy is less common, and is thought to be associated with pollination by Lepidopterans (butterflies and moths) (Webb and Lloyd 1986; Barrett and Harder 2005).

Some angiosperms display stylar or stigma-height polymorphisms, with more than one herkogamous type occurring in the same breeding population. Heterostyly (reciprocal herkogamy) is an evolutionarily advanced stylar polymorphism, where the anther height of one morph complements the stigma height of another morph, and vice versa (Lloyd and Webb 1992a). Both heterostyly and stigma-height polymorphisms are controlled by one or two Mendelian loci (Lloyd and Webb 1992b). Although stylar polymorphisms have been extensively studied, the majority of angiosperms display a unimodal distribution of style length (Barrett et al. 2000).

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Although stylar polymorphisms exhibit Mendelian inheritance (reviewed in Barrett et al. 2000), style length is more often controlled by multiple genes each with smaller effects, as is generally the case for metric traits. Several field and greenhouse studies have demonstrated continuous variation in style length (see Shore and Barrett 1990; Mitchell and Shaw 1993; Carr and Fenster 1994; Robertson et al. 1994; Lennartsson et al. 2000; Motten and Stone 2000; Caruso 2004). Many of these studies examined continuous variation in herkogamy as a mechanism of reproductive assurance in self-compatible species (e.g., Lennartsson et al. 2000; Motten and Stone 2000).

The role of continuous variation of style lengths in outcrossing plants is largely unexplored. A continuum including both approach and reverse herkogamy would not be necessary to reduce self pollination. Instead, stylar variation within species may reflect contrasting selection by two or more dissimilar pollinators. Baker (1964) and Grant and Grant (1983) hypothesized that this may be the case for *Mirabilis froebelii*, which is visited by hawkmoths and hummingbirds. A second explanation for continuous variation in herkogamy could be a developmental relationship between flower age and herkogamy. For example, dichogamous flowers may first display reverse herkogamy during male phase, and then styles may lengthen to produce approach herkogamy during female phase. Thirdly, stylar variation may be maintained via gene flow between species or populations that have diverged in style length (see Campbell and Aldridge 2006). Finally, the optimal stigma-anther separation may be near zero. In this case, the left-hand tail of the herkogamy distribution may cross zero resulting in reverse herkogamy.

The placement of sex organs is only one aspect of floral design that is influenced by pollinators. Species that rely on similar functional groups of pollinators such as long-

tongued bees or hummingbirds often have flowers with similar colour, shape and arrangement of sex organs (Fenster et al. 2004; but see Waser et al. 1996; Wilson et al. 2004). For example, moth pollination is not only associated with reverse herkogamy, but also with white or cream coloured flowers, strong scent, and long narrow corolla tubes (Fægri and van der Pijl 1972).

Strong selection imposed by pollinators for particular combinations of floral traits may result in high genetic correlations among traits, i.e., genetic integration (see Conner and Sterlling 1995). For example, high genetic correlations of both stigma-and anther-height with corolla tube length in *Raphanus raphanistrum* ensure contact between sex organs and flower-probing insect visitors (Conner and Via 1993). Different patterns of floral integration may exist among plant species visited by different functional groups of pollinators, providing a tool for predicting which pollinators impose strong selection (Conner et al. 1995; Herrera et al. 2002). However, developmental or pleiotropic relations among traits may also constrain floral adaptation (Ashman and Majetic 2006).

Here, I investigate pollination and floral variation in *Polemonium brandegeei*. *Polemonium brandegeei* is a sub-alpine perennial herb, ranging from northern New Mexico to southern Montana along the Rocky Mountains (Davidson 1950). The flowers emit a heavy sweet fragrance and the foliage produces a strong skunky odour. The cream-white flowers have long, narrow floral tubes, have been hypothesized to be pollinated by hummingbirds (Grant and Grant 1968), and are self-sterile (Worley unpublished data). Preliminary observations of floral visitors by A. Worley indicated hawkmoth (*Hyles lineata*) pollination. Also, both approach and reverse herkogamous plants were present in the same breeding populations. *Polemonium brandegeei*'s closest relative, *P. viscosum,*

is pollinated by bumblebees and large flies (Galen 1989), has blue, approach-herkogamous flowers, and is very similar genetically (Worley et al. 2009). Thus, changes in pollinators appear to have coincided with recent floral divergence between these sister species.

As a first step towards understanding floral evolution in *Polemonium brandegeei*, my general objectives were to identify potential pollinators and to characterize phenotypic and genetic variation in floral traits. This study included three specific objectives. (1) I documented floral visitors to *P. brandegeei* in natural populations over three field seasons, to determine whether they conform to the hypothesized pollinators (hummingbirds; Grant and Grant 1968) and preliminary observations (hawkmoths; A. Worley, personal observation). (2) My second objective was to characterize phenotypic variation in floral design in three natural populations. My measurements allowed us to determine whether variation in floral design, particularly stigma-anther separation, of *P. brandegeei* was continuous or polymorphic, and whether herkogamy was correlated with corolla dimensions. I also compared floral dimensions in *P. brandegeei* with other species pollinated by hummingbirds and hawkmoths. (3) My third objective was to determine the heritability of and genetic correlations among *P. brandegeei*'s floral traits. These data allowed us to determine the extent to which phenotypic variation in floral traits, particularly herkogamy, has a genetic basis. As well, genetic correlations enabled us to comment on the integration of floral characters.

2.2 MATERIALS AND METHODS

2.2.1 Study populations

This study included a total of five field populations of *Polemonium brandegeei,* visited from 2000-2006. These populations are located near the center of the species range, and included a range of elevation, moisture and soil types (see Table 2.1). The three primary sites were Taylor Canyon and Deer Mountain, Colorado, and Vedauwoo recreational area, Wyoming. Geographic locations (latitude and longitude), approximate elevations, dates each field population was visited, and associated species are presented in Table 2.1.

The southernmost site, Taylor Canyon, was located approximately 25 km north of Gunnison, Colorado. Individuals of *P. brandegeei* were found in the crevices of rock faces, and at the base of trees. Taylor Canyon was relatively dry and warm, and had little available soil. Deer Mountain was located within Rocky Mountain National Park, along Deer Ridge trail. This population was situated on a slope (~30°), with a moderate layer of soil. Twin Sisters Mountain was also located in Rocky Mountain National Park. I visited Twin Sisters to observe pollinators in 2006, because it was the first site where hawkmoths (*Hyles lineata* Fab.) were observed visiting *P. brandegeei* (A. Worley personal observation). The northernmost site, Vedauwoo recreation area, was located approximately 65 km west of Cheyenne, Wyoming. Vedauwoo is moist, and has a thick soil layer compared to the other sites.

Table 2.1. Name, location (latitude and longitude, elevation), approximate number of individuals, and dates visited of

Polemonium brandegeei field sites used in this study.

Population Name	Latitude/Longitude (Elevation)	N	Associated Species	Dates Visited
Taylor Canyon, CO	39°34'33"N 104°22'26"W (2700 m)	100	*Pinus contorta* *Oenothera cespitosa*	May 23-27, 2004 May 25-31, 2005
Deer Mountain, CO	40°46'57"N 105°53'01"W (2500 m)	200	*P. contorta* *Ribes cereum*	May 28-June 7, 2004 June1-11, 2005 June 6-12, 2006
Twin Sisters Mountain, CO	40°23;04"N 105°35'03"W (3483 m)	150	*Phacelia sericea* *Eritrichum aretoides*	June 16-22, 2006
Vedauwoo, WY	44°29'39"N 116°18'50"W (1900 m)	500	*Populus tremuloides* *Aster spp* *Carex spp*	June 8-13, 2004 June 12-19, 2005 June12-17, 2006
Lone Tree Gulch, CO	38°30'17" N 107°11'26"W (2743 m)	100		August, 2000

Plant for the greenhouse experiments were raised from seeds collected in August

2000 in Lone Tree Gulch, approximately 15 km west of Saguache, Colorado. This site

was geographically close to Taylor Canyon, and had similar habitat conditions. Between

2001 and 2004 a wild fire destroyed the population at Lone Tree Gulch. Therefore field

measurements and floral visitor observations were not collected at this population.

2.2.2. Floral visitors

During peak flowering periods of 2004-2006 floral visitors were observed in

Taylor Canyon, Deer Mountain, Vedauwoo, and Twin Sisters (Table 2.1). I documented

floral visitors for 5-11 days per population, three times per day (7:30am, 12:30pm, and

7:30pm) for a minimum of 30 minutes. I also made observations past dusk (8:00pm to

10:30pm) at the Vedauwoo population. These evening observations were not done at the

other populations, due to limited accessibility after dusk. Finally, casual observations

were made throughout the day. I spent a total of 340.5 hours documenting floral visitors

to *Polemonium brandegeei* among all field populations.

The floral visitors were documented and, when possible, photographed, video

recorded, and/or captured for identification and to confirm the presence of pollen on their

bodies. I identified hummingbirds with the aid of binoculars, and captured insects.   In

addition, I noted whether the visitors contacted the stigma and anthers because effective

pollination requires consistent physical contact between the visitor and both sex-organs.

Finally, I adapted the "index of pollinator effectiveness" of Boyd (2004) as the product of

the total number of observed foraging bouts and the average number of plants visited per

foraging bout. Since *Polemonium brandegeei* is self-sterile, pollen transfer between

individual plants is required for successful pollination. Thus my index captured both the

frequency of foraging bouts and the potential that each bout contributed to reproductive

success.

2.2.3 Floral morphology

    To examine corolla shape and sex-organ position in *Polemonium brandegeei*, I

measured floral characters on randomly selected plants (n = 50 - 100) at the three primary

field populations. Measurements included flower length and width, corolla-tube length

and width, as well as the height, exsertion and relative positions of stigmas and anthers

(Figure 2.1). The flowers are protandrous so a "male" flower (before the stigma lobes

reflexed and became receptive) and "female" flower (after the stigma became receptive)

were measured on each plant to determine if herkogamy changed with flower age.

Finally, the distribution of tube lengths in *P. brandegeei* was compared with the bill

length for the hummingbird, *Selasphorus platycercus* (from Waser 1978) and proboscis

length for the hawkmoth *Hyles lineata* (from Grant 1983).
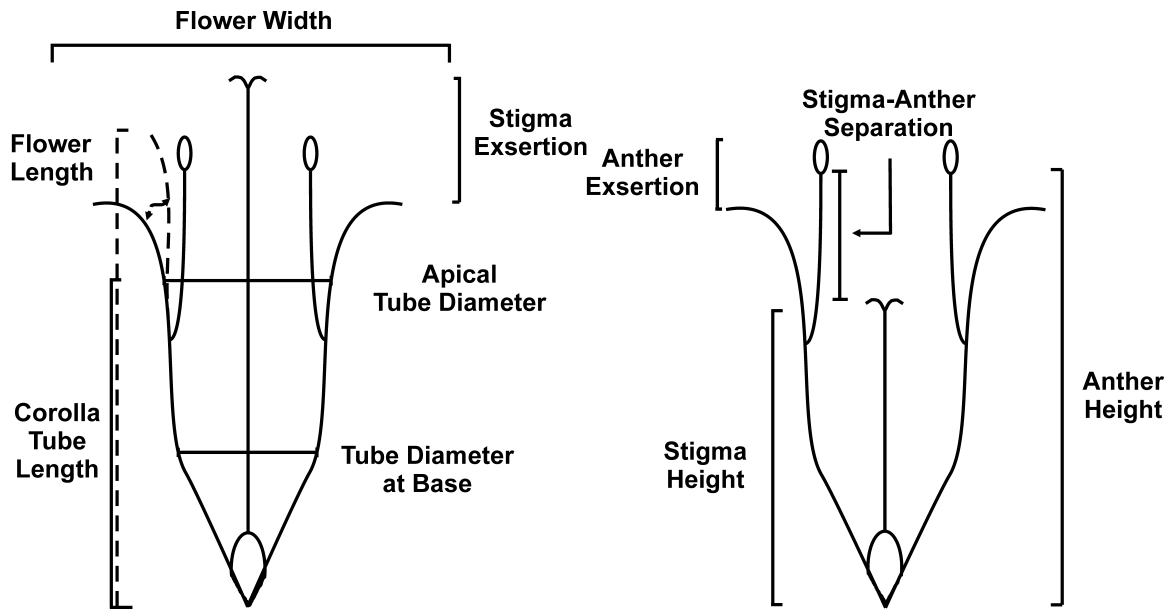
Figure 2.1 Diagram of approach (left) and reverse (right) herkogamous

flowers, with precise location of floral measurements.

To determine the narrow sense heritabilities and genetic correlations among

Polemonium brandegeei's floral traits, I used a greenhouse crossing experiment with

extreme and intermediate phenotypes. The parental generation consisted of 121

P. brandegeei plants representing 22 maternal families from seed collected at Lone Tree

Gulch, CO. I measured two flowers from the first two inflorescences produced by each

plant. All measurements were identical to those made in field populations. I chose 47

plants as parents, based on their style length. Parents with stigmas 1.50 mm or more

above the anthers were classified as "exserted." Plants with stigmas 1.50 mm or more

below the anthers were "recessed." Plants with stigmas between 1.50 mm and -1.50 mm

were classed as "intermediates;" these plants had an average stigma-anther separation of

0.75 mm. I performed a total of 36 reciprocal crosses that were roughly evenly

distributed among stigma-height categories, comprising both assortative and

disassortative crosses (Table 2.2). This crossing strategy has been shown to increase both

the power and precision of narrow-sense heritability estimates (Falconer and Mackay,

1996; Lynch and Walsh, 1998)

Table 2.2 Summary of 36 reciprocal crosses made based on stigma position relative to the
anthers, or stigma-anther separation (SA). A positive SA value indicates an exserted
stigma, while a negative SA value indicates a recessed stigma. Numbers in parentheses are
number of surviving families per cross type.

Stigma-Anther Separation (SA)	Recessed (≤ -1.50mm)	Intermediate (~ ± 0.75mm)	Exserted (≥ 1.50mm)
Recessed (≤ -1.50mm)	6 (5)		
Intermediate (~ ± 0.75mm)	6 (4)	6 (4)	
Exserted (≥ 1.50mm)	7 (6)	4 (3)	7 (5)

I planted eight seeds from each cross, 344 seeds in total. I chose the number of

offspring to plant using power-estimation curves (Lynch and Walsh 1998) to cover a

wide range of possible heritabilities, and an anticipated 75% germination rate. Due to a

lower than expected germination rate (~60%), and unexpectedly high greenhouse

temperatures, many individuals of the offspring generation died before flowering. A total

of 134 plants (average of 4.9 plants per cross) from the offspring generation reached

flowering, and flower measurements identical to those of the parental generation were collected.

Growing conditions were similar for the parental and offspring generation. Seeds were stratified at 4°C for 2-3 weeks in an incubator, then planted in plug trays, and moved to the greenhouse. Natural lighting was supplemented with sodium lamps for a photoperiod of about 14 hours, receiving 75-125 μmol of light. The temperature typically ranged from 22-30°C, but did reach temperatures > 35°C. I raised seedlings in a mixture of peat and Osmocote® Plus slow-release fertilizer (10:10:10) in conical Deepots^{TM} (800mL conical pot), and bottom watered by suspending the Deepots^{TM} in water holding trays. This allowed optimal water availability for the plants. The Deepots^{TM} containing the plants remained in the same greenhouse and conditions as the plug trays.

2.2.4 Statistical analysis of floral morphology

Before analysis, all morphological characters were tested for departures from normality with the Kolmogorov-Smirnoff test in SAS 9.1.2 (SAS institute 2004). Morphological data was made sufficiently normal by a log_{10} transformation, with the exception of stigma-anther separation. Since stigma-anther separation contained negative values, the constant 6 was added, and then the sum was square root transformed to meet the statistical assumptions of normality.

2.2.5 Phenotypic variation

Female and male flowers were compared with a Pearson's product moment correlation. I also determined the mean difference in stigma-anther separation between females and males. Since the difference between female and male flowers was slight (see Results), I averaged their measurements for subsequent analyses.

I used principal components analysis to summarize variation in floral design. I analysed a random sample of 50 plants from each of the three primary field populations, as well as from both greenhouse generations. The final ordination was run from a covariance distance matrix because all variables were on the same scale (Legendre and Legendre 1998). I performed the ordinations in CANOCO 4.52 developed by ter Braak and Schaffers (2004).

I reduced the original nine floral traits to five based on correlations among traits, the weighting of eigenvectors in preliminary analyses, and the probable function of strongly correlated traits in pollination (see below). The elimination of redundant, highly correlated variables from the ordination allowed us to examine the traits which explained the maximum amount of variation. Composite variables of floral traits were not used, as I sought to determine the influence of actual floral characters on overall floral variation. The traits analyzed were corolla-tube length (= tube length), apical-tube diameter (= tube diameter), style length, anther height, and stigma-anther separation.

Four of the final five traits were initially represented by two measurements that were strongly correlated. I retained apical-tube diameter over basal-tube diameter, because this trait is likely more important in pollination. When sex organs are positioned near the mouth of the tube, the apical diameter of the corolla tube determines the

proximity of a visitor's mouth parts to the anthers and stigma, and thus the probability of pollen removal and deposition. Similarly, corolla-tube length was retained over other measures of total corolla length because the "fit" between the length of the corolla tube and the length of a visitors mouth parts has been hypothesised to be functionally important (see Grant and Grant 1968). Finally the measures of sex organ length were retained over sex-organ exsertion, because style length and anther height were direct measurements of organ size.

I initially performed two separate ordinations to determine if plants grown in the greenhouse were morphologically similar to those from field populations. The first ordination consisted of only field plants, while the second contained only greenhouse plants. Both ordinations examined the same floral traits. Overall variation explained in the two analyses was nearly equal (92% for greenhouse and 93% for field plants), while the eigenvector loadings differed by no more than ± 0.05. Therefore, the field and greenhouse measurements were pooled into a single data set for the final PCA.

To determine if floral morphology differed significantly among the Taylor Canyon, Deer Mountain, and Vedauwoo populations, a multivariate analysis of variance (MANOVA) was conducted. When the MANOVA revealed significant differences in floral traits, I performed a canonical discriminant functions analysis to determine which traits contributed most strongly to differences among populations. The MANOVA analysis was performed in SAS 9.1.2 (SAS institute 2004), while the discriminant functions analysis was performed in CANOCO 4.52 (ter Braak and Schaffers 2004).

2.2.6 Genetic variation

I determined the narrow-sense heritabilities (h^2) and additive genetic correlations (r_a) with the program VCE REML version 5.1 (Neumaier and Groeneveld 1998; available at ftp.zgr.fal.de). I performed this analysis on the traits that explained a large amount of floral variation, as determined by the principal components analysis. VCE estimates variance components using restricted maximum-likelihood (REML) and pedigree information incorporating parent-offspring, as well as full- and half-sibling relationships. The REML approach is preferred to traditional variance partitioning (i.e. analysis of variance) because it makes no assumptions about crossing design and is robust to unbalanced and unconventional crossing designs (Shaw 1987; Falconer and Mackay 1996; Lynch and Walsh 1998).

I accounted for variation in flower size by including the dry weight (mass) of flowers as a covariate (cf. Robertson et al. 1994), although including mass as a covariate did not significantly alter the genetic parameters. This allowed us to detect variation in flower shape independently of variation in overall flower size. I assessed the significance of both the heritabilities and genetic correlations with one-and two-tailed one sample *t*-tests, respectively, with the standard errors produced by VCE. Significance thresholds were determined with the sequential Bonferroni correction factor to control for *Table*-wise type I error (Rice 1989).

I also calculated the coefficient of additive variation (evolvability) for each floral trait as $CV_a = 100\left(\dfrac{\sqrt{V_a}}{\bar{x}}\right)$, where V_a is the additive genetic variance and $\bar{x}$ is the trait mean (cf. Houle 1992). Houle (1992) has described CV_a as a more informative estimate of a trait's potential response to selection, because genetic variation is standardized by the

trait mean. Estimation of evolvability for stigma-anther separation was complicated by the fact that values ranged from positive to negative. As a result, a mean of zero is possible, which would result in an infinitely large CV_a. Therefore, I added the absolute value of the most negative value (5.26 mm) to each value of stigma-anther separation. This shifted the entire distribution of stigma-anther separations to the right of zero, and resulted in a conservative measure of CV_a.

2.3 RESULTS

2.3.1 Floral visitors

The visitation indices for each of the three primary populations varied with respect to year of observation, and visiting taxa (Figure 2.2). The two components of this visitation index, the total number of observed visits and the average number of plants visited per visiting bout, for each population are in Appendix 2.1. I observed consistently high visitation rates by hoverflies (Diptera) over three years of observations (Appendix 2.1). However, the mean number of plants visited per bout was low ($\bar{x} = 2$ across all three years and all three field populations), resulting in a lower visitation index (Figure 2.2). In addition, hoverflies collected pollen by hovering above the flowers, rarely contacting the stigma. This visitation behaviour would further reduce the potential for pollen movement by hoverflies. Bees (Hymenoptera) were also relatively frequent visitors, but usually only visited 2-4 plants. Beetles (Coleoptera), non-sphingid moths and butterflies (Lepidoptera) were infrequent visitors and contacted few flowers or plants per visit.
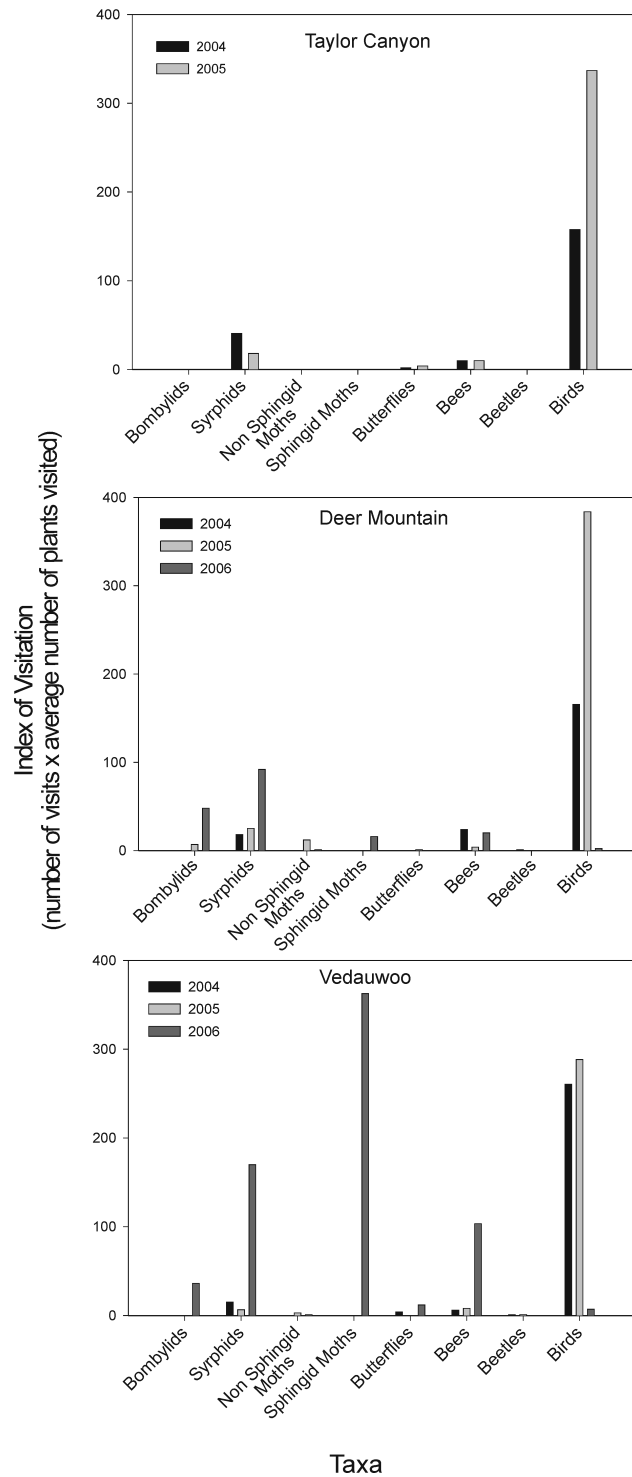
Figure 2.2   Visitation indices (=number of observed visitation x average number of plants visited per foraging bout) by eight major groups of visitors. Indices are presented across three flowering seasons, and three field populations (Deer Mountain, Taylor Canyon, Vedauwoo). I did not conduct floral visitor observations at Taylor Canyon in 2006.

Hummingbirds (*Selasphorus platycercus* Swainson; Trochiliformes) and

hawkmoths (*Hyles lineata* and *H. gallii*; Sphingidae) appear to be important pollinators

of *Polemonium brandegeei* (Figure 2.2). Hummingbirds were observed visiting multiple

plants of *P. brandegeei* in 2004 and 2005 in all three of the primary populations.

However, considerably fewer visits by hummingbirds were observed in 2006

(Figure 2.2). Hawkmoths had the highest visitation index in 2006 at Vedauwoo (Figure

2.2). I also observed a single hawkmoth at Deer Mountain, which visited 30 flowers.

Casual observations at Twin Sisters Mountain (also in Rocky Mountain National Park)

revealed nine additional hawkmoth visits in 2001, and two visits in 2006, indicating that

hawkmoths were active in the area. When present, hawkmoths visited many plants in

each foraging bout (Appendix 2.1), a pattern that provides ample opportunity for pollen

transport. My visual observations indicated that hummingbirds and hawkmoths

consistently contacted both sex-organs, making them likely vectors of pollen transport.

2.3.2 Phenotypic variation in floral morphology

The stigma-anther separation of female and male flowers was strongly correlated

with each other ($r = 0.910$, $P < 0.001$, $n = 83$), and displayed a mean difference of only

0.26 mm (standard error: 0.011). By contrast, stigma-anther separation ranged from -

2.77 to 5.80 mm in this group of plants. Therefore, I averaged the female and male

flowers for each individual plant for subsequent analyses (see below).

The final five measures of floral shape explained a large proportion of total floral

variation in the multivariate analysis (Figure 2.3). The first two eigenvalues (axes)

extracted from the analysis accounted for 93% of the total variation (69% and 24%, respectively). Style length and anther height were most closely associated with the first axis of variation, $r = 0.675$ and $r = 0.604$, respectively. The loadings for corolla-tube length and diameter indicated a general gradient of flower size and shape along the first principal component (PC1). Corolla tubes were short and narrow at one extreme, and long and wide at the other extreme (Figure 2.3). To confirm that PC1 reflected variation in size, I ran another ordination including dry mass. In this analysis, mass loaded onto PC1 fairly strongly ($r = 0.623$), and there was a 5% reduction in overall variation explained. The other traits loaded with a similar weighting as in the previous ordination. There were no groupings of discrete phenotypes, indicating that herkogamy and the other traits comprising *P. brandegeei*'s floral design were not polymorphic. However, the ordination did indicate that flowers from the parental greenhouse generation were generally smaller than flowers from the field (Figure 2.3).

	Axis 1	Axis 2
Tube Length	0.413	-0.226
Tube Diameter	0.085	-0.036
Stigma-Anther Sep.	0.033	0.786
Style Length	0.675	0.437
Anther Height	0.604	-0.372
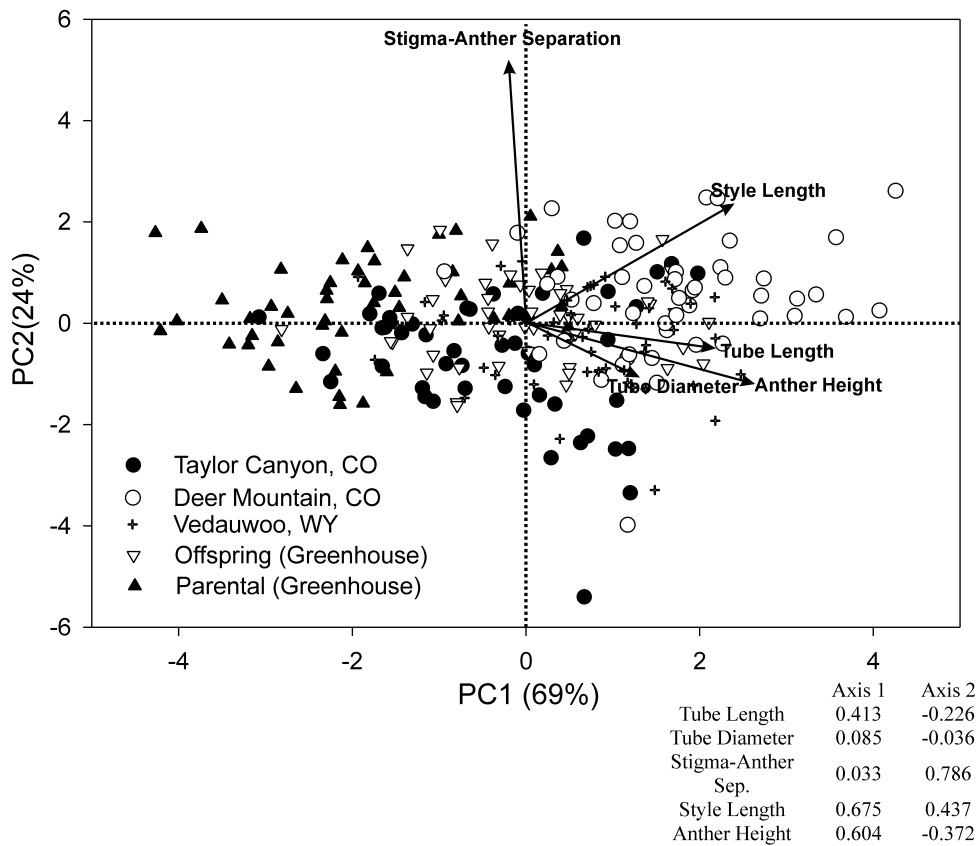
Figure 2.3   Ordination diagram from the first two principal components of 50 individuals of *Polemonium brandegeei* from each field site (Deer Mountain, Taylor Canyon, Vedauwoo) and both parental and offspring generations of greenhouse plants.   The original 9 floral traits have been reduced to five.   Tube Length is the length of the corolla tube, Tube Diameter is the apical diameter of the corolla tube, Stigma-Anther Separation (Stigma-Anther Sep.) is the separation between the anthers and stigma, Style Length is the length of the style, and Anther Height is the height of anther presentation from the base of the flower.   See Figure 1 for precise locations of measurements.   The first two principal components accounted for 69% and 24% (cumulative 93%) of the total variation, respectively.   Loadings of eigenvectors on both axes are also indicated in the bottom left of figure.

Stigma-anther separation (= herkogamy) displayed continuous variation, which was largely independent of variation in corolla size. This independence is reflected in a low correlation with the first axis of variation in the PCA ($r = 0.033$), and a high correlation with the second axis of variation ($r = 0.786$; Figure 3). Variation in stigma exsertion, which reflects style length, was correlated with the degree, and type of herkogamy, whereas anther exsertion (and anther height) was uncorrelated with herkogamy (Figure 2.4). This resulted from most anthers being positioned slightly below the opening of the corolla tube. Ranked plots of stigma-anther separation for the three primary field populations and greenhouse plants indicated that extreme values for approach and reverse herkogamy were roughly evenly distributed around zero (Figure 2.5). However, there were approximately 80 approach: 20 reverse herkogamous plants in each population (Figure 2.5). Means and standard errors for all floral traits in the three field populations are in Appendix 2.2.
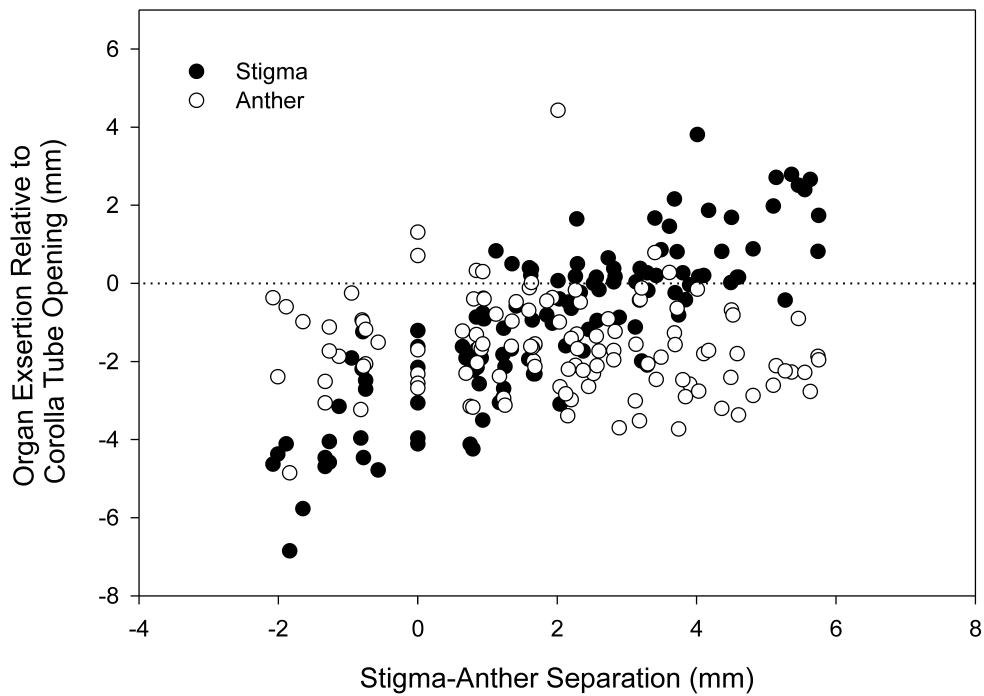
Figure 2.4 Variation in stigma and anther exsertion across the range of stigma-anther separations in the parental greenhouse generation. An identical pattern was observed in the offspring generation and field data (not shown). The stigma-anther separation ranges from reverse (negative SA values) to approach (positive SA values). Stigmas within ± 0.25 mm of anthers were approximated to zero.

Floral traits differed significantly among populations of *Polemonium brandegeei*, as determined through MANOVA (Wilk's $\lambda = 0.927$; $F_{16, 940} = 2.28$, $P = 0.003$). Therefore, I conducted a canonical discriminant functions analysis to determine which variables are best at distinguishing among the three analysed field populations (Taylor Canyon, Deer Mountain, and Vedauwoo). The discriminant analysis maximizes among population variance along the first discriminant axis, which in the present study accounted for 69% of the total variation. Along this axis, corolla-tube width and sex-

organ length were strongly correlated ($r = 0.597$-0.692). Corolla-tube length was primarily associated with the second axis of variation ($r = 0.652$), accounting for the remaining variation (31%). Therefore, the three field populations differed primarily with respect to corolla dimensions, rather than level of herkogamy ($r = 0.150$ for stigma-anther separation on the second discriminant axis). This result is reflected in similar distributions of herkogamy for each population (Figure 2.5).
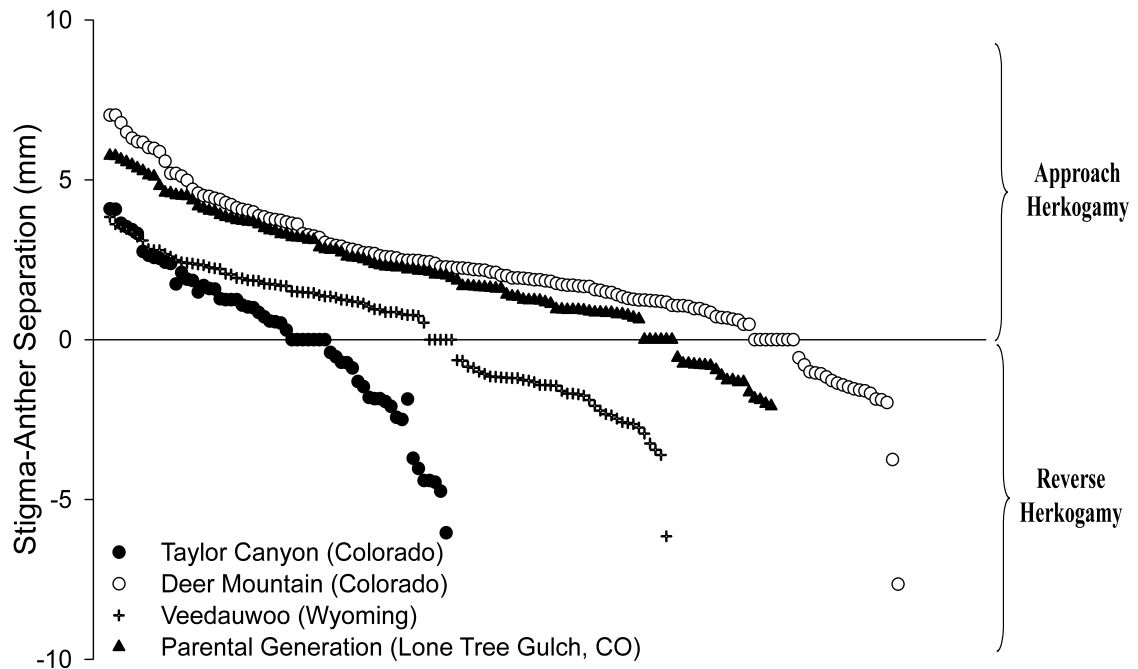
Figure 2.5 Ranked stigma-anther separation of three field populations and the parental generation of greenhouse grown *Polemonium brandegeei*. Stigma-anther separation (distance from top of the anthers to the bottom of the stigma) is ranked from highest to lowest distances. Highest ranking individuals are on the left of the horizontal axis, while the lowest rankings are on the right. Measurements above horizontal line are from approach herkogamous flowers, while those below the line are reverse herkogamous.

A graphical comparison of corolla-tube length and the mouth parts of pollinators indicated that most plants had tubes slightly longer than the mean *S. platycercus* bill length (mean of 35 male and 50 female bills = 17.85mm; Waser 1978). In contrast, the mean *H. lineata* proboscis length (38mm; Grant 1983) was well beyond the distribution of corolla tube lengths in *P. brandegeei* (Figure 2.6).
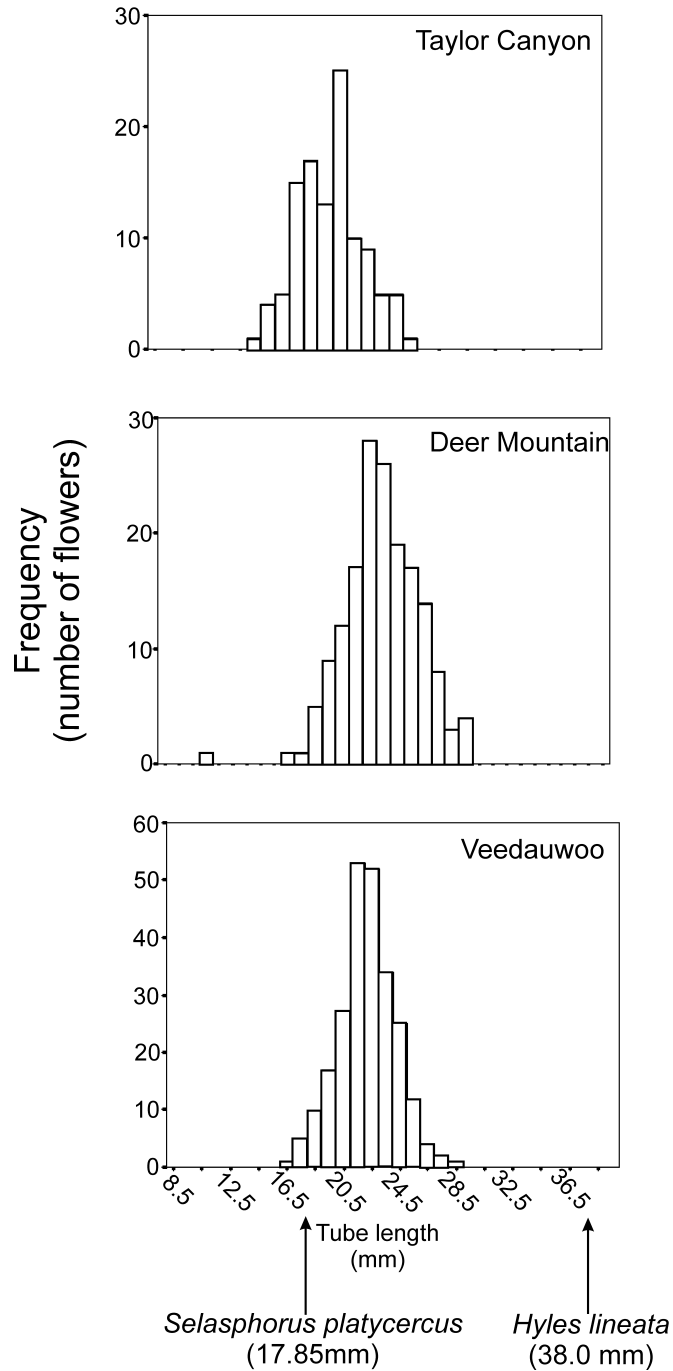
Figure 2.6   Frequency distribution of corolla tube length from three field populations of *Polemonium brandegeei*.   Bill length of *Selasphorus platycercus* (Waser, 1978) was averaged between two means for male and female birds (17.0mm and 18.7mm, respectively).   *Hyles lineata* proboscis length was from Grant (1983).

2.3.3 Genetic variation in floral morphology

All floral traits of *Polemonium brandegeei* exhibited heritable variation (Table 2.3). Heritabilities varied from very low (tube diameter: $h^2 = 0.044$) to high (stigma-anther separation: $h^2 = 0.851$). A similar pattern was observed in the additive genetic variance and coefficients of variation (Table 2.3). The largest additive variance was attributed to stigma-anther separation ($V_a = 4.636$), which coincided with a coefficient of variation four to twelve times greater than that of the other traits ($CV_a = 36.88$). The diameter and length of the corolla tube displayed the lowest additive variances, and the smallest coefficients of variation (tube diameter: $CV_a = 3.04$; tube length: $CV_a = 3.39$).

Table 2.3   Narrow-sense heritabilities (diagonal) and additive genetic correlations (above diagonal) of floral traits in *Polemonium brandegeei* with the effects of mass (flower size) removed as a covariate. Values in bold differed significantly from zero after a sequential Bonferonii correction was applied.

Tube Length	Tube Diameter	Style Length	Anther Height	Stigma-Anther Separation	
0.125[**]	**0.997**[***]	**0.309**[*]	-0.040	**-0.177**[***]	Tube Length
	0.044[**]	**0.314**[*]	0.005	-0.211[‡]	Tube Diameter
		0.293[***]	0.246	**0.442**[***]	Style Length
			0.238[**]	**-0.653**[**]	Anther Height
				0.851[***]	Stigma-Anther Separation

* P < 0.05, ** P < 0.01, ***P < 0.001
‡ indicates value that was significant before sequential Bonferonni correction

The additive genetic correlation between the two measures of corolla size (tube length and diameter) was high and positive ($r_a = 0.997$), whereas correlations involving sex-organs were more variable (Table 2.3). Style length showed moderate positive correlations with measures of corolla size (both $r_a \approx 0.3$), but anther height was not correlated with either corolla dimension (Table 2.3). Therefore, once variation in flower size (mass) was accounted for, variation of anther height appeared to be independent of corolla length and width. Corolla-tube length and diameter were negatively correlated with the separation of stigmas and anthers ($r_a \approx -0.2$). However, the correlation between tube diameter and stigma-anther separation was no longer significant after sequential Bonferonni correction.

Interestingly, stigma-anther separation was positively correlated with style length, but negatively correlated with anther height (Table 2.3). This association indicated that genotypes with longer styles and low anthers tend to display approach herkogamy (exserted stigmas), whereas genotypes with shorter styles and higher anthers displayed reverse herkogamy (inserted stigmas). These additive genetic correlations were consistent with respect to the magnitude and direction of the phenotypic correlations in the field and greenhouse plants (compare Figure 2.3 and Table 2.4).

Table 2.4 Mean trait values and standard errors for offspring generation, additive genetic variances (V_a), and evolvabilites (CV_a) for the offspring generation. Means are given in millimetres.

Floral Trait	Mean (SE)	V_a	CV_a
Tube Length	21.14 (0.16)	0.513	3.39
Tube Diameter	3.45 (0.04)	0.011	3.04
Style Length	21.80 (0.27)	2.757	7.62
Anther Height	21.36 (0.27)	2.899	7.97
Stigma-Anther Separation	5.84 (0.14)[*]	4.639	36.88

[*]Mean of adjusted value used to obtain CV_a actual mean (SE) of stigma-anther separation = 1.50 (0.14).

2.4 DISCUSSION

Herkogamy is a widespread adaptation that promotes efficient pollen transfer (Webb and Lloyd 1986). However, few studies have considered continuous variation in herkogamy, which is very likely the norm rather than the exception. Natural populations of *Polemonium brandegeei* displayed continuous variation in both the type and degree of herkogamy (stigma-anther separation). A ratio of approximately 80:20 approach to reverse herkogamous individuals occurred in all three study populations. Herkogamy in *P. brandegeei* was highly heritable, largely independent of flower age, and was primarily determined by variation in style exsertion. Based on visitation rates and behaviour,

hummingbirds and hawkmoths were likely important pollinators of *P. brandegeei*. These pollinators displayed marked annual fluctuations in their relative abundances over my three years of observations. The observed range of approach to reverse herkogamous flowers may reflect the combined effects of selection to reduce self-pollination and gamete wastage (Barrett 2002), and divergent selection on style length by hummingbirds and hawkmoths.

2.4.1 Floral visitors

The flowers of *Polemonium brandegeei* were visited by a morphologically diverse fauna. Although several types of visitors may transfer pollen among individuals of *P. brandegeei*, morphological and behavioural characteristics of both the animals and flowers likely reduced pollen transfer by flies, butterflies and beetles. Bees and syrphids visited on numerous occasions (especially Vedauwoo in 2006), but never visited more than four flowers, often on the same plant. In addition, syrphids rarely contacted both sex organs, further reducing the potential for pollen transfer. Observations of pollinator behaviour, number of visitations, and plants visited indicated that hummingbirds (*Selasphorus platycercus*) and hawkmoths (*Hyles lineata, H. gallii*) may be important pollinators of *P. brandegeei*. This is reflected in the calculated "visitation index" which accounts for the frequency and duration of visits by each taxon (Figure 2.2). Both hummingbirds and hawkmoths seem likely to have imposed selection on *P. brandegeei*'s floral morphology.

Hummingbirds were abundant in 2004 and 2005 and uncommon in 2006, whereas hawkmoths were only observed in 2006 mostly at Vedauwoo, but also at Deer Mountain

and Twin Sisters. The slightly later observation periods in 2006 (Table 2.1) may explain

the reduction in hummingbird observations during that year (Figure 2.2, Appendix 2.1).

However, my observations in 2004 and 2005 spanned almost the entire flowering season,

without observing hawkmoths. Therefore it is unlikely that hawkmoths were significant

pollinators during these years. Similar temporal variation in hawkmoth frequencies

(*Hyles lineata*) has been reported by other researchers (Campbell et al. 1997; Sime and

Baldwin 2003), and marked temporal fluctuations in frequency seem to be a feature of

hawkmoth biology (A.R. Westwood personal communication). Fluctuations in the

relative abundances of hummingbirds and hawkmoths would likely impose a

heterogeneous selection regime on the flowers of *P. brandegeei* (cf. Fenster et al. 2004).

2.4.2 Phenotypic variation in floral morphology

   Polemonium brandegeei did not exhibit polymorphic variation in stigma-height,

but rather continuous variation in both stigma-anther separation (=herkogamy) and

corolla-tube dimensions. The distribution of herkogamy was consistent among all

populations measured. Hawkmoths are thought to be associated with narrow corolla

tubes that cause the slender proboscis to contact a recessed stigma (Webb and Lloyd

1986). Hummingbirds select for wider tubes that allow full insertion of their bills so that

the exserted sex-organs deposit pollen on their foreheads (Grant and Grant 1965; Grant

and Grant 1968). In *P. brandegeei*, these phenotypes occur as extremes in a continuum

of corolla shapes. This variation may reflect contrasting selection by hummingbirds and

hawkmoths.

To further assess how well *Polemonium brandegeei* and other species with similar pollinators conform to the above expectations, I compiled a survey of published floral dimensions (Table 2.5). Both hawkmoth- and hummingbird-pollinated species displayed both approach and reverse herkogamy. However, hawkmoth-pollinated species tended to display inserted sex organs, whereas hummingbird-pollinated flowers had at least one exserted sex organ (Table 2.5). In *P. brandegeei*, the filaments are attached to the corolla tube so that the anthers are usually presented near the mouth of the tube. Approach herkogamy is required to achieve both separation of stigma and anthers, and the exserted style favoured by hummingbirds. Similarly, reverse herkogamy is required to separate the stigma and anthers, and to display the inserted stigma favoured by hawkmoths. Each herkogamous arrangement would reduce sexual-interference, while promoting efficient pollen transfer by one of these two pollinators. Published floral dimensions for species pollinated by both hummingbirds and hawkmoths are rare. However, existing data indicated a range of exserted to inserted sex organs similar to those observed in *P. brandegeei* (Baker 1964; Boyd 2002; Boyd 2004; Grant and Grant 1983; Wolfe et al. 2003).

Table 2.5 Summary of 19 (of 196) studies that reported at least two of: apical corolla diameter, tube length, stigma-anther separation, and sex organ exsertion.
included are two additional studies by Grant and Grant (1968) and Grant (1983). All studies reported visitation/pollination by hummingbirds and/or *Hyles lineata*
of Western North American plant taxa. Values given are population means or ranges among populations.

Taxa	Apical Corolla Diameter	Corolla Tube Length	Herkogamy (stigma-anther separation)	Sex Organ Exsertion/Insertion	Reference
Polemonium brandegeei	3.7 - 5.8 mm (mean = 4.90 mm)	18 - 29 mm (mean = 22.48 mm)	-7.5 - 8.3 mm (mean = 1.65)	Stigma: -5.39 - 7.82mm Anther: -0.85 - 3.72 mm	current study
Hummingbird Pollinated					
22 species	1.5 - 7.0 mm (mean = 3.85 mm)	10 – 33 mm (mean = 22.08 mm)	--	--	Grant and Grant, 1968
Ipomopsis aggregata	3.61 mm	25.01 mm	Reverse	Stigma: -1.20 mm Anthers: 0.21 mm	Grant and Wilkin, 1988 Campbell, 1996
Marginatocereus marginatus	6.0 mm	22.4 mm	14.86 mm[c]	Stigma: 0.50 mm[c] Anther: -14.28 mm[c]	Dar *et al*., 2006
Melocactus [a,b] *curvispinus*	2.76 - 3.17 mm	24.48 - 25.89 mm	0.58 - 1.78 mm	Stigma: slightly exs. Anther: inserted	Nassar and Ramírez, 2004
Mimulus cardinalis	3.90 mm	25-30 mm	6.0 mm [c]	Exserted	Grant and Grant, 1968 Bradshaw *et al*., 1998
Nicotiana [a] *glauca*	6.93 - 8.55 mm	35.17 - 42.59 mm	Approach	Exserted	Schueller, 2007
Penstemon *barbatus* [b]	6.2 mm	37.0 mm	Reverse	Exserted	Mitchell and Shaw, 1993
pinifolius [b]	4.0 mm	37.0 mm	Reverse	Exserted	Lange *et al*., 2000
centranthifolius [b]	4.94 mm	25.04 mm	--	Inserted	Lange *et al*., 2000

Hawkmoth Pollinated	Apical Corolla Diameter	Corolla Tube Length	Herkogamy (stigma-anther separation)	Sex Organ Exsertion/Insertion	Reference
28 species	--	10 - 170 mm (mean = 49.9 mm)	--	--	Grant, 1983
Datura stramonium	--	74.7 - 99.5 mm	-4.04 - 3.28 mm [d]	Stigma: ins. to exs. Anther: inserted	Motten and Stone, 2000
Diervilla lonicera	1.2 mm	7.4 mm	Reverse	At/near opening of corolla tube	Schoen, 1977
Ipomopsis tenuituba	2.6 - 2.8 mm	32.1 - 33.0 mm	Approach	Inserted	Grant and Grant, 1965 Campbell *et al.*, 1997
Mirabilis longiflora	2.0 mm	100 - 105 mm	Approach	1.5 - 2.0 mm	Grant and Grant, 1983
Phlox superba	-- "narrow"	26 - 33 mm	~ 0.0 mm	Inserted	Grant and Grant, 1965 Strakosh and Ferguson, 2005
Hawkmoth and Hummingbird Pollinated					
Istertia laevis	6.5 mm	41.0 mm	~ 9.0 mm[c]	Stigma: slightly exs. Anthers: inserted	Wolff *et al.*, 2003

[a] range of means reported from more than one population, [b] hummingbird/hawkmoth is not sole pollinator, but determined as a "primary pollinator", [c]Calculated from data given by cited study, [d]Variation related to selfing rate, "--" data not available

In contrast to the sex organs, the corolla tube in *Polemonium brandegeei* was more comparable to other species pollinated by hummingbirds than to species pollinated by hawkmoths (Table 2.5). Most *P. brandegeei* flowers had corolla tubes longer than *S. platycercus'* bill length (Figure 2.6). Therefore, hummingbirds pushed their heads deep into the flowers, thus increasing contact between the sex-organs and the forehead (Grant and Grant 1965; Grant and Grant 1968; Lertzman and Gass 1983). The proboscis of *H. lineata* is considerably longer than *Polemonium brandegeei's* corolla tube (Figure 2.6), and would likely transport pollen rather than the forehead or thorax (see Grant and Grant 1965). Therefore, *P. brandegeei*'s tube length may not be as critical under hawkmoth-pollination as under hummingbird-pollination.

The discriminant functions analysis indicated that the differences in floral design among the field populations were primarily in corolla dimensions, rather than in stigma-anther separation. Consistency in selection pressures may explain why the distribution of herkogamy was similar among all populations. The smallest flowers were in Taylor Canyon, and the largest in Deer Mountain, possibly reflecting a moisture or nutrient gradient among these three sites (see study site descriptions for details). Similarly, *Achillea millefolium*, *Hypochaeris radicata,* and *Polemonium viscosum* produced larger flowers in habitats with high soil moisture than in habitats with low soil moisture (Galen 2005; Lambrecht and Dawson 2007).

2.4.3 Genetic variation in floral morphology

       Significant heritable variation in the floral traits of *Polemonium brandegeei*

indicated a genetic basis to the phenotypic variation. Developmental effects contribute to

variation in stigma-anther separation (herkogamy) in some species (e.g., Lennartsson et

al. 2000). However, my comparisons of flowers in female and male phase eliminated this

possibility in *P. brandegeei*. In fact, herkogamy showed the highest heritability

measured (h^2 = 0.851; CV_a = 36.88). This value was higher than most published

heritabilities for herkogamy, which ranged from h^2 = 0.30 - 0.85 (see Shore and Barrett

1990; Robertson et al. 1994; Carr and Fenster 1994; Motten and Stone 2000; but see

Caruso 2004; Lennartsson et al. 2000). Similar evolvabilities (CV_a) were found by Carr

and Fenster (1994) for stigma-anther separation in *Mimulus guttatus* and *M. micranthus*

(CV_a = 27.7 and 33.4, respectively). However, the large observed variance in herkogamy

may reflect that herkogamy is composed of both style length and anther height measures.

Therefore, the variance of herkogamy may reflect the sum of variances from style length

and anther height, plus twice the covariance between style length and anther height

(Sokal and Rohlf 1995). Corolla-tube length and diameter exhibited the lowest levels of

additive genetic variation and evolvability (CV_a = 3.39 and 3.04, respectively).

       As discussed above (*Phenotypic Variation in Floral Morphology*), contrasting

selection pressures imposed by hummingbirds and hawkmoths could maintain the high

genetic variation in herkogamy, whereas stabilizing selection by hummingbirds may

reduce genetic variation in tube dimensions. It is also possible that gene flow from

Polemonium brandegeei's closest relative, *P. viscosum*, has introduced additional

variation in style length. This explanation seems unlikely because the populations

analysed in the current study are distant from populations of *P. viscosum* (75-100 km, plus ~600-800 m elevation), with the possible exception of the Deer Mountain population (approximately 12-15 km, plus ~400 m elevation). However, flower morphology and variation in stigma height in the Deer Mountain population is very similar to the other two populations (Figure 2.3 and 2.5). Furthermore, gene flow from *P. viscosum* would likely also affect other traits that differ between the species such as tube diameter, flower colour, and vegetative morphology.

High genetic correlations may reflect strong and consistent selection for the integration of floral traits (Herrera et al. 2002; Waitt and Levin 1998; Conner 1997) or constraints imposed by pleiotropy (Ashman and Majetic 2006). My study revealed high genetic and phenotypic correlations between tube length and diameter, whereas other genetic correlations involving corolla measurements were low to moderate (Table 2.3). The moderate positive correlation between tube diameter and style length results in phenotypes more suited to hawkmoths (narrow tubes, short styles) or hummingbirds (wider tubes, longer styles). Similar phenotypic correlations have been found in other studies of plant taxa pollinated by hummingbirds and hawkmoths (Campbell 1989; Grant and Temmels 1992). However, the strong positive correlation between tube width and length indicates that selection for wide tubes will also result in long tubes. Thus, the correlation may slow the evolution of optimal phenotypes for hawkmoths (narrower, longer tubes) and hummingbirds (wider, shorter tubes; cf. Conner 2006).

Plants grown under greenhouse conditions often display slight differences in floral traits compared to those from field populations. For example, my field populations had slightly larger flowers than greenhouse plants. This could result from the greenhouse

plants being derived from a different population than those used for field measurements. Alternatively, this size difference could be due to environmental conditions differing between the field and greenhouse.

Greenhouse-based estimates of genetic parameters may overestimate heritabilities and genetic correlations due to reduced environmental variation (Conner et al. 2003). I cannot rule out this possibility from my study. However, levels of total phenotypic variation were comparable in the field and greenhouse (Figure 2.3 and 2.5, Appendix 2.2), indicating that heritabilities may also be comparable. Similarly, genetic and phenotypic correlations within the present study were consistent in magnitude and direction, as the phenotypic correlations among greenhouse and field populations (compare Figure 2.3 with Table 2.3). Therefore, extreme bias in my estimation of genetic parameters of greenhouse-grown plants seems unlikely (for a discussion of the similarity of phenotypic and genotypic correlations see Waitt and Levin (1998)).

2.4.4 Implications for floral evolution

Variation between approach and reverse herkogamy in *Polemonium brandegeei* (Figure 5) seems likely to reflect contrasting selection imposed by hummingbird and hawkmoth pollinators. The continuous variation of style length in this species means that many individuals display an intermediate phenotype, with the stigma either slightly above or below the anthers (Figure 2.4 and 2.5). Recent debate about the prevalence and importance of specialized pollination syndromes (Waser et al. 1996; Wilson et al. 2004) has led to a reassessment of shifts between pollinators and the presence of apparently "intermediate" floral phenotypes (see Fenster and Marten-Rodriguez (2007) for a

discussion regarding self-compatible species). The intermediates in *P. brandegeei* may represent a compromise between two extreme phenotypes, which are best suited to hummingbird or hawkmoth pollination (see Campbell and Aldridge 2006). Alternatively, it is possible that the optimal stigma-anther separation is near zero. I plan to test these possibilities by quantifying selection imposed by hummingbirds and hawkmoths on *P. brandegeei* flowers. I expect a negative correlation between fitness and herkogamy under hawkmoth pollination, and a positive correlation between fitness and herkogamy under hummingbird pollination.

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Appendix 2.1. Total number of observed visitations (top three panels) and mean number of plants visited per foraging bout of eight major visitor groups of *Polemonium brandegeei*. Data is presented for the three analyzed field populations (Taylor Canyon, Deer Mountain, Vedauwoo), for three flowering seasons (2004-2006). Observations were not made for Taylor Canyon in 2006.
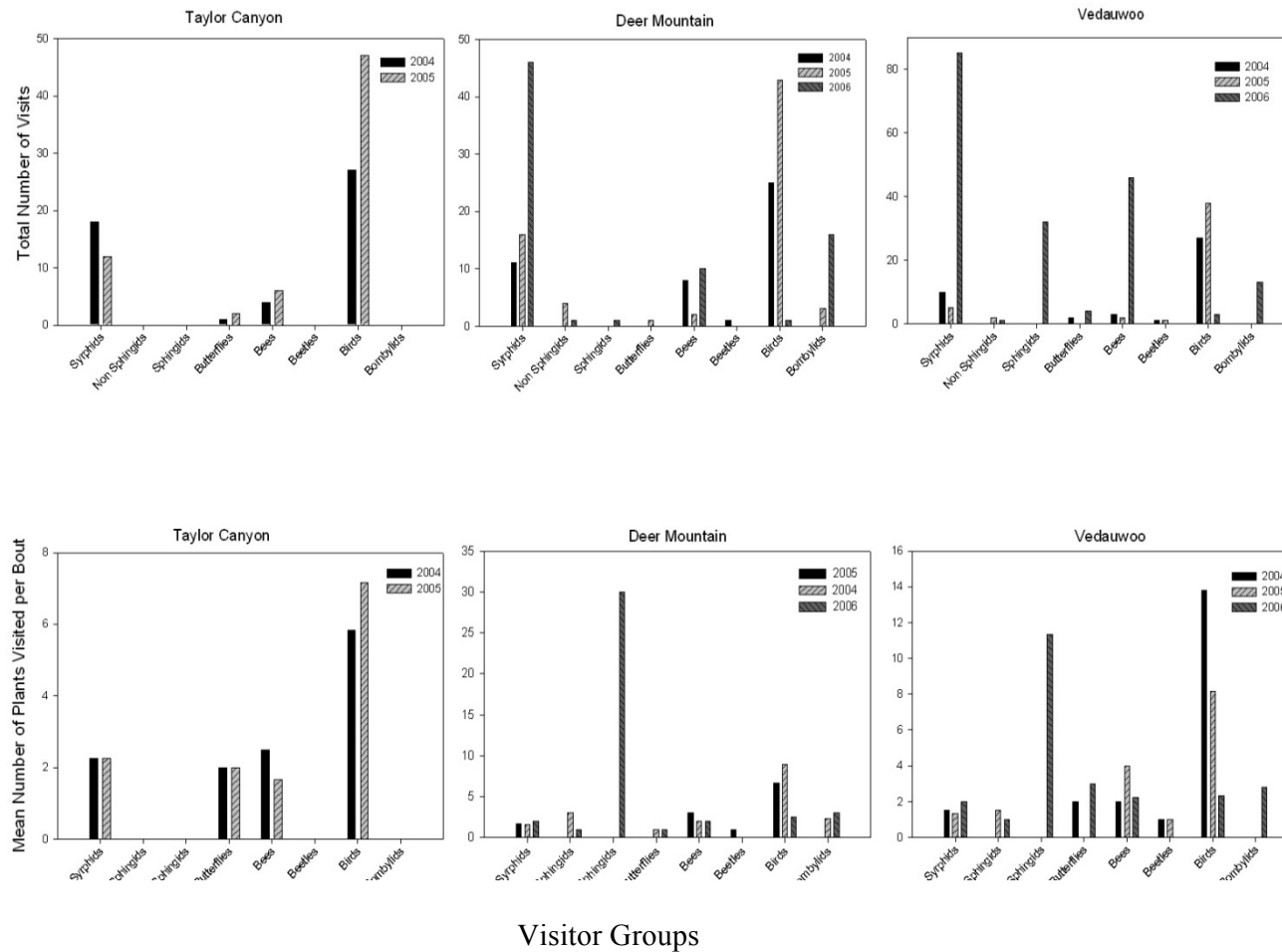
Appendix 2.2. Mean and standard errors (in parentheses) of *Polemonium brandegeei* floral measurements by population. See Figure 1 for precise location of measurements. All measurements were in millimeters.

Trait	Population		
	Taylor Canyon $N = 110$	**Deer Mountain** $N = 164$	**Vedauwoo** $N = 243$
Flower Width	14.25 (0.20)	16.17 (0.20)	17.80 (0.17)
Flower Length	27.66 (0.28)	32.67 (0.27)	30.60 (0.17)
Tube Length	20.85 (0.22)	24.44 (0.21)	22.16 (0.13)
Basal Tube Diameter	2.36 (0.11)	2.39 (0.08)	2.92 (0.03)
Apical Tube Diameter	4.65 (0.14)	5.04 (0.09)	5.02 (0.05)
Stigma-Anther Separation	1.61 (0.23)	2.05 (0.18)	1.30 (0.18)
Style Length	22.32 (0.33)	28.50 (0.45)	24.98 (0.22)
Anther Height	22.27 (0.30)	26.53 (0.27)	25.67 (0.15)

CHAPTER 3. PATTERNS OF POLLEN REMOVAL AND DEPOSITION IN *POLEMONIUM BRANDEGEEI* (POLEMONIACEAE) OVER TWO FLOWERING SEASONS, IN MULTIPLE NATURAL POPULATIONS

3.0 CHAPTER SUMMARY

The shape, size, colour, and arrangement of floral parts within flowers (collectively floral design) have evolved largely to promote mating success via animal-mediated pollen transfer. I studied the timing (diurnal or nocturnal) of pollen transfer in *Polemonium brandegeei*, and how floral traits affected the movement of pollen. I quantified the number of pollen grains remaining in anthers (pollen removal) and deposited on stigmas (pollen deposition) of plants receiving visits during either the day (7:30 am – 8:00 pm) or night (8:30 pm – 7:30 am) in natural populations over two flowering seasons. I used general linear models to analyze the effects of floral traits on pollen movement. Pollen removal and deposition occurred both diurnally and nocturnally, with more pollen movement occurring diurnally with increased floral visitations in 2006. This increase in pollen movement may be attributed to increased activity of small-bodied insects, and increased deposition of self pollen in warm weather. Nonlinear effects of style length significantly affected pollen removal, with more pollen being removed from flowers with intermediate style lengths. Pollen deposition was slightly more complex, with herkogamy and anther height affecting pollen deposition. Overall, pollen movement was variable between years and populations, but sex organs consistently influenced the removal and deposition of pollen grains.

3.1 INTRODUCTION

The movement of pollen among individual plants is essential for reproduction in self-incompatible flowering plants. Multiple factors influence successful pollen transfer. First, in animal pollinated species, attracting adequate numbers of visitors is important to ensure sufficient amounts of pollen transfer for seed set. Second, effectiveness of pollen transfer varies among animal visitors. For example, some insect visitors actively collect and consume pollen that is required for plant reproduction (Hargreaves et al. 2012; Harder and Routley 2006). Third, pollen must be placed on a region of the visiting animals body where it can be deposited on receptive stigmas of other flowers. Fourth, deposition of self -pollen can also affect the reproductive output of flowering plants (Karron and Mitchell 2012). Self-pollen deposition can occur autonomously or because pollinators transfer pollen from other flowers on the same plant, or within the same flower. All of the above components of pollen transfer are influenced by abiotic factors, such as temperature and wind speed (Anderson and Johnson 2007).

Many flowering taxa experience temporal and geographic variation in the relative abundance and composition of pollinators (Herrera et al. 2006; Johnson 2006). As a result, patterns of pollen removal and deposition can vary widely across flowering seasons and geographically separate populations. For example, wide variation in the rate of pollen deposition occurred across 6 populations and 7 flowering seasons in *Ipomopsis aggregata* (Price et al. 2007). In addition, considerable variation occurred within populations over the course of a single flowering season due to changes in the relative abundance of pollinators and less effective floral visitors (Price et al. 2007). Similarly, variation in the abundance of diurnal bird and nocturnal hawkmoth pollinators of *Isteria*

laevis contributed to increased variation in female reproductive success (Wolff et al. 2003). In some species, variation in the effectiveness among a diverse and variable assemblage of floral visitors can introduce variation in the removal and deposition of pollen (Adler and Irwin 2006). This variation in effectiveness can even lead to a discordance between the amount of pollen removed and deposited (Wilson and Thomson 1991). However, other studies have found more consistent assemblages of pollinators, which may provide a more consistent selective environment for floral traits (Sahli and Conner 2007). Therefore, effects of time and location on pollen movement likely vary among species, but the quantification of basic patterns provides initial insight into selection on floral design.

In the present study, I examined the effects of floral and inflorescence variation on the removal and deposition of pollen over two consecutive flowering seasons, in natural populations of *Polemonium brandegeei* (A. Gray) Greene. My objectives were to determine the timing (day versus night) of pollen movement (pollen removal and deposition), and examine the effects of floral traits on pollen removal and deposition under natural pollinator and floral visitor assemblages.

The following predictions are based on my descriptions of floral variation in *Polemonium brandegeei*, and documented associations between floral traits and pollinators from the literature (Chapter 2). I predicted that (1) when daytime pollinators (particularly hummingbirds) were abundant, pollen transfer would primarily be diurnal. (2) In contrast, when nocturnal or crepuscular pollinators (hawkmoths) were more abundant, pollen transfer would occur during the evening/night. Further, (3) I expected more pollen to be removed from flowers with long-corolla tubes, and more pollen to be

deposited on flowers with exserted (approach herkogamy) stigmas under hummingbird visitation. (4) Under hawkmoth pollination, I expected flowers displaying recessed stigmas would have more pollen removed and deposited. (5) Pollen-collecting insect visitors were predicted to effectively remove pollen, but contribute little to pollen deposition.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design and data collection

I quantified pollen removal and deposition among individuals of *Polemonium brandegeei* in natural populations. To determine the timing of pollen transfer, I manipulated when plants could be visited. The 2004 flowering season consisted of a pilot study, that was refined for the 2005 and 2006 flowering seasons. All three of the study seasons coincided with the floral measurements and observations of flower visitations presented in Chapter 2.

The first step in quantifying pollen movement was to determine the timing (i.e. diurnal versus nocturnal) of pollen removal and deposition. I performed a series of pollinator exclusion experiments (Table 3.1). These experiments included three manipulative treatments of individual plants which differed in when plants were exposed to pollinators: *Day* plants were exposed to day-flying floral visitors from 7:30 am to ~8:00 pm (daylight hours) while the remaining time they were enclosed within a tightly woven mesh bag to prevent visitation. *Night* plants were exposed to potential pollinators from ~8-8:30 pm to 7:30 am (included dusk and dawn), and covered for the remaining hours. *Control* plants remained covered over the entire course of the exclusion trial, and

provided data on self-pollen deposition due to contact between stigma and anthers or plant movement. The exclusion trials lasted 48 hours, therefore each *Day* and *Night* treatment-plant was covered and exposed twice.

In 2004 and 2005 exclusion trials were performed in Taylor Canyon and Deer Mountain, Colorado, and Vedauwoo, Wyoming. In 2006 I performed exclusion trials in the Deer Mountain and Vedauwoo populations. During the 2004 pilot season, mesh bags were loosely fitted around experimental inflorescences, to prevent visitation. However, this technique was abandoned after concern that the bags were catching the wind, and knocking pollen out of the experimental flowers. In 2005 and 2006, a mesh teepee fitting around the entire plant was used to exclude floral visitors, reducing the effect of wind. Further, I ensured that a balance between approach and reverse herkogamous plants were included to compare the patterns of pollen movement in these floral designs. I present data and analyses from the 2005 and 2006 study seasons below.

Two flowers on each experimental plant were designated as "focal flowers" and marked with acrylic paint just prior to opening. After the 48-hour period of the exclusion trial was complete, all anthers from each focal flower were stored in 70% ethanol and the stigmas mounted in fuschin-stain jelly on a microscope slide (Beattie 1971) for later quantification. Pollen remaining in anthers was used as an indicator of pollen removed. The numbers of grains remaining in anthers (pollen removal) and deposited on stigmas were averaged from both focal flowers for analysis.

To examine how variation in floral traits affected pollen removal and deposition, I measured floral dimensions in both a female-phase (stigma open and receptive) and male-phase flowers (anthers dehisced) on the same inflorescences as the focal flowers. I

measured corolla tube-length, tube-width at the base and apex of the flower, height of sex-organ presentation relative to the base of the flower, and flower mass (dry weight of corolla, calyx, androecium and gynoecium; see Figure 2.1 for precise locations of measurements). I also measured several aspects of floral display, including: the number of inflorescences and number of open flowers per inflorescence, and the number of flowers in female and male phase.

Table 3.1   Sample sizes for pollinator exclusion experiment for 2004-2006 field season. Field seasons 2004 and 2005 included data from all three field populations (Taylor Canyon, Deer Mountain, and Vedauwoo), while only two populations (Deer Mountain and Vedauwoo) were visited in 2006. Note: "intermediate" (-0.5 to 0.5 mm) stigma-anther separations are included in *Approach* category.

		2004	2005		2006	
	Herkogamy:	*No Distinction*	*Approach*	*Reverse*	*Approach*	*Reverse*
Treatment	**Control**	41	71	12	19	9
	Day	60	66	52	14	14
	Night	60	74	37	16	12

Pollen grains were dislodged from the anthers by placing them in a sonicator for 5 minutes. The amount of pollen remaining in anthers was determined with a Multisizer III particle counter (Beckman Coulter). Anthers from each flower were suspended in 25 mL of electrolyte solution (Isotone II®), and three 500 μL subsamples were averaged for the final estimate. Pollen deposition was quantified by manually counting fuschin-stained

grains under light magnification with the aid of image capturing software (Image-Pro express version 4.5.1). All pollen grains on the slide were counted, including those along the sides of the style, on the stigma, and those dislodged from the style. Therefore, my quantification of pollen deposition reflects both self and imported pollen.

Abiotic factors may affect pollen movement directly, or through effects on pollinator activity. I calculated the average high and low temperatures, and average wind speed for the three study populations. The temperature and wind speeds for Taylor Canyon and Vedauwoo were obtained from the Weather Underground database (www.weatherunderground.com, accessed January 17, 2012), and the Almanac database for Deer Mountain (www.almanac.com, accessed January 17, 2012).

3.2.2 Statistical analyses

Pollen removal and deposition was analyzed using general linear models (Proc GLM) in SAS v 9.2.1. The mixed model procedure (Proc MIXED), which uses a restricted maximum likelihood approach, was also used. However, both techniques gave identical results; therefore, the more straightforward general linear models are presented.

Pollen remaining in the anthers and pollen deposited on stigmas were analyzed as dependent variables. Treatment type (Control, Day, Night) was a categorical fixed effect, while population (Deer Mountain, Taylor Canyon, Vedauwoo) was a categorical random effect. Separate analyses were conducted for each year of the study because one population was not examined in 2005. Floral and inflorescence characters were added to the model as covariates as both linear and quadratic terms. I also explored all two-way interactions among covariates, and class variables and covariates. A manual reverse-

stepwise elimination process was employed to remove nonsignificant interaction terms, and covariates (Sokal and Rohlf, 1994). Finally, least-squares means were calculated and compared via *t*-tests to examine differences among treatment and population effects. To meet assumptions of normality and stabilize variances, data for the pollen remaining in anthers and pollen deposited on stigmas were square-root transformed.

To examine the influence of significant covariates on pollen removal and deposition, estimates of the slope of relationships between the covariate and dependent variable were obtained. In many analyses, more than one covariate and or class effect was significant. To best portray the influence of specific covariates on pollen removal or deposition, I adjusted the data in my figures to account for significant effects not portrayed in the figure. Predicted values were first generated with partial regression coefficients and observed values of the focal covariate. The partial regression coefficients and mean values of the remaining significant covariates were also included in the calculation of predicted values. Residuals from the model were then added to the predicted values to generate adjusted values (cf. Worley and Harder 1996).

Outlier values were found through preliminary exploration of the data. An extremely small value for anther height was recorded in the Deer Mountain population (< 10.0 mm), and had a disproportionate influence on the relationship between anther height and pollen deposition. The removal of this single data point changed the relationship from positive to negative. As this value was well outside the distribution of anther height from all three populations, over three years of measurements, I present analyses and results without the extreme value of anther height. Similarly, an individual with 23 inflorescences was removed from the Vedauwoo population, that was well

beyond the maximum of 15 inflorescences found across all other individuals. Finally, one individual with an unusually large number of flowers (39) in Deer Mountain was removed.

3.3 RESULTS

3.3.1 *Environmental conditions*

The dates each population was visited, and the environmental data over those periods are presented in Table 3.2. The average maximum temperature and wind speed varied among years and populations. The warmest study period overall was in 2006, with the highest average temperature occurring in Deer Mountain. The fastest average wind speed occurred in Vedauwoo, during the 2006 flowering season.

Table 3.2. Average maximum and minimum temperature, and average wind speed for dates visited for three study populations. See Chapter 2 for detailed locations and habitat information of study populations.

Population	Dates Visited	Average Maximum Temperature (°C)	Average Minimum Temperature (°C)	Average Wind Speed (km/hour)
Deer Mountain, CO	June 1 – 11, 2005	24.9	7.8	11.9
	June 6 – 12, 2006	34.4	13.4	13.0
Taylor Canyon, CO	May 25 – 31, 2005	20.9	1.6	6.3
Vedauwoo, WY	June 12 – 19, 2005	21.8	6.3	19.1
	June 12 – 17, 2006	28.2	8.4	21.2

3.3.1 Pollen removal - pollen grains remaining in anthers

I used the amount of pollen remaining in anthers as an indication of pollen removal. Plants with less pollen in their anthers were assumed to have experienced greater removal. Similar patterns of pollen removal occurred in the 2005 and 2006 flowering seasons. In both years significant differences occurred among all three treatments (Table 3.3), with the greatest removal occurring during the day and the least in control plants (for all pairwise comparisons, $t > 2.5$, $P < 0.01$, Figure 3.1). Considerably higher pollen removal was observed in 2006 compared to 2005, especially in the day and night treatments (Figure 3.1). These results supported my prediction that greater pollen removal would occur during peak floral visitation periods (see Chapter 2).

Pollen removal differed among populations only in 2005 (Table 3.3). Pollen removal was greatest in Taylor Canyon, intermediate in Deer Mountain, and lowest in Vedauwoo (for all pairwise comparisons, $t > 3.2$, $P < 0.001$, Figure 3.1). The variation in pollen removal among populations was not initially predicted.

In both years, style length was the only floral trait to significantly influence pollen removal. Both linear and quadratic terms of style length were significant, and the effect of style length did not differ significantly among populations or treatments. While I predicted that sex-organ position would affect pollen removal, I did not expect plants displaying intermediate values of style length to have the least amount of pollen remaining in anthers (Figure 3.2).

Table 3.3 General linear model describing the effects of population, treatment, and floral traits on pollen removal (pollen remaining in anthers) in *Polemonium brandegeei*, during the 2005 and 2006 field seasons.

Effect	2005	2006
Population (random)	$F_{2, 301} = 32.06***$	$F_{1,75} = 1.57$
Treatment	$F_{2, 301} = 17.84***$	$F_{2, 75} = 36.98***$
Population ×Treatment	$F_{4,301} = 1.85$	$F_{2,75} = 2.46$
Style Length	$F_{1, 301} = 5.89*$	$F_{1, 75} = 12.00**$
(Style Length)^2	$F_{1, 301} = 6.29*$	$F_{1, 75} = 11.49**$
R^2	0.30	0.61

* $P< 0.05$, **$P<0.01$, ***$P<0.0001$
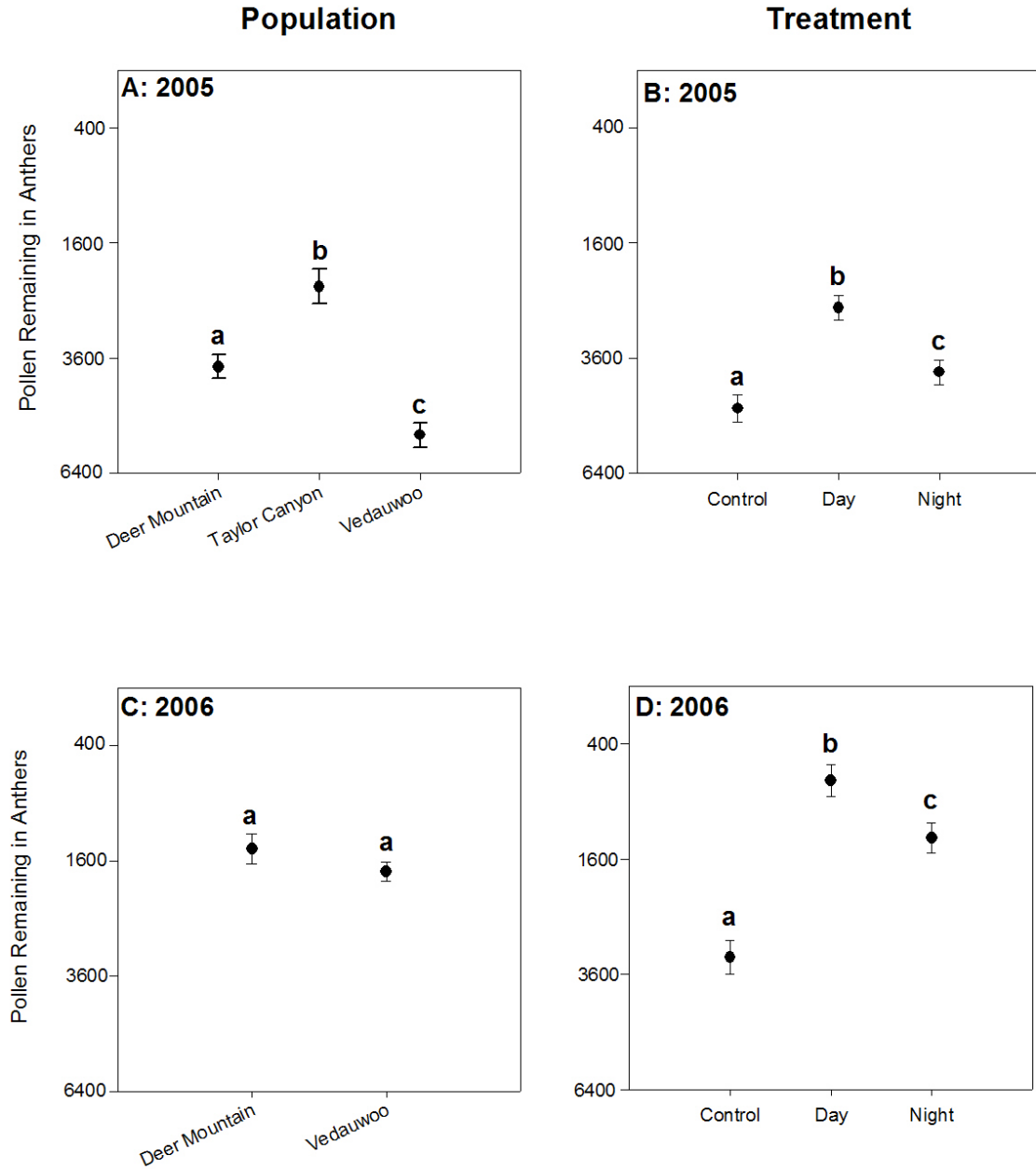
Figure 3.1 Square-root pollen remaining in anthers across populations (left panels) and treatments (right panels) for 2005 (A and B) and 2006 (C and D) flowering seasons. Data points are least-squares means, error bars represent ± 1 standard error. Data points denoted by different letters are significantly different at $\alpha = 0.05$. Note inverted axes describing pollen remaining in anthers.
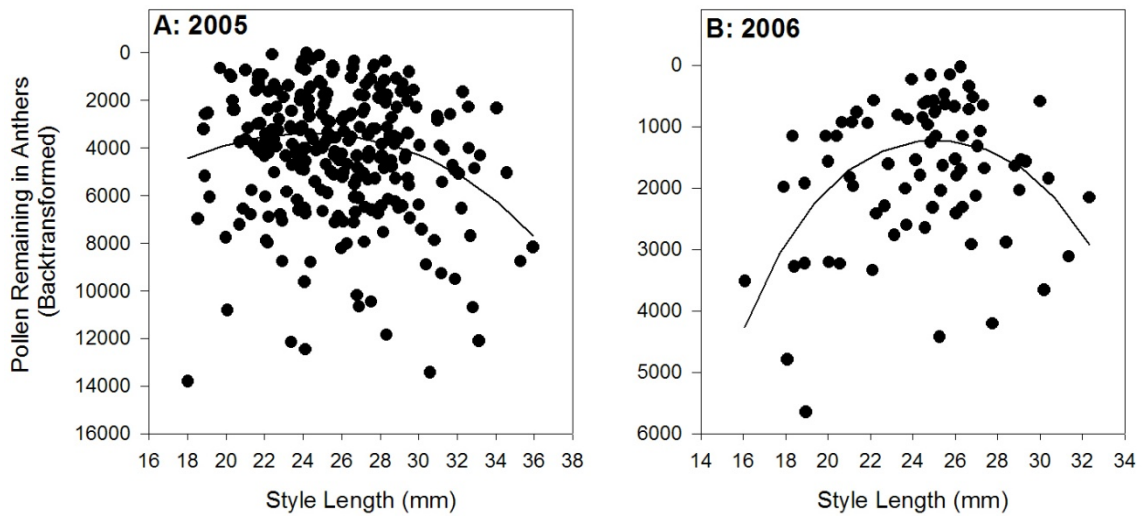
Figure 3.2 Effect of style length on pollen removal (pollen remaining in anthers) in *Polemonium brandegeei* during the 2005 (panel A) and 2006 (panel B) flowering season. Data points represent adjusted values, and lines represent predicted values. Estimate of b (SE) for 2005: style: -7.49 (4.24), style^2: 0.16 (0.07). Estimate of b (SE) for 2006: style: -18.86 (4.69), style^2: 0.38 (0.09) Note difference in scale between pollen remaining in anthers from 2005 and 2006, and inverted axis of Pollen Remaining in Anthers.

3.3.2 Pollen deposition – pollen grains deposited on stigmas

Factors affecting pollen deposition were different in each year (Table 3.4). Pollen deposition in 2005 was significantly different across treatments, populations, and the differences among treatments varied across populations (population × treatment interaction). Herkogamy and anther height also influenced pollen deposition in 2005. Although overall levels of pollen deposition were comparable between years (see Figure 3.3), pollen deposition in 2006 was only influenced by herkogamy, and the overall model was marginally nonsignificant ($P = 0.056$).

In 2005, I detected differences in pollen deposition across populations and treatments. Overall, I observed greater pollen deposition in Taylor Canyon, in the day and night treatments relative to the unvisited controls. In these same treatment types, Deer Mountain had intermediate pollen deposition, and Vedauwoo exhibited the least amount of pollen deposition (for all pairwise comparisons, $t > 2.3$, $P < 0.05$). These general differences paralleled those observed for pollen removal. However, all three populations displayed comparable stigma loads on day treatment flowers (all $t > -1.35$, $P > 0.17$; Figure 3.3). Day and night treatments differed from controls in Deer Mountain and Vedauwoo (all $t > -3.92$, $P < 0.0001$), but not in Taylor Canyon. Rather, only the night treatment differed in Taylor Canyon (all $t > -3.04$, $P < 0.04$). In 2006, I did not detect any significant differences among populations or treatments with respect to pollen deposition (Table 3.4).

Table 3.4 General linear model analysis of floral traits affecting pollen deposition in *Polemonium brandegeei*, during the 2005 and 2006 field seasons.

Effect	2005	2006
Population	$F_{2, 301} = 8.16**$	$F_{1, 75} = 0.53$
Treatment	$F_{2, 301} = 35.75***$	$F_{2, 75} = 0.31$
Population ×Treatment	$F_{4, 301} = 4.15**$	$F_{2, 75} = 0.63$
Stigma-Anther Separation	$F_{1, 301} = 7.30**$	$F_{1, 75} = 8.30*$
(Stigma-Anther Separation)^2	$F_{1, 301} = 15.62***$	--
Anther Height	$F_{1, 301} = 0.86$	--
Anther Height × Population	$F_{2, 301} = 8.04**$	--
(Stigma-Anther Separation)^2 × Treatment	$F_{1, 301} = 5.65**$	--
R^2	0.41	0.15

*$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$
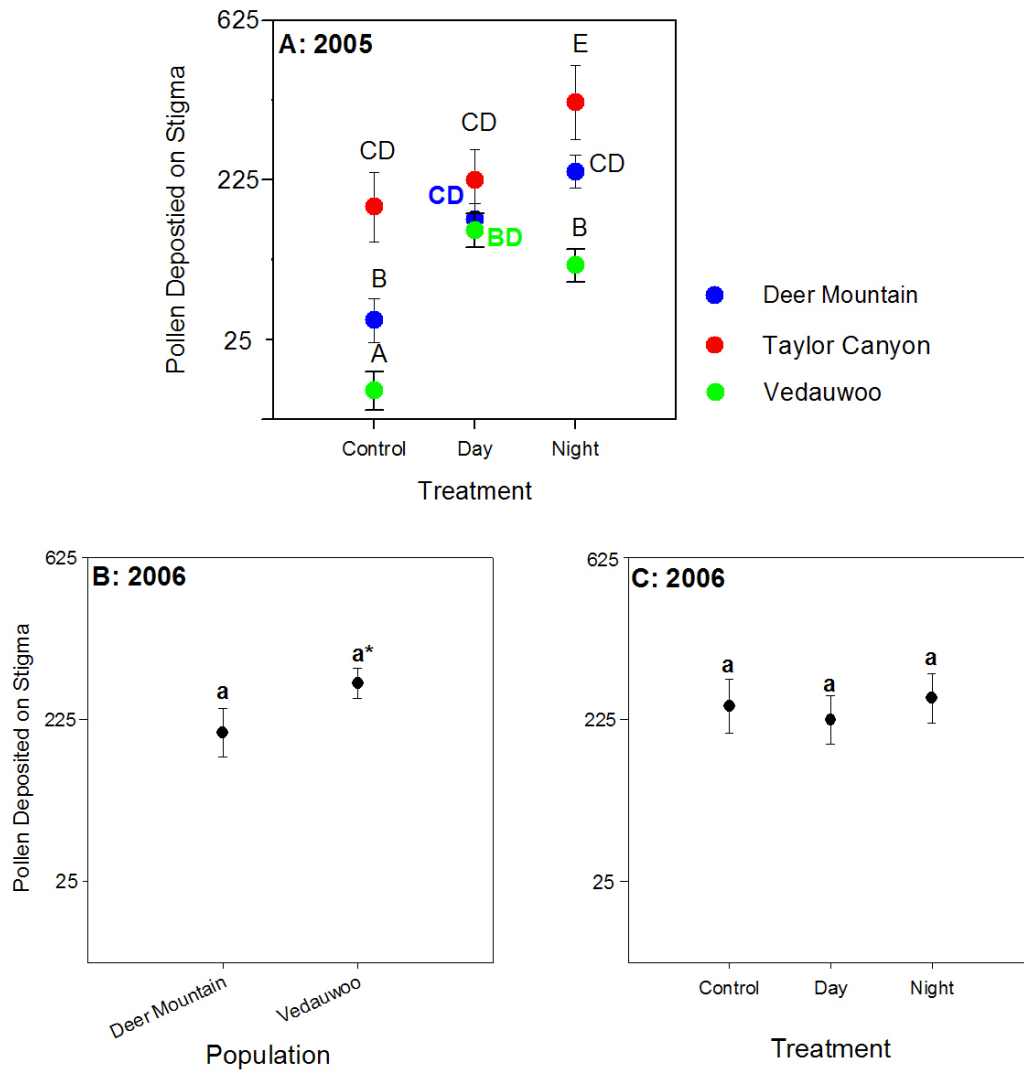
Figure 3.3 Least square mean comparison of pollen deposition across populations and treatments in 2005 (Panel A). Pollen deposited on stigmas across populations (Panel B) and treatments (Panel C) in 2006. Data points are least-squares means, error bars represent ± 1 standard error. Data points denoted by different letters are significantly different at α = 0.05. The contrast denoted with * was marginally nonsignificant ($P = 0.0936$).

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The effect of herkogamy on pollen deposition changed between 2005 and 2006. The function describing the effects of stigma-anther separation in 2005 was quadratic in day and night treatments and linear in the control treatment. However, in 2006, the effect of herkogamy was linear in all three treatments (Figure 3.5). Therefore, in 2005 slightly recessed stigmas received the greatest pollen loads, while in 2006 more negative values of herkogamy received more pollen.
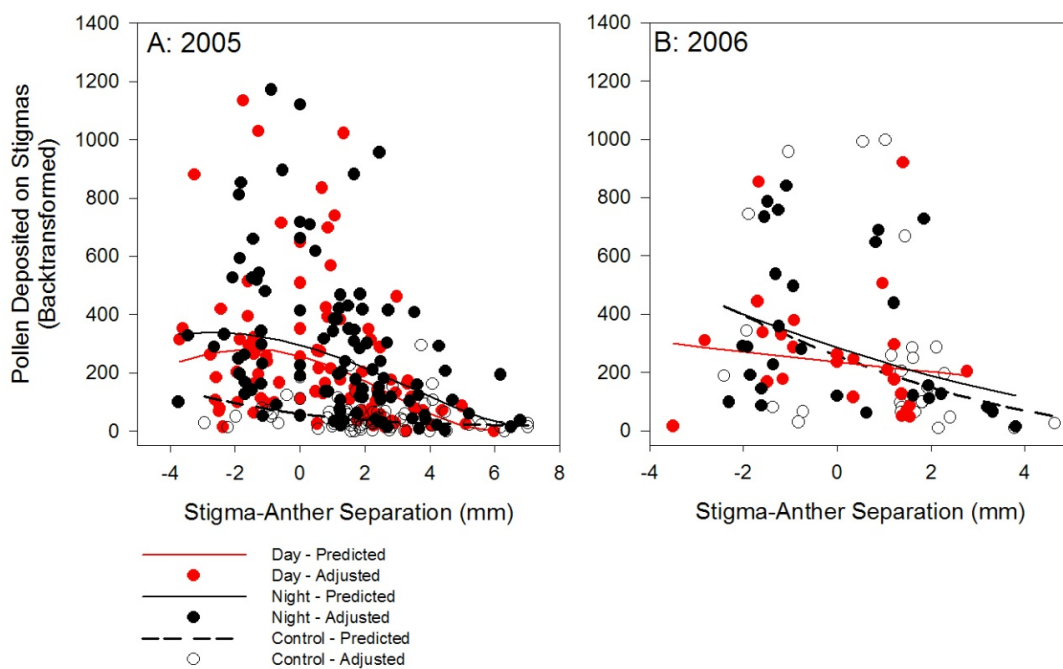
Figure 3.4 Effects of stigma-anther separation on pollen deposition during 2005 (quadratic relationship) in Taylor Canyon (panel A) and 2006 (panel B) flowering season (linear relationship). Estimates of b (SE) for herkogamy in 2005: Control: 0.52 (0.08), Day: -0.29 (0.11), Night: -0.18 (0.07). Estimates of b (SE) for herkogamy in 2006: Control: -1.93 (0.76), Day: -0.56 (0.22), Night: -1.56 (0.77). Lines are predicted functions, and points represent adjusted values that account for all three treatments (Control, Day, and Night).

In 2005, the effect of anther height on pollen deposition varied across populations.
Anther height was negatively related to pollen deposition in Deer Mountain, but
positively related in the Taylor Canyon and Vedauwoo population (Figure 3.6). The
distribution of anther height differed across populations (Appendix 3.1). However, all
slope estimates were significant and significantly different from each other ($t > 2.48$,
$P < 0.05$).
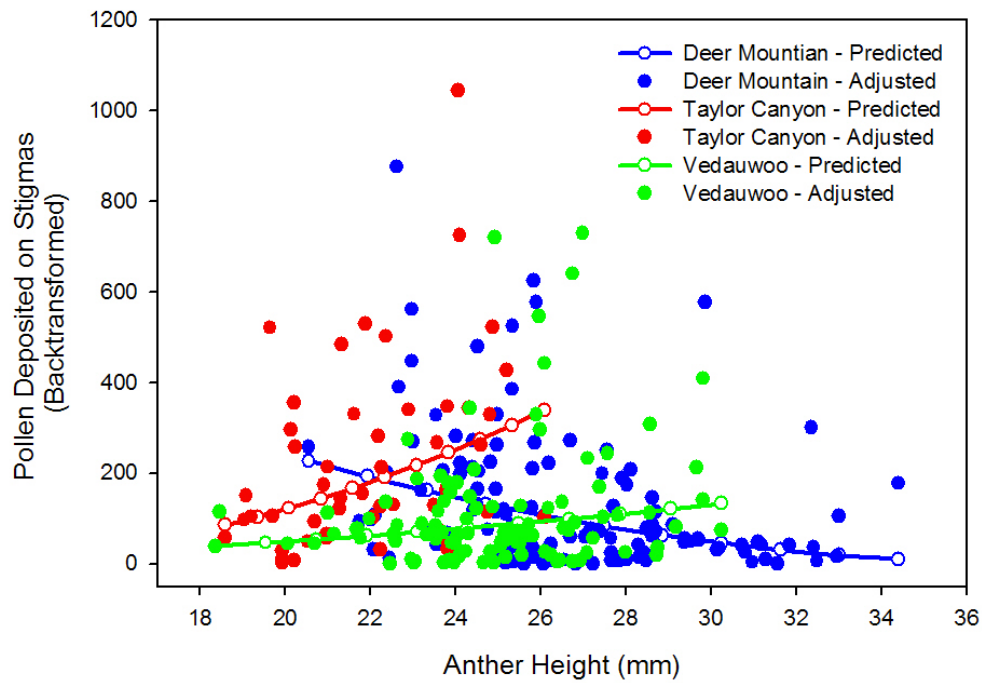
Figure 3.5 Predicted linear functions (solid lines with open circles) and adjusted
values (points) of the effects of anther height on pollen deposition. Data is from the
2005 flowering season, for three populations to highlight the interaction between
anther height and population. Estimates of b (SE) for anther height: Deer Mountain: -
0.82 (0.18), Taylor Canyon: 1.03 (0.41), Vedauwoo: 0.45 (0.20). Note: estimate for
Vedauwoo was marginally significant ($P = 0.044$).

3.4 DISCUSSION

Pollen removal and deposition are critical components of reproductive success in flowering plants. I measured pollen remaining in anthers (pollen removal) and deposited on stigmas in plants exposed to pollinators during the day, night, or not visited at all. Quantifying pollen removal and deposition in unvisited plants allowed me to gauge the rate of mechanical self-pollen movement, relative to plants visited during the day or night. Therefore, my measures of pollen movement reflect pollen that contributed to reproductive success (outcross pollen) and pollen that was wasted (self pollen) or consumed by floral visitors. Further, I sought to determine which floral traits affected pollen movement, over two consecutive flowering seasons in natural populations of *P. brandegeei*.

3.4.1 Temporal and spatial variation in pollen movement – removal and deposition

Rates of pollen removal and deposition generally increase with visitation rates (Sahli and Conner 2011). Differences in pollinator activity partially explain variation in pollen movement in *P. brandegeei*. Greater pollen removal during the day was consistently observed across sites and flowering seasons. This pattern of removal corresponded with my observation of the greatest floral visitor activity during daylight hours (Chapter 2; Kulbaba and Worley, 2008). Similarly, an increase in the number of visits by syrphid flies, bees, and bombylid flies in 2006 likely contributed to the increased rate of removal in that year.

Increased visitor activity in 2006 did not translate into increased pollen deposition, perhaps due to the high frequency of pollen-collecting insects. For example,

pollen collecting bees and syrphid flies can remove large amounts of pollen, which is then consumed. As this pollen is not transferred to stigmas on subsequent visits, these "pollen thieves" do not positively contribute to plant reproduction (Wilson and Thomson, 1991) because the fraction of pollen deposited on conspecific stigmas can be very small (Thomson et al. 2000; Wolf and Leide 2006).   Consumption of pollen without pollination service reduces reproductive output, and potentially fitness, in flowering plants (Hargreaves et al. 2010).

Although pollen movement was highest during the day, significant amounts of pollen were removed and deposited nocturnally in both years. Interestingly, nocturnal visitors were not observed over the 2005 flowering season. The detection of nocturnal pollen movement raises the possibility of unobserved crepuscular or nocturnal visitors. Although hawkmoths were not observed in 2005, they were common in 2006 and likely contributed to pollen movement. However, the warmer and dryer conditions in 2006 may have also contributed to the overall higher removal and deposition of pollen during this flowering season (see below).

In addition to insect activity, abiotic factors can greatly influence pollen movement (Strauss and Whittall, 2006). In *P. brandegeei,* changes in temperature and wind speed may have contributed to variation in pollen movement between years and populations. The flowering season in 2006 was characterized by warmer temperatures and moderately more wind than in 2005, resulting in dried, loosely aggregated pollen that was easily dislodged. Disturbance by floral visitors or wind likely agitated the style/stigma and may have caused pollen loss and self-pollen deposition. These effects may explain why plants in the 2006 control treatments had less pollen remaining in

anthers than control plants in 2005, and why deposition was similar in control plants and plants exposed to pollinators.

Floral visitations can vary across populations and time. Pollinator assemblages can vary in relative abundance or even presence and absence across years (Herrera et al. 2006). Consequently, spatial and/or temporal variation in pollinators can result in a matching variation of pollen removal and deposition (Waites and Agren 2004). For example, in 2005, I frequently observed hummingbirds visiting *P. brandegeei* flowers in all three populations. Bird visitations were highest in the Vedauwoo population (Chapter 2). Although hummingbirds likely contributed to diurnal pollen deposition, I did not observe proportionally higher rates of deposition in Vedauwoo. Rather, plants in Taylor Canyon had more pollen removed and deposited. This was unexpected as pollinator activity was not highest in this population.

Similarly, most hawkmoth visits were observed in 2006 during the night in Vedauwoo, and closer to midday in the higher elevation Deer Mountain population (Chapter 2). Variable behaviour by hawkmoths across elevation gradients have been documented previously (Campbell et al. 1997; Sime and Baldwin 2003). Contrary to my predictions, however, I did not detect greater pollen removal or deposition in night visited plants in Vedauwoo or in day visited plants in Deer Mountain. Therefore, other floral visitors or deposition of self pollen caused by abiotic factors may have obscured the influences of hawkmoth activity.

3.4.2 Effects of floral design on pollen removal

The amount of pollen removed from flowers depends largely on the prevalence of pollinators and pollen collecting visitors, and how these animals interact with floral traits. In both flowering seasons, style length was an important floral trait affecting pollen removal. I predicted that plants with shorter styles would have more pollen removed from their anthers relative to plants with longer styles, because a recessed stigma poses less interference to pollen removal by a floral visitor than an exserted stigma (Sahli and Conner, 2007). Indeed, short styled *Solanum carolinense* functioned primarily as a pollen donor relative to long-styled individuals when bees were frequent visitors (Quesada-Aguilar et al. 2008). In contrast, *P. brandegeei* flowers with intermediate style lengths had the least amount of pollen remaining, in control and treated plants. The effect of style length on pollen removal may partly reflect mechanical interference between sex organs. Long styles may dislodge pollen from anthers, even when the stigma is exserted beyond the anthers, due to agitation by floral visitors or wind.

Corolla dimensions can play a large role in the attraction and orientation of pollinators during floral visitation. Interestingly, I did not find corolla dimensions to significantly affect pollen removal. While long corolla tubes have been shown to restrict or exclude small-bodied visitors likes bees and flies (Castellanos et al. 2004; Ford and Johnson 2008), the anthers of *P. brandegeei* are presented at or slightly exserted past the opening of the corolla tube (Chapter 2; Kulbaba and Worley, 2008). Therefore, my prediction that plants displaying flowers with relatively long tubes would have more pollen removed under increased hummingbird visitation was not met. While, access to

nectar at the base of the tube may be restricted by long corolla-tubes, floral tube length

may have little influence on which visitors can access pollen in *P. brandegeei*.

3.4.3 Effects of floral design on pollen deposition

Pollen deposition was a complex process in *P. brandegeei*, with two floral traits

influencing the number of grains deposited on stigmas in 2005. Confounding self pollen

deposition in 2006 due to the dry and warm weather likely obscured patterns caused by

pollinators in this flowering season. However, herkogamy (stigma-anther separation)

significantly affected pollen deposition in both study years, and may influence both

sexual interference and pollen receipt.

The close proximity of female and male sex organs may lead to an interference

between the gender functions within a flower (Webb and Lloyd 1986). That is, the

processes of pollen deposition and pollen removal may conflict, and potentially reduce

reproductive output (Barrett 2002). Flowers that present stigmas and anthers close

together may experience autonomous self-pollen deposition, or facilitated self-pollination

when a floral visitor agitates the flower. The spatial separation of sex organs

(herkogamy) increases the distance between stigmas and anthers, reducing the potential

for sexual interference and the resulting selfing and gamete wastage (Webb and Lloyd

1986). However, when pollinating animals are small (e.g. bees and flies), flowers must

display a small value of sex-organ separation to function both as pollen donors and

recipients (Conner and Rush 1996). Therefore a trade-off between the benefits of

imported and exported pollen and the negative effects of selfing may exist.

In *P. brandegeei*, herkogamy is highly variable among individual plants. Some

phenotypes may carry a higher risk of sexual interference. For example, stigmas recessed

below the anthers may receive more self pollen than stigmas exserted beyond the anthers,

as has been observed in other species (Mucia 1990). In my study of *P. brandegeei*,

control treatment flowers with recessed stigmas (reverse herkogamy) received more

pollen than exserted stigmas, likely due to mechanical dislodgement of self pollen.

Further, in both years of my study recessed stigmas to intermediate values of herkogamy

(close proximity of anthers and stigmas) resulted in high rates of pollen deposition

(Figure 3.5). However, this increased rate of deposition likely reflected a mixture of self

and outcrossed pollen. Recessed stigmas across all three treatments was similar, in that

recessed stigmas received more pollen deposition. However, more pollen was deposited

on the recessed stigmas of the day and night treatment plants than the controls (Figure

3.5). These results highlight the potential role of herkogamy for sexual interference, but

also in pollen import in self-sterile *P. brandegeei*.

The interaction effect between anther-height and population on pollen deposition

in 2005 is interesting and perplexing. The different patterns of pollen deposition among

populations may partially reflect differences in mean anther height in these populations.

For example, flowers in the Deer Mountain population displayed the largest mean anther

height of the three study populations (Appendix 2.2, 3.1). While the exsertion of anthers

should increase the spatial separation of sex organs when accompanied by reverse

herkogamy, these sex organs are brought into closer proximity with approach herkogamy.

Accordingly, pollen deposition was highest in the low to intermediate values of anther

height in my study (Figure 3.6). However, the effects of herkogamy were already

accounted for in my analysis of pollen deposition (Table 3.4). Therefore, the role of anther height in pollen deposition is not presently clear in my study.

3.4.4 Conclusions

The general patterns of pollen movement in *P. brandegeei* are varied and complex. The removal and deposition of pollen changes across populations, and in accordance with observed fluctuations in weather conditions and observed visitation frequency. However, some generalities can be described. Pollen removal appears to occur both diurnally and nocturnally. Weather conditions also played a role in the removal of pollen in 2006. Similarly, pollen deposition occurred during both the night and day treatments, but the treatment effect was only significant during the 2005 flowering season.

Sex organs played an important role for both the removal and deposition of pollen. Style length affected the patterns of pollen removal in both 2005 and 2006, but the nature of the effect changed slightly across years. Herkogamy also influenced pollen deposition. Plants with stigma-anther separation between -2.0 and 1.0 mm received the most pollen. However, these quantifications of pollen loads also include self pollen. Therefore, the potential for sexual interference is high in *P. brandegeei* and requires further study.

Small-bodied visitors such as bees and syrphids may be both pollinators and pollen thieves. While previous studies have shown these insects to be capable of consuming large amount of pollen (Wilson and Thomson 1991). However, some legitimate and effective pollen transfer likely occurs by these insects (Herrera 2005). The

relative roles of hawkmoths and hummingbirds are investigated in Chapter 5 and 6,

respectively. Further studies are required to fully understand the influence of small

bodied insect visitors on the floral evolution of *P. brandegeei*.

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Appendix 3.1 Summary statistics for each of three study populations, divided over the 2005 and 2006 flowering seasons. Values reported are: Mean, standard error (SE), minimum value (Min.), maximum value (Max.), and the coefficient of variation (CV).

Trait	2005 Deer Mountain					2006 Deer Mountain				
	Mean	SE	Min.	Max	CV	Mean	SE	Min.	Max.	CV
Flower Width	16.01	0.24	9.52	22.72	16.23	14.07	0.54	9.30	18.83	18.45
Flower Length	32.78	0.34	16.35	40.64	11.20	28.94	0.83	19.80	35.25	13.68
Tube Length	24.42	0.27	11.57	30.75	11.70	22.18	0.74	11.70	27.49	16.07
Basal Tube Diameter	2.06	0.04	1.12	3.65	19.73	2.45	0.05	1.95	3.10	10.72
Apical Tube Diameter	4.63	0.07	2.26	5.98	15.50	3.96	0.12	3.01	5.26	14.70
Herkogamy	2.23	0.21	-3.75	7.87	99.90	0.59	0.33	-2.01	3.81	271.89
Style Length	28.35	0.29	16.30	35.94	10.81	24.64	0.80	17.92	32.34	15.56
Anther Height	26.52	0.31	8.86	34.41	12.59	24.67	0.74	15.12	31.24	14.45
Mass	8.24	0.15	4.70	14.10	19.30	5.97	0.22	4.30	8.40	17.97
Number of Inflorescences	6.54	0.34	1.00	15.00	54.97	4.91	0.70	1.00	12.00	68.59
Inflorescence Height	18.83	0.38	10.00	29.20	21.74	16.90	0.95	9.00	30.00	26.95
Number of Flowers	9.41	0.38	2.00	23.00	43.49	8.96	0.72	4.00	18.00	38.31

Trait	2005 Vedauwoo					2006 Vedauwoo				
	Mean	SE	Min.	Max.	CV	Mean	SE	Min.	Max.	CV
Flower Width	17.43	0.24	11.66	25.07	13.67	15.66	0.29	10.92	19.29	13.81
Flower Length	29.89	0.24	23.47	36.62	8.05	28.17	0.46	18.05	36.09	12.38
Tube Length	21.74	0.19	16.63	26.91	8.74	21.24	0.45	8.71	28.99	15.88
Basal Tube Diameter	2.90	0.06	1.91	4.51	20.13	2.31	0.06	1.62	3.44	18.50
Apical Tube Diameter	5.16	0.07	3.23	6.87	14.44	4.37	0.09	2.92	6.36	15.47
Herkogamy	0.46	0.20	-3.61	4.46	431.31	0.26	0.24	-3.50	4.64	715.52
Style Length	24.55	0.23	18.83	29.43	9.53	24.17	0.46	15.58	31.36	14.27
Anther Height	24.96	0.23	18.35	30.24	9.32	25.15	0.45	14.33	33.27	13.66
Mass	9.17	0.21	5.80	18.80	23.27	5.17	0.22	2.00	8.70	31.53
Number of Inflorescences	3.44	0.21	1.00	11.00	60.97	4.00	0.36	1.00	13.00	68.30
Inflorescence Height	17.84	0.37	9.20	29.10	20.62	16.60	0.44	10.00	23.50	19.83
Number of Flowers	12.71	0.36	5.00	26.00	28.19	7.30	0.37	2.00	14.00	38.05

Trait	2005 Taylor Canyon				
	Mean	**SE**	**Min.**	**Max.**	**CV**
Flower Width	14.43	0.24	10.76	18.12	11.73
Flower Length	28.22	0.37	23.18	34.36	9.30
Tube Length	21.06	0.31	16.69	25.47	10.66
Basal Tube Diameter	1.70	0.04	1.03	2.40	16.15
Apical Tube Diameter	4.14	0.09	2.53	5.37	14.74
Herkogamy	0.77	0.27	-3.71	4.46	252.58
Style Length	22.60	0.35	17.09	28.38	11.15
Anther Height	21.86	0.30	14.95	26.08	9.71
Mass	5.95	0.16	4.10	8.90	19.03
Number of Inflorescences	5.10	0.45	1.00	12.00	63.41
Inflorescence Height	14.07	0.44	7.20	22.90	22.51
Number of Flowers	6.47	0.41	1.00	12.00	45.67

CHAPTER 4. DEVELOPMENT OF MOLECULAR MARKERS AND POPULATION GENETICS OF *POLEMONIUM BRANDEGEEI* AND *P. VISCOSUM* (POLEMONIACEAE)

4.0 CHAPTER SUMMARY

Microsatellite markers were isolated in *Polemonium brandegeei* to be used in future studies of paternity analysis, mating system evolution, population structure, and hybridization. A total of 19 microsatellite loci were identified, and six loci were used in a preliminary genetic diversity study in two populations each of *Polemonium brandegeei* and *P. viscosum*. I found 39 alleles across the six loci (average 7 per locus), with overall levels of observed heterozygosites ranging from 0.067 to 0.867 in *P. brandegeei* and 0.000 to 0.666 in *P. viscosum*. The remaining 13 primers are reported, but require further design and optimization. The reported markers will aid in future studies of *P. brandegeei* and *P. viscosum* reproductive and population biology.

4.1 INTRODUCTION

The genus *Polemonium* L. (Polemoniaceae) includes between 19 and 42 species (Worley et al 2009; Grant 1989; Davidson 1950). *Polemonium* is of particular interest to evolutionary biologists as several closely related taxa display contrasting floral morphologies and pollination strategies (Grant and Grant 1965). *Polemonium brandegeei* (A. Gray) Greene displays floral traits associated with both hawkmoth

and hummingbird pollinated species and is frequented by both types of pollinators (Chapter 2). However, *P. brandegeei*'s close relative, *P. viscousm* Nutt. is primarily pollinated by bees and large flies (Galen 1991) and displays a markedly different floral morphology (Grant 1989). However, to fully resolve the selection imposed by pollinator on hermaphroditic plants, estimates of selection through female (seeds set) and male (seeds sired) are required. While estimates of female fitness are relatively straightforward, measuring selection through male function often requires a DNA based marker. To this end, genetic markers have become invaluable to measure selection through male function (seeds set).

Microsatellite markers have become a key component to studies examining selection by pollinators on floral traits. Microsatellite markers are short tandem repeats consisting of 2-6 base pairs, and can be repeated up to 100 times in a sequence of DNA (Avise 2004). Being co-dominant markers, individual alleles at a particular locus differ in the number of these repeats as a result of mutations and other errors during DNA replication. Microsatellites are highly variable, and experience a higher overall mutation rate relative to other regions of the genome (10^{-3} to 10^{-4}; Avise 2004), that makes them ideal markers to resolve relationships between parents and offspring.

To explore the basic population genetic parameters and how a shift in pollinators has influenced the floral design of *P. brandegeei*, I developed microsatellite markers to study patterns of paternity under hawkmoth and hummingbird pollination (Chapters 5 and 6).

4.2. MATERIALS AND METHODS

Polemonium brandegeei genomic DNA from the Taylor Canyon population was extracted with a FastPrep® homogenizer and FastDNA® extraction kits (QBioGene, Solon, Ohio). Microsatellite loci were isolated using a biotin/streptavidin enrichment process (Khasa et al. 2000). Genomic DNA was digested separately with restriction endonucleases Hae III, Rsa I, and Alu I (each with PshA1), and oligonucleotide adaptors M28 (5'-CTCTTGCTTGAATTCGGACTA) and M29 (5'-TAGTCCGAATTCAAGCAAGAGCACA) were added using T4 DNA ligase. Linker-adapted fragments were then enriched by two rounds of hybridization with 5' biotin (GA)10, (TG)10, (CATA)5, and (GATA)5 followed by purification with streptavidin paramagnetic bead (M270S, Invitrogen, Carlsbad, CA). After amplification with linker specific primer M28 and digestion with Eco R1, the enriched genomic DNA fragments were cloned into plasmid vectors (pGEM3Z+, Promega) and single colonies containing microsatellites were identified by dot blot hybridization. Inserts from positive colonies were amplified with m13 universal forward and reverse primers, treated with Exonuclease I and Antarctic alkaline phosphatase (New England Biolabs), and then sequenced from one or both orientations using ABI 3730 capillary electrophoresis (NAPS Service, University of British Columbia). Primers were then manually designed using Primer 3.0 (v. 0.4.0, Rozen and Skaletsky 2000).

PCR amplification of microsatellite loci was performed in 25 µL volumes containing 0.2 NEB Taq polymerase (New England Biolabs, Ipswich, MA), 0.25µM of both primers, 1.5mM MgCl$_2$, 0.2mM of each dNTP. PCR reactions began with a 3 minute denaturation step at 94°C, followed by 35 cycles of 30 seconds at 94°C and 30

seconds at 55°C, followed by 1 minute 30 seconds at 72°C. The PCR cycle completed

with a final extension step of 5 minutes at 72°C. Amplification products were run on an

ABI 3730 Genetic Analyzer and manually scored in GeneMapper 4.0 (Applied

Biosystems, Foster City, CA).

We sampled 15 individuals from two populations of both *Polemonium brandegeei*

(Taylor Canyon, CO: 39°34'33"N, 104°22'26"W; and Vedauwoo, WY: 44°46'57"N,

116°18'50"W) and *P. viscosum* (Cottonwood Pass, CO: 38°46'34"N, 106°13'3"W; and

Loveland Pass, CO: 39°39'48.92" N, 105°52'44.94" W). Therefore, we screened a total

of 60 individuals. Of the 19 identified loci, six (Pbra6, Pbra18, Pbra21, Pbra27, Pbra43,

and Pbra13t) were selected for a population study of genetic diversity, and therefore had

dye-labels appended to the forward primer (6-FAM, PET, HEX, NED; Applied

Biosystems, Foster City, CA). As *P. brandegeei* and *P. viscosum* are both self-sterile, we

tested the six loci for deviations from Hardy-Weinberg equilibrium, evidence of linkage-

disequilibrium, as well as the number of observed and expected heterozygotes in

GenePop v3.4 (Raymond and Rousset 1995).

4.3 RESULTS

A total of 39 alleles were found across the six dye-labelled primers (Table 1).

One locus (Pbra27) in *Polemonium brandegeei* and two loci in *P. viscosum* (Pbra27 and

Pbra13t) showed significant departures from Hardy-Weinberg equilibrium (Table 2). This

may be a result of heterozygote deficiency, potentially resulting from the presence of null

alleles and/or population structure. None of the 15 pairwise comparisons among loci

displayed significant linkage disequilibrium.

Table 4.1 Primer sequence and configuration of 19 microsatellite loci developed for *Polemonium*

brandegeei. T_a is the optimized annealing temperature

Locus	Primer Sequence	Repeat Motif	T_a (°C)	Microsatellite Size Range or expected size (bp)	GenBank acc. Number
Pbra6	F: GCCTGCTGCTACTATGACTGG R: GACACCATGGATGGACCTCT	$(CA)_8$	56	234-342	HQ197909
Pbra10	F: TAGGGCAGCGCGTCTCTC R: CCCAAAATCACTTAAGTGATTTTC	$(GA)_{21}$	--	95	HQ197910
Pbra18	F: CCCTAGAATTGGTCGAACTG R: GAGGCTAAGCAAATTAGGAAAACA	$(TG)_7$	56	167-175	HQ197911
Pbra21	F: GGAAAACAGAGGCAACGAGA R: CTCCGAAGCCCAGACATC	$(TC)_5$	55	151-174	HQ197912
Pbra27	F: GAATTCTATGTTTTACCTGTGTT R: TGCAATAAAACCTCTCTCTCT	$(TC)_{14}$	54	166-253	HQ197913
Pbra43	F: GAGAGACAGATCGGGCAGAG R: CCTCTCTATTTTCCCCTCGATT	$(TC)_9$	55	103-244	HQ197914
Pbra2t	F: GAATTCTGTTAGAGTTCACGTG R: GAATTCCCTTCTGTCTGTCT	$(TCTG)_5$	--	174	HQ197915
Pbra5t	F: TAGATGTTCCCAACGGCTTG R: TTTGGGCGAATCCAAATAAA	$(CATA)_6$	--	151	HQ197916
Pbra16t	F: CTTTCACGATAGTAGTAGTAG R: AATGGATCGGTCTCACTTGT	$(CTA)_{22}$	--	223	HQ197917
Pbra9	F: GGTTAAACGTTGTCAATTATGTG R: CTCGGTAGATGCTATGTTATA	$(TG7GA)_{23}$	--	279	HQ197908
Pbra11	F: CTTCTCGACAGTAAATCTCTCT R: AACTAGTAACAGTCGGTGAAC	$(GA)_{25}$	--	218	HQ197902
Pbra17	F: CGCTTGGTCTACTATGTACAA R: TTAGTATAACAGGACAATGGGAAA	$(TG)_{13}(GA)_n$	--	483	HQ197903
Pbra18.1	F: CAAAAGAGAAAAGACGGTGTCA R: GTGTACCCAAGTGGCACAAA	$(TG)_{>14}$	--	728	HQ197918
Pbra18.2	F: GGTTTGGTGTATTTGTGTTTGAA R: CTATAAGGCCATCCACTATATAA	$(CAA)_{36}$	--	308	HQ197919
Pbra22	F: ATCGAGTGCTCCGATCTTG R: GAATTCCAGGCAAGTTACAG	$(TC)_{26}$	--	152	HQ197904
Pbra33	F: CTCCACCCTAAAACCACCA R: TCTGCGGTACCATCTCTC	$(TC)_{36}$	--	140	HQ197905
Pbra39	F: GAATTCTCCAATGCAAATTAAC R: CTGTCCCATTGAATACACCTT	$(TG)_{19}$	--	135	HQ197906
Pbra11t	F: TCGGTCGTATCGCTAGAATG R:TGACATAAATTTTACCTTCATTGTTGA	$(GTA)_{16}$	--	184	HQ197921
Pbra13t	F CCCGTCAACCAGGGATAGTA R TCATGTTTGGGTAAACAATCCA	$(CATA)_{18}$	54	105-240	HQ197922

Table 4.2 Summary of allele counts, and expected and observed heterozygosity for two populations of *Polemonium brandegeei* and *P. viscousm*.

| | *Polemonium brandegeei* | | | | *Polemonium viscosum* | | | |
| | Taylor Canyon, CO | | Vedauwoo, WY | | Loveland Pass, CO | | Cottonwood Pass, CO | |
Primer	No. of Alleles	H_O/H_E	No. of Alleles	H_O/H_E	No. of Alleles	H_O/H_E	No. of Alleles	H_O/H_E
Pbra6	3	0.333/0.535[†]	2	0.800/0.496	3	0.466/0.604	4 (1)	0.400/0.540
Pbra18	3 (1)	0.467/0.387	3	0.733/0.545	3	0.400/0.439	3	0.267/0.239
Pbra21	5	0.533/0.664	4 (1)	0.666/0.522	4	0.666/0.655	3	0.467/0.480
Pbra27	3	0.733/0.680	3 (1)	0.400/0.615*	5 (1)	0.600/0.640	3 (1)	0.200/0.649*
Pbra43	8 (2)	0.600/0.829	2	0.067/0.186	6	0.400/0.690	4	0.667/0.671
Pbra13t	9 (2)	0.867/0.885	2	0.200/0.287	7	0.600/0.834*	1	0.000/0.000[n/a]

H_O / H_E bserved and expected heterozygosity, respectively.

* Significant departure from Hardy-Weinberg equilibrium ($P < 0.05$).

† Marginally did not deviate from Hardy-Weinberg equilibrium ($P = 0.0514$).

[n/a] Could not test for departure of Hardy-Weinberg equilibrium, allele was fixed.

4.4 DISCUSSION

Of the 19 microsatellite loci reported, six were characterized in two species of *Polemonium*. The remaining loci will be further explored (e.g. primer design and optimization), for use in future population studies. These markers will be used for population levels of genetic diversity, parentage assignment in pollination experiments (Chapter 5 and 6), and studies examining hybridization between *P. brandegeei* and *P. viscosum*. These six primers developed for *P. brandegeei* have been demonstrated to consistently cross-amplify in *P. viscosum*.

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CHAPTER 5. SELECTION ON FLORAL DESIGN IN *POLEMONIUM BRANDEGEEI* (POLEMONIACEAE): FEMALE AND MALE FITNESS UNDER HAWKMOTH POLLINATION

5.0 CHAPTER SUMMARY

Plant-pollinator interactions promote the evolution of floral traits that attract pollinators and facilitate efficient pollen transfer. The spatial separation of sex-organs, herkogamy, is believed to limit sexual interference in hermaphrodite flowers. Reverse herkogamy (stigma recessed below anthers) and long narrow corolla-tubes are expected to promote efficiency in male function under hawkmoth pollination. I tested this prediction by measuring selection in six experimental arrays of *Polemonium brandegeei*, a species that displays continuous variation in herkogamy, resulting in a range of recessed to exserted stigmas. Under glasshouse conditions, I measured pollen removal and deposition, and estimated selection gradients (β) through female fitness (seeds set) and male fitness (siring success based on 6 polymorphic microsatellite loci). Siring success was higher in plants with more nectar sugar and narrow corolla-tubes. However, selection through female function for reverse herkogamy was considerably stronger than was selection through male function. Hawkmoths were initially attracted to larger flowers, but overall preferred plants with reverse herkogamy. Greater pollen deposition and seed set also occurred in reverse herkogamous plants. Thus, reverse herkogamy may be maintained by hawkmoths through female rather than male function. Further, my results suggest that pollinator attraction may play a considerable role in enhancing female function.

5.1 INTRODUCTION

Floral traits affect mating opportunities because they attract pollinators and/or facilitate the efficient movement of pollen. Quantitative traits such as flower size can be correlated with nectar volume or sugar content, providing a means for pollinators to estimate potential rewards (Bell 1985; Glaettili and Barrett 2008; Kaczorowski et al. 2008). In addition, flower color (Meléndez-Ackerman et al. 1997) and olfactory cues (Raguso 2008) may appeal to specific pollinators or functional groups of pollinators (cf. Fenster et al. 2004). Other aspects of floral design, including flower shape and the positioning of sex-organs, typically facilitate efficient pollen transfer but are not necessarily associated with pollinator attraction. These traits orient pollinators, so that pollen is consistently placed where it can be deposited on the stigmas of subsequently visited flowers rather than consumed or lost (Fenster et al. 2009). Traits such as nectar guides or landing pads affect both attraction and efficiency; phenotypes that enhance both functions may be under stronger selection than those that only increase one aspect of successful pollination. However, I know of no studies that have examined this possibility.

The majority of flowering plants are hermaphroditic, and may simultaneously function as females and males. Therefore, selection on attraction and efficiency may occur through either gender function, and even be in contrast among genders (Campbell 1989; Ashman and Morgan 2004). Indeed, conflicting selection among gender functions has been predicted to be common (Morgan 1992), but evidence for gender conflict is sparse (Delph and Ashman 2006; Sahli and Conner 2011, but see Campbell 1989). Further, Bateman (1948) predicted that selection through male function should be

stronger than through female function. This postulate too is controversial, in part because

studies examining direct measures of male fitness lag behind those using surrogate

measures of siring success (Conner 2006; but for a recent example see Sahli and Conner

2011). More studies that directly estimate both female and male fitness are required to

fully resolve the prevalence of gender conflict, and the relative magnitude of selection

through female and male functions in flowering plants.

A variety of traits influence attraction and female fitness in hawkmoth pollinated

species. In natural settings, hawkmoths display an innate attraction to lightly colored

flowers (Raguso and Willis 2003) with particular olfactory cues (Riffell et al. 2008).

However, the relative roles of corolla color and scent are still unclear (Goyret et al.

2008). Efficient pollen transfer is associated with long, narrow corolla-tubes or nectar

spurs (Nilsson 1988; Alexandersson and Johnson 2002; Brunet 2009). Long flowers

promote stronger contact between long-tongued hawkmoths and floral sex-organs

because rewards (usually nectar) are produced at the base of the flower or spur. This

increased contact often promotes efficient pollen deposition. However, selection by

hawkmoths has primarily been detected through female fitness alone, or with indirect

estimates of male fitness such as pollen removal or pollinator visitation rates (e.g.

Campbell et al. 1997; Sletvold and Ågren 2010; Vanhoenacker et al. 2010). Direct

measurements of male fitness are notoriously difficult to obtain, and apparently have

never been estimated for hawkmoth pollinated species. Thus, the understanding of

hawkmoth-mediated selection lags behind that of other groups, such as bees and or

hummingbirds, even though hawkmoths are important pollinators across a variety of

plant families (Grant 1983; Thomson and Wilson 2008).

One floral trait of particular importance for efficient pollen transfer in hermaphroditic plants is the spatial separation of sex-organs, herkogamy. Although herkogamy may reduce self-pollination and subsequent inbreeding depression, it occurs in many species with genetic self-incompatibility (reviewed in Barrett 2003). Therefore, Webb and Lloyd (1986) proposed that herkogamy may function to reduce the interference between female and male functions, limiting the wastage of gametes. In approach herkogamy, the stigma is presented above or beyond the level of the anthers, so that pollinators (and outcrossed pollen) contact the stigma first when visiting a flower (Barrett 2003). In contrast, reverse herkogamy presents the anthers above a recessed stigma, ensuring the anthers are contacted before the stigma. Approach herkogamy is common and associated with a diversity of pollinators. Reverse herkogamy is less common, and it is thought to be associated with long-tongued pollinators, such as hawkmoths, when accompanied by a long, narrow corolla tube (Webb and Lloyd 1986). This association is hypothesized because the long slender proboscis of a moth would more likely contact a recessed stigma (reverse herkogamy) contained within a narrow tube, than it would an exserted stigma (Barrett 2002). Further, pollen removal should be greater when the anthers are presented above the stigma, because physical interference from the stigma and style would be reduced. However, the functional association between reverse herkogamy and hawkmoth pollination has not been explored empirically.

Surprisingly, causes and consequences of continuous variation in herkogamy in self-incompatible species have received little attention (but see Forrest et al. 2011), even though continuous variation in herkogamy is very widespread. This contrasts with the

majority of studies examining selection on herkogamy, which have focused on the 10% of flowering species that display polymorphisms (e.g. heterostyly) in sex-organ arrangement (Barrett et al. 2000; Barrett 2003), or on species in which reduced herkogamy results in selfing (e.g. Fishman and Willis 2007). In outcrossing species, selection may sometimes favor reduced herkogamy if close proximity of stigma and anthers is required to ensure that small pollinators contact both sex organs, as in *Raphanus raphanistrum* (Sahli and Conner 2011). Generally, selection should favor a phenotype that will reduce the interference between female and male functions, and still allow outcrossed pollen to reach the stigma. Interestingly, stabilizing selection for approach herkogamy occurs in *Ipomoea wolcottiana* under hawkmoth pollination (Para-Tabla and Bullock 2005). This result contradicts the expectation of reverse herkogamy in hawkmoth-pollinated taxa outlined above (Webb and Lloyd 1986). Additional effort is required to quantify selection on herkogamy in outcrossing species, and to determine whether and under which conditions lepidopteran pollinators select for reverse herkogamy.

I measured selection on floral traits of the self-sterile, sub-alpine perennial *Polemonium brandegeei,* under pollination by captive individuals of the hawkmoth *Hyles gallii*. *Polemonium brandegeei* displays tubular corollas that are cream-white in color, and emit a strong, heavy, sweet smell. Populations of *P. brandegeei* exhibit continuous heritable variation in style length, that results in a range of approach (exserted stigma on long style) to reverse (recessed stigma on short style) herkogamy (Kulbaba and Worley 2008). Hawkmoths (*Hyles lineata*, *H. gallii*) and hummingbirds (*Selasphorus platycercus*) are both pollinators of *P. brandegeei*. I proposed previously that hawkmoths

maintain reverse herkogamy in *P. brandegeei* populations (Kulbaba and Worley 2008; Chapter 2).

To fully evaluate the interaction between hawkmoths and floral design, I adopted a strategy similar to that suggested by Campbell (2009). I selected individual plants based on herkogamy (approach or reverse) to assemble experimental arrays of *P.brandegeei*, pollinated exclusively by captive *H. gallii* under controlled conditions. This approach allowed manipulation of a single floral trait, while the remaining traits were allowed to vary naturally (Campbell 2009). Since visits by hawkmoths occurred in the absence of other visitors, I was able to directly measure hawkmoth-mediated selection on floral design. To my knowledge, this was the first study to directly measure selection by hawkmoths through both female and male fitness.

My main objective was to assess how floral dimensions and nectar rewards affect attraction, pollen movement, and fitness under hawkmoth pollination. Secondary objectives were to describe hawkmoth foraging behaviour on and among inflorescences of *P. brandegeei*, and to compare selection through female and male fitness. I expected that (1) hawkmoths would be more attracted to plants with greater rewards (larger flowers corresponding to higher nectar volume and/or sugar content), but would not respond to variation in corolla shape or sex-organ positioning (floral design). Rather, (2) I expected variation in floral design to affect male and female fitness through effects on efficient pollen removal and deposition. Specifically, I predicted that (3) hawkmoths would select for long and narrow corollas displaying reverse herkogamy, corresponding to the flowers of other hawkmoth-pollinated taxa (Nilsson 1988; Strakosh and Ferguson 2005; Kulbaba and Worley 2008).

5.2 MATERIALS AND METHODS

5.2.1 Floral traits and experimental design

To generate experimental populations (hereafter referred to as "arrays") of *P. brandegeei* flowers, I grew individual plants in Premier Pro Mix® medium with Osmocote Plus® slow-release fertilizer (10:10:10) in 800 mL Deepots®. I raised the plants in a greenhouse (temperature range: 23°C to 27°C) under a 16 hour photoperiod, with seeds obtained from Lone Tree Gulch, Southern Colorado (38° 30′ 17″ N, 107° 11′ 26″ W).

Plants were arrayed as in Figure 5.1. This arrangement produced an even distribution of approach and reverse herkogamous plants, and an equal probability of a hawkmoth encountering an approach or reverse herkogamous plant on entering the array. Arrays consisting of 12-18 plants each (depending on available phenotypes) were placed in a controlled environmental flight chamber (Pernal and Currie 2001) with a 12 hour diurnal period, including a 30 minute simulated sunrise and sunset "illumination" and "dimming" period, respectively. Daytime temperature was 24°C, and night time temperature was 17°C with a relative humidity of ca. 60%. Pupae of *Hyles gallii*, a known pollinator of *P. brandegeei*, were obtained from Worldwide Butterflies (Cornwall UK) and covered with damp peat moss until emergence as flying adults. I introduced 2-3 adult *H. gallii* to the same flight chamber containing the array plants, and allowed them to forage on the plants for 48 hours. A total of 6 arrays (94 plants), and 14 individual *H. gallii* adults were used for this study. Arrays were run consecutively because I had only a single flight chamber, and unique hawkmoths were used in each array. Captive

H. gallii were similar in both mass and proboscis-length to those from natural

populations.
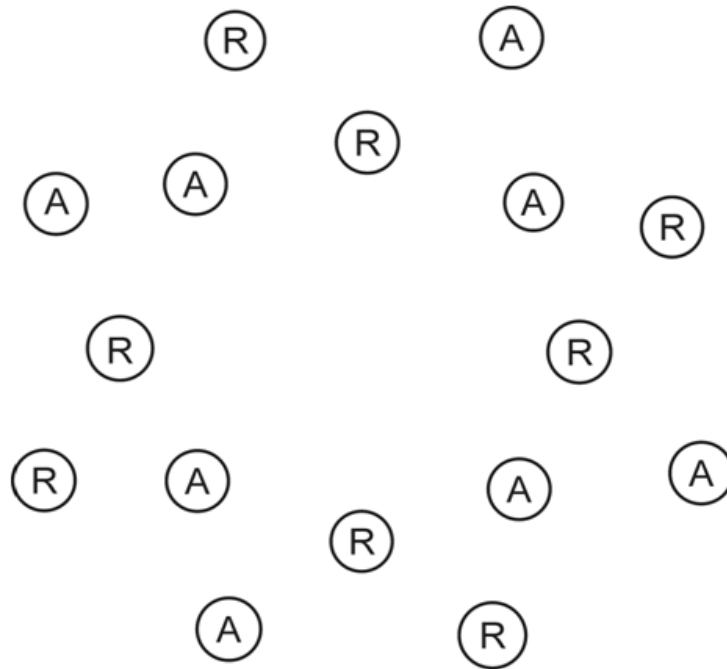
Figure 5.1 Arrangement of approach (A) and reverse (R) herkogamous plants in

the experimental array populations. Distance between plants was 45 cm.

I chose plants from different maternal families (seed source plants no closer than

1.5 m) based on their herkogamous phenotype for inclusion in an array (total of six

arrays). Herkogamy was directly measured as the position of the stigma relative to the

nearest anther (Figure 5.2). However, because stigma-anther separation is a continuous

trait, my array populations contained a range of phenotypes (reverse herkogamy:

approximately -4.0 to -0.2 mm; approach herkogamy: approximately 0.2 to 4.6 mm),

which was comparable to the range seen in natural populations. Inflorescences were standardized to 7 freshly opened flowers, also to reflect the natural display size (mean of 6.97, $n = 369$ across three natural populations; Kulbaba and Worley 2008). Thus 84-126 flowers were available to hawkmoths in each array.
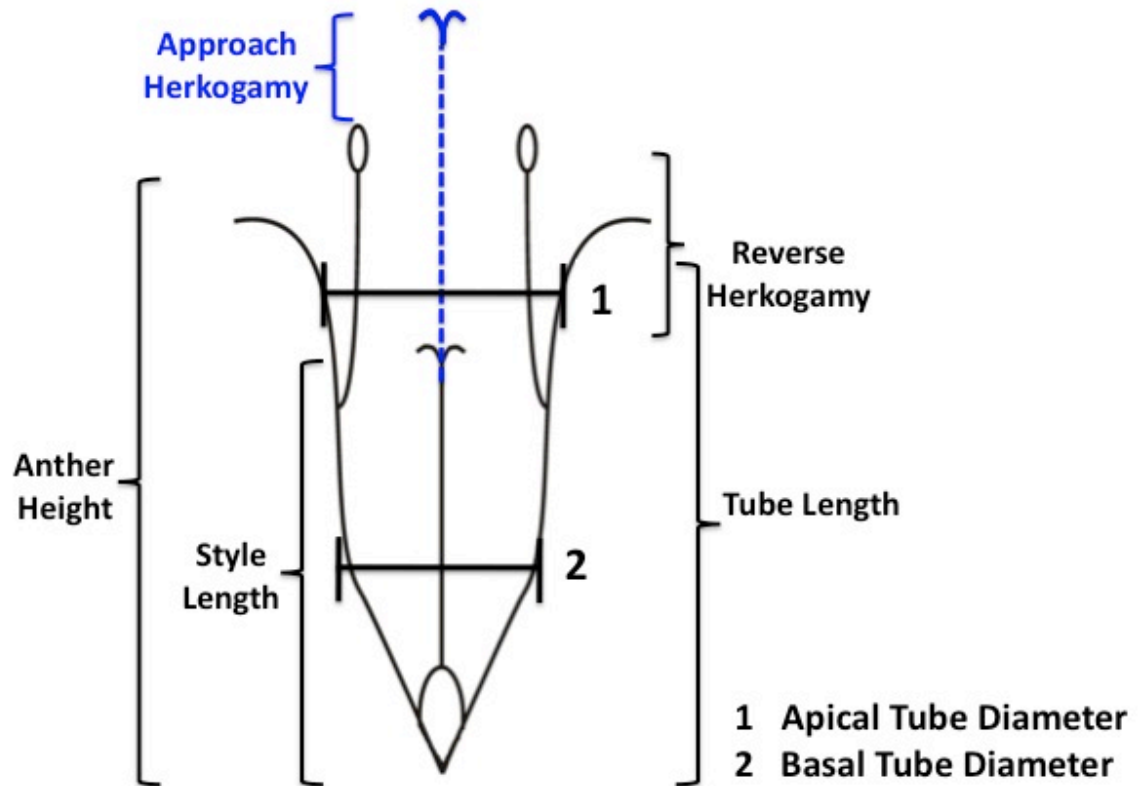
Figure 5.2 Diagram of floral measurements. Dashed line indicates style position of an approach herkogamous flower, and a solid line indicates style of a reverse herkogamous flower.

I measured additional floral traits to determine how floral design affects female and male fitness in *P. brandegeei*. These traits were: corolla tube-length, corolla tube-width at the base and apex of the flower, height of sex-organ presentation relative to the

base of the flower, flower mass (dry weight of corolla, calyx, androecium and gynoecium), nectar volume, and nectar sugar content (Figure 5.2). Floral traits were selected for detailed examination because they had a large influence on phenotypic variation in floral dimensions and/or seemed likely to affect the attractiveness of flowers, or pollen transfer efficiency by hawkmoths. Further, floral dimensions were heritable in a greenhouse crossing experiment, and could potentially respond to selection (Kulbaba and Worley 2008). I obtained floral dimensions and nectar measurements just before the extra flowers were removed to standardize flower number, so that measured flowers were a similar age to flowers visited by hawkmoths. Floral dimensions were measured on flowers used in the array and were made to the nearest 0.1 mm with digital calipers. Nectar measurements were destructive and were therefore made on flowers that had been removed. *Polemonium brandegeei* is weakly protandrous, with stigmas becoming receptive 6-24 hours after anthers dehisce. All flowers visited by hawmoths in my arrays were functionally hermaphroditic, with dehisced anthers and mature stigmas.

Nectar volume was determined by removing the nectar with calibrated 2 μL micropipettes (Drummond Scientific, Broomall, Pennsylvania). Nectar was removed from unvisited flowers that were on the same inflorescence as the experimental flowers. These samplings were taken as representative of the inflorescence because a pilot study in natural populations showed greater variation in nectar volume among plants than within plants. The extracted nectar was dried on clean filter paper for later quantification of sugar content. I determined the sugar concentration of extracted nectar via the colorimetric anthrone technique (McKenna and Thomson 1988). I performed two replicate analyses of the same nectar sample and used the mean (Kearns and Inouye

1993). Finally, I compared the nectar sugar content of the array plants with 10 samples

from Taylor Canyon, Colorado (39° 34′ 33″ N, 104° 22′ 26″ W) to see if greenhouse and

field plants differed with respect to floral reward. Seed from Lone Tree Gulch, Colorado

was unavailable due to a fire at this population. Taylor Canyon, Colorado was the closest

population of *P. brandegeei* resembling Lone Tree Gulch, in elevation and general

habitat features (Kulbaba and Worley 2008).

5.2.2 Hawkmoth behaviour and fitness estimates

Pollinator behaviour was observed by recording *Hyles gallii* foraging bouts with a

video camera. Each bout consisted of consecutive visits to one or more flowers; bouts

began when a hawkmoth visited the first flower and ended when the hawkmoth exited the

array and rested without flight for at least 5 minutes, or ceased visiting flowers. The

number of visits to each plant was recorded, as was the sequence of plants visited within

each *H. gallii* foraging bout, to determine if hawkmoths display a preference while

foraging. Since pollinator movements within an inflorescence can indicate the potential

for geitonogmous pollen transfer, I recorded whether moths moved to higher or lower

flowers while foraging within an inflorescence. I scored the total number of flowers

visited/probed for each plant ("flowers visited per plant"), and the number of times an

individual plant was visited over the 48 hour array period ("visits per plant"). Each visit

to a plant could include probes of multiple flowers. Unfortunately, I was unable to record

flower handling time as I could not consistently observe flower entry and exit given the

camera angle.   However, I did record the total length of each foraging bout. Therefore, I

calculated the average time spent per flower by dividing the length of an individual

foraging bout by the total number of flowers visited in that bout. Finally, I observed the

location of pollen on the bodies of hawkmoths with the aid of a stereomicroscope.

After the standardized foraging period the plants were returned to the greenhouse

and allowed 24 hours for ovule fertilization. Pilot experiments had shown 24 hours to be

sufficient for fertilization (Kulbaba unpublished data). I then collected the anthers and

stigmas from 5 of the 7 experimental flowers. I stored anthers in 70% ethanol, and later

determined the number of pollen grains remaining with a Multisizer 3 particle counter

(Beckman-Coulter, Fullerton, California). I mounted stigmas on fuchsin jelly slides

(Beattie 1971), and manually counted the number of pollen grains deposited on the

stigma from digital images (Image-pro express, Media Cybernetics Inc.). I was unable to

distinguish between self and outcross pollen; therefore counts of pollen deposition reflect

both combined.

I measured fitness through both female and male functions. Female fitness for

individual plants was the number of seeds set. Male fitness was the number of seeds

sired by each plant. Offspring from each array were raised in a greenhouse to the

seedling stage under similar conditions to the array plants. I extracted DNA from dried

leaf tissue and genetically screened both parents and offspring from each array (up to five

offspring per array plant) with six polymorphic microsatellite loci as described in

Kulbaba and Worley (2011; Chapter 4). Microsatellite genotypes were manually scored

with genemapper software 4.0 (Applied Biosystems, Foster City, California) for parents

and offspring, and then analyzed with the program Cervus v 3.03 (Kalinowski et al.

2007) to determine the most likely paternal parent. Cervus uses a maximum-likelihood

approach (Meagher 1986) based on Mendelian segregation probabilities of parent and offspring genotypes to assign parentage. These probabilities are first determined through a simulation performed by Cervus v 3.03. The simulation parameters were as follows: 10 000 cycles, complete sampling of 17-11 candidate fathers (reflecting the array size minus the known mother because *P. brandegeei* is self-sterile), 0.750 as the proportion of loci typed, 0.01 as the rate of typing error (calculated from repeat genotyping), 0.95 for the strict confidence level and 0.80 for the relaxed confidence level since I did not have to consider pollen donors outside my closed experimental populations (cf. Nishizawa et al. 2005; Hodgins and Barrett 2008a).

5.2.3 Statistical analysis

I used general linear models employing analysis of covariance (ANCOVA; Proc GLM and Proc GENMOD) to assess factors influencing hawkmoth behaviour, patterns of pollen transfer, and fitness measures. Array number was included in all ANCOVAs as a fixed factor. Both two-and-three-way interactions among the explanatory variables (fixed effect and covariates) were analyzed, as were quadratic terms to explore the possibility of stabilizing or disruptive selection. Non-significant interactions and effects were removed from the model in a reverse step-wise fashion (Sokal and Rohlf 1995). This general approach was used in all subsequent analyses involving multiple explanatory variables. I also examined the consistency of trends found with ANCOVA by conducting multiple regressions on each array. All trends and relationships described

were significant in at least three of the six replicate arrays, and were consistent in all replicate arrays (i.e. the signs of slope estimates were consistent).

In many analyses, more than one explanatory variable was significant. To best portray the influence of specific covariates on dependent variables, I adjusted the data in my figures to highlight the effects of statistically significant floral dimensions. Predicted values were first generated with partial regression coefficients and observed values of the focal covariate. The partial regression coefficients and mean values of the remaining significant covariates were also included in the calculation of values. Residuals from the mixed model were then added to the predicted values to generate adjusted values (cf. Worley and Harder 1996). All analyses and generation of adjusted values were performed in SAS 9.1.2 (SAS Institute 2004).

5.2.3.1 Hawkmoth behaviour - I analyzed hawkmoth behaviours to assess pollinator preferences. The sequence of plants visited, number of times a plant was visited, and the mean number of flowers visited per plant were treated as dependant variables in general linear models (Proc GLM). Plants unvisited in a bout were treated as missing data, and not included in calculation of mean number of flowers visited per plant. All plants within arrays were visited within the 48 hours that the arrays were available to hawkmoths. Measured floral traits were included as explanatory covariates. All three dependent variables were square-root transformed and covariates were $\log_{10}$ transformed to improve normality of residuals.

To determine if hawkmoths foraged longer on certain floral phenotypes, I calculated the "average phenotype" of plants visited in a foraging bout. Each plant

phenotype was weighted by the proportion of total flower visits that it received in a bout. Average phenotypes were calculated for flower size (mass), herkogamy, and nectar sugar content and volume. The average time spent per flower (total time of foraging bout / total number of flowers visited in that foraging bout) was analysed as a dependant variable, with the "average phenotypes" included as explanatory factors in a general linear model (Proc GLM).

5.2.3.2 Female and male function - I first sought to identify floral traits important for pollen removal and deposition under hawkmoth pollination. Pollen remaining in anthers and deposited on stigmas were treated as dependent variables after $\log_{10}$ transformation to meet assumptions of normality. Floral traits, number of visits received by a plant, and the mean number of flowers visited per plant were included as explanatory variables.

To detect selection on floral traits under hawkmoth pollination, I examined associations between floral traits and female (seeds set) or male (seeds sired) fitness. The number of seeds set and number of seeds sired were analyzed as dependant variables. Floral traits, number of visits received by a plant, and the mean number of flowers visited per plant were included as explanatory variables (covariates). Seed set was square-root transformed to improve normality, and analyzed with a general linear model (Proc GLM). However, I analyzed the number of seeds sired with a generalized linear model (Proc GENMOD) along a Poisson probability distribution, as the data best followed this distribution. Ideally, I would have included pollen export and import as explanatory variables. However, I was unable to differentiate between self and outcross pollen.

Therefore, I individually examined the relationships between seed set and pollen deposition, and siring success and pollen removal.

Selection gradients were calculated as the linear regression coefficients between standardized fitness measures and floral traits. I standardized fitness by dividing individual fitness measures by mean fitness (Lande and Arnold 1983). Explanatory variables (floral traits) were standardized to a mean of one, by dividing individual trait values with the mean trait value (Hereford et al. 2004). Both linear (β) and quadratic selection gradients (γ) were examined for all floral traits, to allow for both directional and stabilizing/disruptive selection, respectively. I included the linear terms of effects in models that included quadratic terms (γ). Although female fitness measures (seeds set) required square-root transformation before analysis, I report selection gradient estimates from the untransformed data, because estimates from transformed fitness measures are biased (Lande and Arnold 1983). However, reported P-values are from the transformed ANCOVA (Mitchell and Shaw 1987; Caruso 2000). Finally, I used Welch's t-test (assuming unequal variance) to statistically examine the relative magnitude of selection gradients on traits through female and male function.

5.2.3.3 Fitness surfaces - To further examine potential multivariate or correlated selection, I analysed nonlinear multidimensional fitness surfaces. I used the projected pursuit regression described in Schluter and Nychka (1994) to fit spline curves to my fitness data without making assumptions about the shape of the function. A single projection was fit to my multidimensional data, with a smoothing parameter (λ) that minimized the general cross validation (GCV) score, a measure analogous to least-square difference. This value of λ was chosen after testing a range of smoothing parameters

(described in Schluter and Nychka 1994). I conducted separate analyses of relative

female, male, and total fitness. Total fitness was the average of relative female and

relative male fitness. I used relative female and male fitness to prevent the larger values

of seed set from dominating variation in total fitness. Floral traits included as covariates

were: basal-tube diameter, herkogamy, nectar sugar, and flower mass. These variables

were indicated as being potentially important by analysis of covariance, or likely

correlated with other traits (e.g. mass). The contribution or loading of each floral trait on

the fitness measures was calculated, and 95% confidence intervals were generated

through bootstrap analysis (1000 replicates).

5.3 RESULTS

My analyses explicitly assessed the possibility of non-linear and multivariate

relationships between floral traits and either pollinator behaviour or plant fitness.

However, all interaction and quadratic terms in the ANCOVA's were non-significant.

These results were mirrored in my projected pursuit regression. Thus, in this study, the

effects of each explanatory variable were linear and independent of other variables

included in the final models.

5.3.1 Pollinator behaviour

On average, moths visited 3.7 plants, 3.4 flowers per plant, and 13 flowers in total

during each foraging bout. This was a small fraction of the available plants and flowers,

given that arrays contained 12-18 plants and 7 flowers per plant for a total of 84-126 flowers per array. Over the 48 hour duration of an array, hawkmoths visited an average of 194 flowers. All array plants were visited in all six arrays. However, both the number of visits received by each plant and the number of flowers visited per plant varied widely (Appendix 5.1). Interestingly, *Hyles gallii* individuals foraged in a "directional" pattern, typically without returning to plants previously visited within a foraging bout. Hawkmoths exited the arrays by flying upwards, and then alighting on the wall of the flight chamber. Within an inflorescence, *H. gallii* was equally likely to move up or down. I observed a total of 1165 movements between individual flowers across the six replicate arrays, and determined that ~53% of visits (615) proceeded in an upwards direction on an inflorescence, while the remaining 47% of visits (550) proceeded in a downward direction ($\chi^2 = 3.67$ for expectation of equal number of movements, $P = 0.075$).

I assessed pollinator preferences by analyzing the sequence of plants visited, the number of visits received by each plant, the number of flowers visited per plant, and estimated handling time per flower. Plants with larger flower mass tended to be visited earlier in a foraging bout (Table 5.1, Figure 5.3) but they did not receive more visits overall than plants with smaller flowers (Table 5.1). Moreover, measures of floral reward (nectar volume, nectar sugar content) were not correlated with flower mass (Appendix 5.2). Contrary to my expectations, the number of visits received by an array plant was not influenced by floral reward, even though variation in nectar reward was higher than for any floral dimension other than herkogamy (Appendix 5.1). In addition, nectar volume and sugar content of experimental and field plants were similar (nectar volume

mean (SE): array plants: 2.66 (0.18) µL, field plants: 2.94 (0.24) µL; $t_9 = 3.6$, $P > 0.05$;

nectar sugar: array plants: 54.13 (2.94) mg; field plants: 42.86 (3.14) mg; $t_9 = 3.4$, $P >$

0.05). Finally, estimated handling time per flower for each foraging bout did not vary

with average floral reward or the weighted average of any other floral traits.

Table 5.1. Analyses of the number and sequence of *Polemonium brandegeei* plants visited by foraging *Hyles gallii*. The position of plants in visit sequence, visits per plant, and number of flowers visited per plant were square-root transformed prior to analysis for slope estimates *b* (SE). Initial models included all floral measurements and non-significant covariates were deleted using backwards elimination.

Effect	Position in Visit Sequence	Visits per Plant	Number of Flowers Visited Per Plant
Array	$F_{6,87} = 2.21$	$F_{5,87} = 1.94$	$F_{5,87} = 2.27$
Herkogamy b (SE)	n.s.	$F_{1,87} = 28.28***$ **-0.141 (0.027)**	$F_{1,87} = 24.70***$ **-0.134 (0.034)**
Mass b (SE)	$F_{6,87} = 5.31**$ **-1.231 (0.391)**	n.s.	n.s.
R^2 of model	0.29	0.20	0.24

*$P < 0.050$, **$P < 0.001$, ***$P < 0.0001$
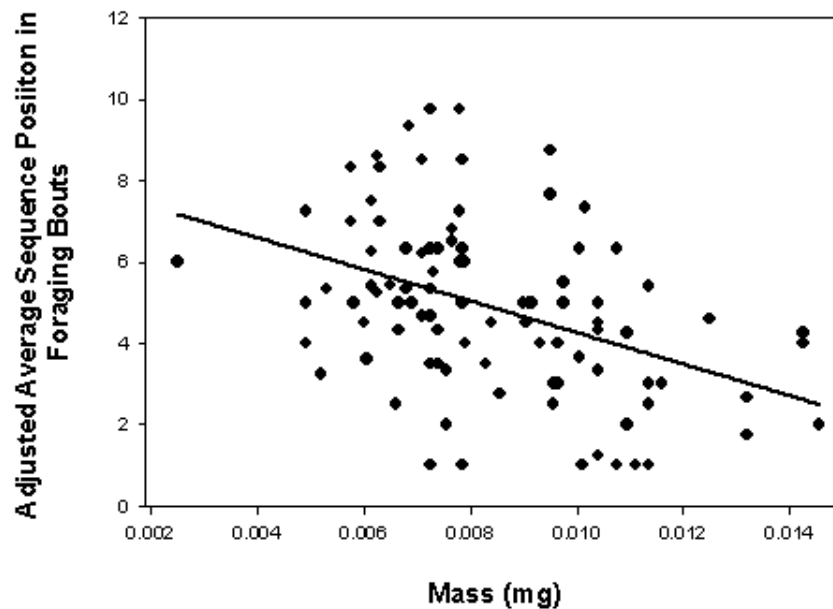
Figure 5.3 The effect of flower mass on the average position of plants within hawkmoth visit sequences. Plants with lower values of average sequence position in foraging bouts were visited earlier. Data points are adjusted to account for the effects of differences among replicate arrays. Solid line is the predicted sequence position.

Stigma-anther separation (herkogamy) was the sole variable influencing the number of times a plant was visited and the number of flowers visited per plant (Table 5.1). Plants displaying reverse herkogamy received more visits from hawkmoths than did plants displaying approach herkogamy (Figure 5.4A). This result was contrary to my predictions because I expected hawkmoths to respond to floral reward but not to floral dimensions, with the possible exception of flower size.
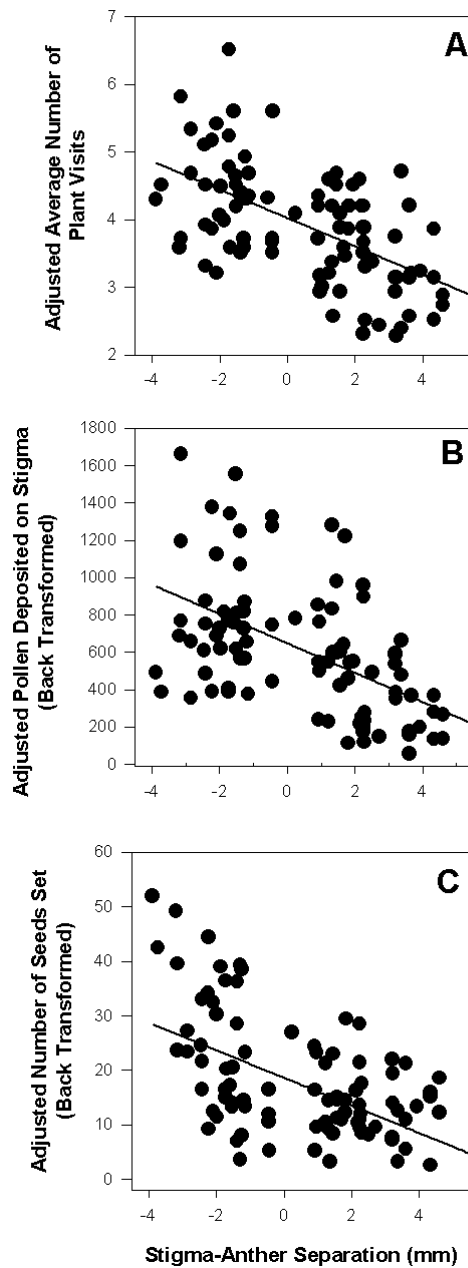
Figure 5.4 Effect of stigma-anther separation (herkogamy) on the number of times a plant was visited by *Hyles gallii* (A), number of pollen grains deposited on the stigmas of *P. brandegeei* array plants (B), and the number of seeds set (C) by array plants after hawkmoth pollination. All points are adjusted to account for the effects of other significant covariates and differences among replicate arrays. Solid lines are the predicted functions for each relationship.

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Table 5.2. Analyses of traits affecting female function (pollen deposition and seed set) in experimental populations of *Polemonium brandegeei* after pollination by *Hyles gallii*. Slope estimates, *b* (SE), are in bold and are from untransformed analyses. Selection gradients, *β* (SE), are in normal typeface and are based on untransformed, but standardized data. Initial models included all floral measurements and visits per plant. Non-significant covariates were deleted using backwards elimination.

Effect	Pollen Deposition	Seeds Set
Array	$F_{5,87} = 1.71$	$F_{5,87} = 5.33*$
Tube Length	$F_{1,87} = 17.85***$	
b (SE)	**2.61 (0.621)**	n.s.
β (SE)	0.095 (0.02)	
Herkogamy	$F_{1,87} = 6.62**$	$F_{1,87} = 5.38**$
b (SE)	**-0.040 (0.017)**	**-0.782 (0.52)**
β (SE)	-0.070 (0.030)	-1.05(0.43)
Number of Plant Visited	$F_{1,87} = 6.22**$	$F_{1,87} = 3.33^{\dagger}$
b (SE)	**0.139 (0.056)**	**0.509 (0.29)**
β (SE)	0.034 (0.015)	0.445 (0.24)
R^2 of Model	0.52	0.27

$*P < 0.05, \ **P < 0.001, \ ***P < 0.0001, \ ^{\dagger}P \approx 0.078$

5.3.2 Female function – pollen deposition and seed set

Pollen deposition varied with both floral traits and hawkmoth behaviour. The number of pollen grains deposited on stigmas was positively related to the number of visits received by a plant, and to the length of the corolla tube (Table 5.2). Pollen deposition was also higher in plants with reverse herkogamy and lower in plants with approach herkogamy (Table 5.2; Figure 5.4B). These independent effects of pollinator

preference and floral dimensions on pollen movement were consistent with my expectations.

Seed set (female fitness) varied slightly across arrays and was influenced by herkogamy but not by any other traits (Table 5.2). The effect of array may reflect differences in the number of moths among arrays. As was the case for visits per plant and pollen deposition, seed set was highest in plants with reverse herkogamy and lowest in plants with approach herkogamy (Figure 5.4C), resulting in a very strong negative selection gradient (β = -1.05). Selection on herkogamy through female function was stronger than through male function (Welch's t = -1.14, $P < 0.05$; Appendix 3.3). The effect of herkogamy was in line with my predictions, but I also expected visits per plant and tube length to affect seed set. The effect of tube length did not approach significance ($F_{1, 87}$= 0.05, $P > 0.80$). However, visits per plant had a marginally nonsignificant effect on seed set ($F_{1, 87}$ = 3.33, $P > 0.07$), indicating that pollinator preference may have effects on seed set that are independent of sex-organ position. Power analysis with G*Power 3.1 (Faul et al. 2009) indicated strong statistical power to detect selection through female function (power: 0.96). Although seed set was correlated with total pollen deposition (r = 0.604, P = 0.0028), pollen deposition was not significant in the full ANCOVA model analyzing seed set ($F_{1, 86}$ = 1.31; P = 0.256).

5.3.3 Male function – pollen removal and siring success

Pollen removal by hawkmoths was influenced only by the number of times a plant was visited (Table 5.3). Plants receiving more visits had less pollen remaining in their anthers, and thus more pollen removed, than did plants receiving fewer visits.

Table 5.3 Analyses of traits affecting male function (pollen remaining in anthers; general linear model and seeds sired; generalized linear model) in experimental populations of *Polemonium brandegeei*, after pollination by *Hyles gallii*. Slope estimates, *b* (SE), are in bold and are from transformed data, while selection gradients*, β* (SE), are in normal typeface and are from untransformed but standardized analyses. Initial models included all floral measurements and visits per plant. Non-significant covariates were deleted using backwards elimination.

Effect	Pollen Remaining	Seeds Sired
Array	$F_{5,87} = 1.46$	$\chi_{1,86} = 2.34$
Number of Plant Visits	$F_{1,87} = 7.21**$	$\chi_{1,86} = 3.48^{\dagger}$
b (SE)	**-76.79 (28.56)**	**53.99 (27.4)**
β (SE)	-0.034 (0.01)	0.399 (0.31)
Tube Diameter		$\chi_{1,86} = 4.26*$
b (SE)	n.s.	**-0.345 (0.29)**
β (SE)		-0.260 (0.12)
Total Sugar Content		$\chi_{1,86} = 4.57*$
b (SE)	n.s.	**0.633 (0.25)**
β (SE)		0.378 (0.17)
R^2 of Model	0.21	N/A

$*P < 0.05$, $**P < 0.01$, $^{\dagger}P \approx 0.09$

I genetically screened a total of 355 offspring, and was able to successfully assign

paternity for 275 (77%) at the minimum confidence level of 0.80. The remaining

offspring were not assigned paternal components, and therefore removed from the data

set. I assigned paternity to an average of 3.5 offspring per maternal plant.

The number of seeds sired (male fitness) depended on nectar sugar and corolla-

tube diameter (Table 5.3; Figure 5.5A and 5.5B). Strong selection favored higher sugar

content (β = 0.378), while weaker but significant selection favored narrow tubes (β = -

0.260). The number of plant visits only had a marginally nonsignificant positive

influence on siring success (Wald's χ^2 = 3.48, P = 0.099), which contrasted with the

strong effect of visit number on pollen removal (Table 5.3). Finally, the amount of pollen

remaining in the anthers was not a good predictor of the number of seeds sired

(r = 0.0003; P = 0.998). I had moderate statistical power to detect selection through

male function (power: 0.68) as determined with G*Power 3.1 (Faul et al. 2009).
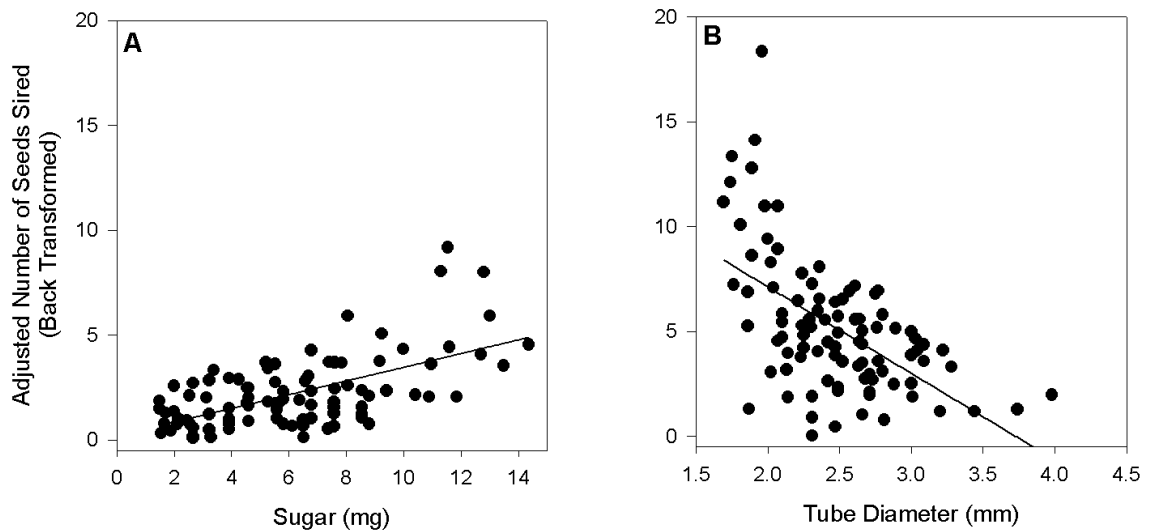
Figure 5.5 Effect of nectar sugar content (A) and corolla tube diameter (B) on male

fitness (seeds sired) of *P. brandegeei* array plants after pollination by *Hyles gallii.*

All points are adjusted to account for the effects of other significant covariates and

differences among replicate arrays. Solid lines are the predicted functions.

5.3.4 Projected Pursuit Regression

The projected pursuit regressions largely supported my multiple regression

analyses.   First, analyses of female, male and total fitness all described primarily linear

fitness functions with two effective parameters (mean parameter value for the three

analyses = 2.002). Secondly, the floral traits identified as important for female and male

fitness from my ANCOVA's approach also had a large influence on fitness through

projected pursuit regression. Herkogamy contributed the most to variation in female

function whereas tube diameter contributed the most to variation in male function (Figure

5.6). However, the coefficient for nectar sugar content along projection 1 was not

statistically significant (Figure 5.6). Interestingly, herkogamy was the only variable that contributed significantly to variation in total fitness. Two individuals were removed that had abnormally high male fitness (15 and 18 seeds sired), relative to the rest of my observations. The extreme values of these individuals compressed the other data points; this increased the apparent linearity of the spline curve. As I was testing for multivariate selection, I did not want to bias the test. Removal of these two points also improved the GCV score from 0.84 to 0.51 indicating a better fit for the spline curve. However, the removal of these points did not qualitatively change the results of the multivariate analysis.

Tube Diameter	0.314		Tube Diameter	0.132		Tube Diameter	0.908
Herkogamy	0.914		Herkogamy	0.849		Herkogamy	0.147
Nectar Sugar	0.317		Nectar Sugar	0.004		Nectar Sugar	0.297
Mass	0.030		Mass	0.314		Mass	0.330
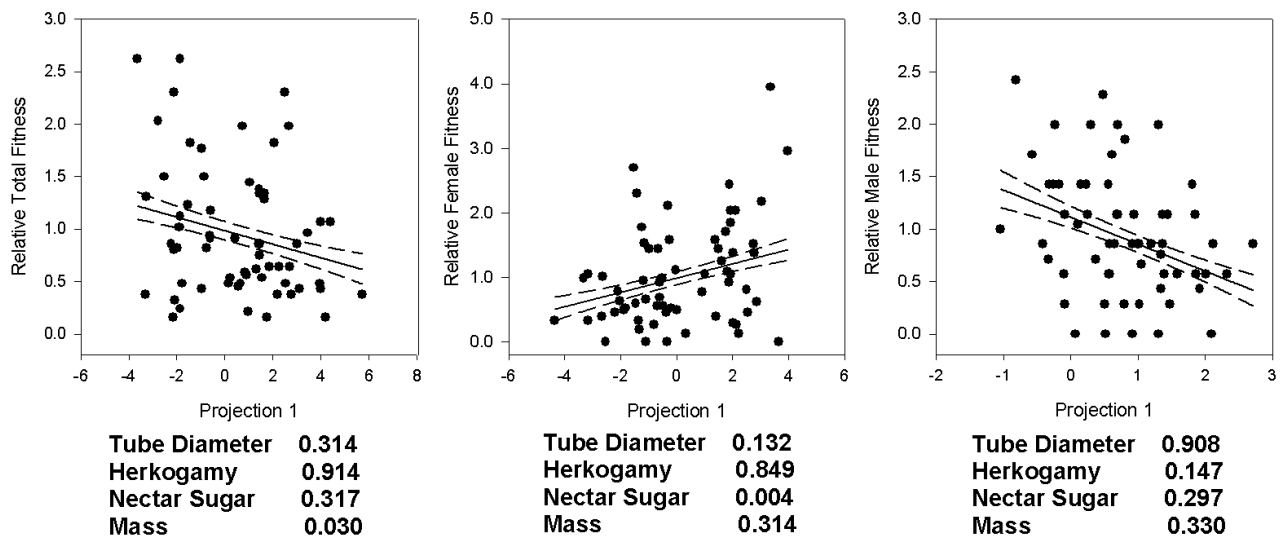
Figure 5.6 Relative total fitness (A), female fitness (B), and male fitness (C) along the first fitted projection from projected pursuit regression analysis. Solid lines represent the mean bootstrap predictive line, and dashed lines represent a 95% confidence interval. Smoothing parameters (λ) values were 6, 6, and 10 for A, B, and C, respectively. Values were chosen based on minimized GCV scores. Values below each panel represent mean bootstrap estimates of variable (floral trait) coefficients. Significance of estimates and confidence intervals were generated from 1000 bootstraps.

5.4 DISCUSSION

The attraction of pollinators and facilitation of efficient pollen transfer are important components of reproductive success in flowering plants. I measured these components under a single pollinator in a controlled setting, using plants with a constant flower number. This approach gave us the statistical power to examine in detail how hawkmoths select on floral design, and represents one aspect of selection that would occur in natural populations.

I detected selection on floral design through both female and male function. Nectar sugar and corolla tube diameter were targets of selection through male function, and relative sex-organ position (herkogamy) was selected through female function. Further, selection through female function on herkogamy was stronger than selection through male function on tube diameter ($t = -1.56$, $P < 0.05$) but not nectar sugar ($t = 4.04$, $P > 0.05$). Plants with recessed stigmas (reverse herkogamy) received more visits, had more pollen deposited, and set more seeds than plants with approach herkogamy. The importance of female function contrasts with predictions in the literature that reverse herkogamy promotes siring success (Webb and Lloyd 1986; Barrett et al. 2000) and the expectation that selection on male function should be stronger than selection on female function (Bateman 1948). My results suggest that recessed stigmas may promote both pollinator attraction and efficient receipt of pollen.

5.4.1 Hawkmoth preferences and foraging behaviour

The preference of hawkmoths for flowers displaying reverse herkogamy was unexpected and, to my knowledge, has not been previously described. Several studies have noted the hovering habit of hawkmoth pollinators; moths remain suspended above flowers, and extend their proboscis to probe flowers (Eisikowitch and Galil 1971; Willmot and Burquez 1996). Therefore, it may be more efficient for a hawkmoth to forage on flowers with fewer exserted sex-organs. The recessed stigmas of reverse herkogamous flowers would not obstruct the foraging target (opening of corolla tube) as much as the exserted stigmas of approach herkogamous flowers. The preference for reverse herkogamy was evident when flower number (floral display) was held constant; additional studies that vary flower number would be required to assess the relative influence of sex-organ position and display size.

Pollinators often prefer larger flowers over smaller ones because information from larger flowers may be more easily processed by insects (Spaethe et al. 2001). Also, large flowers typically offer more floral rewards (Cresswell and Galen 1991; Cresswell 1998; Vaughton and Ramsey 1998), and may therefore provide a visual cue of reward level (Bell 1985; Armbruster et al. 2005). Indeed, hawkmoths have been shown to directly respond to increased reward availability and display (Hodges 1995). I observed an initial preference for large flowers in the sequence of plants visited by *Hyles gallii*. However, I did not observe a correlation between flower size and reward. Therefore, hawkmoths initially cued in on large flowers but, without an associated reward, the initial preference did not translate into a higher total number of visits to plants with large flowers. In my experiment, higher sugar content did enhance siring success and may have increased the

handling time of flowers by hawkmoths. However, I did not observe greater removal of

pollen from plants with higher nectar sugar. Nor did I find the average phenotype of

plants visited within a foraging bout to be a good predictor of the average time spent

visiting a flower. Timed visitation trials with naive and experienced hawkmoths would

be required to conclusively determine all aspects of hawkmoth foraging preferences.

Overall, captive hawkmoths in my study appeared to behave similarly to

hawkmoths in natural settings. Reports on the number of flowers and plants visited by

hawkmoths are rare, but the patterns I observed (13.36 ± 1.49 flowers per bout; Appendix

A) were similar to field observations (10.2 ± 2.2 flowers per bout; Kulbaba and Worley,

2008). On average *Hyles gallii* visited a similar number of *P. brandegeei* flowers per

foraging bout as did its close relative *H. lineata* (11-17 flowers per bout) in natural

populations of *Ipomopsis tenuituba* (Aldridgde and Campbell 2007). Hawkmoths

foraging in natural populations with more plants may be able to show stronger

discrimination among phenotypes than I observed. However, in my study moths never

visited more than half of the available flowers in a bout and seed set appeared to be

pollen limited. Therefore, hawmoths were apparently not compelled to visit unattractive

phenotypes in my arrays. In my artificial *P. brandegeei* populations, hawkmoths did not

revisit the same plant within a bout. This result implies that hawkmoths retain

information about which plants they have recently visited, possibly to avoid flowers

already emptied of nectar.

The foraging behaviour of pollinators can strongly influence both female and

male fitness through effects on outcrossed and self-pollen movement. Visits to multiple

flowers on a plant introduce the potential for geitonogamous (self) pollen transfer. Self-

pollen deposition under bee pollination is minimized when female-phase flowers are positioned below male-phase flowers because bees typically forage from bottom to top and therefore visit female flowers first (Harder et al. 2000; 2004). In my study *Hyles gallii* were equally likely to move upwards or downwards. If this unpredictability is a general feature of hawkmoth pollination, the spatial distribution of floral gender may not affect mating patterns in hawkmoth pollinated species.

5.4.2 Selection on floral design

Based on the literature, I hypothesized that sex-organ position would affect the efficiency of pollen dispersal under hawkmoth pollination. Specifically, I expected stigmas recessed within narrow corolla tubes (reverse herkogamy) to receive more outcrossed pollen than exserted stigmas. Although recessed stigmas sometimes receive more self-pollen than exserted stigmas under moth pollination (Murcia 1990), the effects of herkogamy on pollen deposition and seed set were very similar in self-sterile *P. brandegeei*. This similarity implies that the larger pollen loads received by reverse herkogamous plants reflected an increase in outcrossed pollen. In contrast to herkogamy, increased tube length was associated with higher pollen deposition but had no effect on seed set. Thus, longer floral tubes may increase self-pollen deposition in *P. brandegeei*, as occurs in *Ipomea trichocarpa* when visted by the moth *Enyo lugubris* (Murcia 1990).

My analyses also suggested that herkogamy had direct effects on pollen deposition and seed set that were independent of pollinator attraction. Galen and Newport (1987) hypothesized that selection promoting pollination efficiency should be

stronger than selection for increased floral rewards, which are energetically expensive, and require additional energy expenditure to increase pollen receipt. Selection on herkogamy was very strong in my experiment and I further suggest that selection on traits that enhance both pollination efficiency and attraction may be stronger than selection on traits that enhance either function alone. Sex-organ position in moth-pollinated plants may be one such trait. A full decoupling of attraction and efficiency would require analysis of pollen loads following single visits by hawkmoths to approach and reverse herkogamous plants. One could then determine if the increased pollen deposition on reverse herkogamous flowers is due to a functional association between recessed styles and hawkmoth morphology or repeated visitation (attraction). Additional measurements would allow us also to explore the possibility that correlations with other traits such as ovule number or pollen viability may contribute to the correlation between herkogamy and seed set.

Selection on sex organ position may depend on the size and shape of the corolla. A recessed stigma is most efficient when the corolla tube is shorter than the moth's proboscis, as in *P. brandegeei*, so that the proboscis is the pollen bearing surface (Nilsson, 1988). Although pollen may be deposited towards the mouth, coiling and uncoiling of the proboscis as the moth moves between plants redistributes the pollen along the length of the proboscis (M. Kulbaba, personal observation). By contrast, some hawkmoth pollinated species such as *Gladiolus longicollis* produce a corolla tube longer than the pollinators' proboscis (Alexandersson and Johnson 2002). These longer tubes force the moths' head deeper into the opening of the flower; thus, pollen is transported on the head instead of the proboscis. This situation may explain why stabilizing selection

for approach herkogamy occurs in hawkmoth pollinated *Ipomoea wolcottiana* (Parra-Tabla and Bullock 2005).

Narrow corolla tubes are generally associated with pollination by lepidopterans (Grant 1983; Grant 1985). Narrow tubes may enhance pollen pickup by increasing the proximity, and likelihood of contact, between the anthers and pollen carrying proboscis. To my knowledge, my study was the first to demonstrate selection on tube diameter through siring success (male fitness). However, Campbell et al. (1997) detected hawkmoth-mediated selection for narrow corolla-tubes through female fitness.

Although selection by hawkmoths may promote reverse herkogamy and narrow corolla tubes, approach herkogamy and relatively wide corollas are common in natural populations of *P. brandegeei*. An additional and more frequent pollinator of *P. brandegeei* is the hummingbird *Selasphorus platycercus* (Kulbaba and Worley 2008). Selection through male function for wider corolla tubes occurs in hummingbird-pollinated *Ipomopsis aggregata* (Campbell et al. 1991; Campbell et al. 1996), whereas selection through female function promotes exserted stigmas (Campbell 1989). Wider corolla tubes and exserted stigmas allowed the pollen bearing faces of hummingbirds to better remove and deposit pollen, respectively (Campbell 1989; Campbell et al. 1996). Therefore, when selection by only hawkmoths was measured in my study, it is not surprising that I found negative directional selection on corolla width and herkogamy. An analogous experiment to measure hummingbird-mediated selection on *P. brandegeei* was conducted to determine if hummingbird pollinators impose contrasting selection on corolla shape and sex-organ position (Chapter 6).

In addition to hawkmoths and hummingbirds, *P. brandegeei* is frequented by a diverse array of apparently ineffective floral visitors (Kulbaba and Worley 2008). Pollen theft by less effective visitors could reduce reproductive output by reducing the total available pollen for dispersal (Hargreaves et al. 2010). In other species, interactions with herbivores (Kessler et al. 2011) and resource variation among microsites also influence selection by pollinators (Sánchez-Lafuente et al. 2005). Indeed, environmentally induced variation may often be lower in laboratory than in natural populations (Endler 1986). Thus, the range and distribution of traits I used may have differed from those in natural populations, although mean values were kept similar. All the above factors may moderate the strong selection by hawkmoths that I observed, which exceeded that normally seen in natural populations (Kingsolver and Pfennig 2007). However, my observed selection gradients are consistent with the prediction that hawkmoths maintain reverse herkogamy in natural populations of *P. brandegeei*.

Natural selection may promote the co-occurrence of particular combinations of traits, without affecting the distribution of either trait individually (Brodie 1992). This possibility is best explored through multivariate analyses of fitness surfaces (Schluter and Nychka 1994; Maad 2000). However, in this study, projected pursuit regression indicated linear selection by *Hyles gallii* on *P. brandegeei* floral traits. The results largely confirmed my analyses of covariance, although the effect of nectar sugar content on siring success was not detected through the projected pursuit regression ($P > 0.05$). As in the analysis of covariance, herkogamy had the largest influence on female and total fitness.

5.4.3 Pollinator response, fitness surrogates, and gender-specific selection

Given the logistics of estimating siring success, many studies of natural populations must rely on surrogate measures to estimate male fitness. Surrogates include pollen removal (Maad and Alexandersson 2004), insect visitation rates (Conner and Rush 1996), and pollen dyes (Van Rossum et al. 2010). However, these surrogates sometimes yield biased estimates of male function (Klinkhamer et al. 1994). In my study, reliance on visitation rates or pollen removal would have given an incomplete picture of selection on flower traits through male function. First, selection on tube diameter and sugar content was not apparent in the surrogate measure of male fitness. Second, the removal of pollen by *Hyles gallii* was largely influenced by the number of times a plant was visited, as has been found for many other floral visitors including bees (Galen 1989; Galen 1992), birds (Engel and Irwin 2003), and bats (Arias-Coyotl et al. 2006). However, in my study, visit number only had a marginal effect on siring success. Independence of pollen removal and siring success may commonly occur in species producing granular pollen due to pollen wastage, and loss to pollen consumers (Galen 1992; Kobayashi et al. 1999; but see Ashman 1998).

Direct measurements of female fitness are easy to obtain compared to measures of male fitness, although surrogate measures of female fitness are sometimes necessary in community-wide studies (Conner 2006; Alacórn 2010). In my study, pollinator behaviour and pollen deposition were reliable indicators of selection on herkogamy through female fitness (Table 1 and 3). However, longer corolla tubes increased pollen deposition, but did not increase seed set.

The relative direction and magnitude of selection on floral traits through female and male function is still contentious (Sahli and Conner 2011). Selection through male function should be strongest when seed production is limited by available resources (resource limitation) and siring success is limited by mating opportunities (Bateman 1948). However, the magnitude of selection through female function should increase when seed production is limited by mating opportunities (pollen limitation; Wilson et al. 1994; review in Harder and Aizen 2010). Pollen limitation may be common in natural populations (Ashman and Morgan 2004). In my study, selection through female function on herkogamy was much stronger than observed through male function on corolla width and nectar sugar content, perhaps because greenhouse conditions and limited numbers of pollinators resulted in pollen limitation of seed set. Most of the few studies that examined selection through both gender functions have found no major discrepancies in selection strength (O'Connell and Johnston, 1998; Ashman and Morgan 2004; Sahli and Conner 2011; but see Hodgins and Barrett 2008b), nor have many instances of contrasting selection among gender functions been reported (but see Campbell 1989). The discrepancy in the strength of selection between female and male function found in my study highlights the importance of female function in the evolution of floral design.

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Appendix 5.1 Summary statistics of measured floral traits pooled across all 6 replicate arrays (n = 94). All measurements are in millimetres except mass (g), sugar (mg) nectar volume (μL), and number of visits (counts). As herkogamy contained negative values, I added the absolute value of the most negative value of herkogamy (3.89) to each value of herkogamy to shift the distribution to the right of zero

Trait	Minimum	Maximum	Mean	SE	CV
Flower Width	9.82	22.14	16.09	0.32	13.8
Tube Length	17.13	30.52	22.98	0.34	14.2
Basal Tube Diameter	1.74	3.98	2.56	0.04	16.63
Apical Tube Diameter	3.05	5.87	4.03	0.06	14.54
Herkogamy	-3.89	4.62	0.58	0.24	52.89
Style Length	19.19	36.67	26.26	0.34	12.70
Anther Height	18.28	36.8	25.70	0.39	15.08
Mass	0.003	0.015	0.008	0.0002	27.21
Nectar Volume	0.25	7.79	2.66	0.18	64.26
Sugar	1.48	143. 40	54.13	2.94	52.58
Flower Visits/Bout[†]	2.00	26.00	13.39	0.49	38.98
Plant Visits/Bout[†]	1.00	7.00	3.67	0.11	29.47
Foraging Time (Seconds)[†]	7	196	108.2	7.10	46.84
Avg. Flowers Visited/Plant/Bout[†]	1.3	4.5	3.4	0.097	20.68
Total Flower Visits [††]	112	235	194.17	18.87	9.72

[††] based on n = 1165 observed flower visits across 6 replicate arrays

Appendix 5.2 Pearson's correlations of floral traits and the number of flowers visited. Values are pooled across all six replicate arrays. All values were $\log_{10}$ transformed for normality assumptions except for the number of flower visits which was square-root transformed.

	FW	TL	TS	TF	Style Length	Anther Height	Herkogamy	Nectar Volume	Sugar	Mass	Flower Visits	Plant Visits
FW		**0.621**	**0.443**	**0.416**	**0.618**	**0.569**	-0.111	0.001	0.111	**0.666**	0.126	0.152
TL			**0.379**	**0.491**	**0.754**	**0.939**	**-0.410**	**0.209**	-0.152	**0.702**	**0.244**	0.193
TS				**0.715**	**0.437**	**0.400**	-0.086	0.048	0.008	**0.681**	0.002	-0.025
TF					**0.491**	**0.506**	-0.151	0.041	0.014	**0.693**	0.061	0.004
Style Length						**0.685**	0.145	0.145	0.007	**0.684**	-0.075	0.005
Anther Height							**-0.547**	0.169	-0.138	**0.646**	**0.282**	**0.215**
Herkogamy								-0.122	0.081	-0.126	**-0.463**	**-0.294**
Nectar Volume									0.073	0.175	0.107	0.052
Sugar										0.107	-0.070	0.050
Mass											0.072	0.044
Flower Visits												**0.791**

Boldface correlations are significant at $P < 0.05$

Appendix 5.3   Analyses of selection via female (seed set) and male (seeds sired) fitness in experimental populations of *Polemonium brandegeei* after pollination by *Hyles gallii*. Slope estimates, *b* (SE), are in bold and are from untransformed analyses. Selection gradients, *β* (SE), are in normal typeface and are based on untransformed, but standardized data. Initial models included all floral measurements and visits per plant. Non-significant covariates were deleted using backwards elimination.

Effect	Female Fitness (Seeds Set)	Male Fitness (Seeds Sired)
Tube Diameter		
b (SE)	**-0.057 (0.372)**	**-0.327 (0.149)***
β (SE)	-0.016 (0.187)	-0.327 (0.290)*
Herkogamy		
b (SE)	**-0.049 (0.077)**	**-0.021 (0.026)**
β (SE)	-0.047 (0.039)	-0.026 (0.049)
Sugar Content		
b (SE)	**0.001 (0.005)**	**0.005 (0.002)****
β (SE)	0.0004 (0.0028)	0.005 (0.004)**
Number of Plant Visits		
b (SE)	**0.069 (0.038)**	**0.064 (0.013)***
β (SE)	0.034 (0.020)	0.064 (0.025)*

* $P < 0.05$, ** $P < 0.001$

CHAPTER 6. COMPARING SELECTION ON FLORAL DESIGN IN *POLEMONIUM BRANDEGEEI* (POLEMONIACEAE): FEMALE AND MALE FITNESS UNDER HUMMINGBIRD AND HAWKMOTH POLLINATION

6.0 CHAPTER SUMMARY

Particular floral phenotypes are often associated with specific pollinators or groups of pollinators. However, flowering plants are often visited, and may be pollinated by more than one type of animal. Therefore, a major outstanding question in floral biology asks: what is the nature of selection on individual floral traits when pollinators are diverse? This study examined how hummingbirds (*Archilochus colubris*) selected on the highly variable floral traits of *Polemonium brandegeei,* a plant species pollinated by both hummingbirds and hawkmoths. I then compared the patterns of selection by hummingbirds with my previous study examining selection by hawkmoths (Chapter 5). I documented contrasting selection on herkogamy through female function, with hummingbirds exerting positive selection and hawkmoths exerting negative selection on herkogamy. These pollinators also selected on corolla dimensions, with hummingbirds selecting for longer and wider corolla tubes, and hawkmoths selecting for narrower corolla tubes. Therefore, contrasting selection brought about by varying abundances of hawkmoths and hummingbirds, may account for the high degree of trait variation in *P. brandegeei*. Further, I have documented how floral traits under selection by multiple pollinators can result in either an intermediate "compromise" between the selective pressures (herkogamy), or a "mosaic" of apparently specialized floral phenotypes (corolla dimensions).

6.1 INTRODUCTION

Despite the apparent specialization of floral traits, the flowers of many plant species are pollinated by two or more functional group of pollinators that may each contribute to selection on floral traits (Waser et al. 1996; Ollerton 2006). The response to selection by multiple groups of pollinators can be varied, depending on the strength and nature of selection by each pollinator (Aigner 2001; Sahli and Conner 2011). Therefore, characterizing selection by each functional group of pollinator is an essential first step toward understanding net selection exerted on floral traits, and thus the evolution of floral design.

Sahli and Conner (2011) described four potential responses to variable selection by two hypothetical pollinators. First, the mean trait value may conform to an "intermediate" or "compromise" phenotype between the optima of two pollinators. This result would occur if the pollinators exerted contrasting selection on the same trait. Second, the mean trait value may adaptively specialize to both pollinator groups, if the pollinators select on the same trait in a similar manner. Third, the mean value of a floral trait may specialize to one pollinator over another. This response may occur when a particular trait is more critical for pollination to one pollinator, or the trait optimum for the second pollinator covers a wider trait range. Finally, the simultaneous presence of both pollinators may change the nature of selection. Therefore, selection may be non-additive when two pollinators overlap, compared to when each pollinator occurs alone. These four outcomes may differ among floral traits so that flowers may have a combination of intermediate phenotypes and/or a mosaic of phenotypes apparently specialized to different pollinators (cf. Aigner 2001). Regardless of the response, few

studies have examined the potential for selection by multiple pollinators (but see Sahli and Conner 2011).

Depending on the strength and nature of selection, variation in pollinators may influence genetic variation in floral traits, as well as trait means (Siepielski et al. 2009). Selection may be variable over time, with the magnitude and even direction changing with pollinators (Gómez et al. 2009; Siepielski et al. 2009), and therefore play a significant role in maintaining floral trait variation (see Forrest et al. 2011). For example, variation in the pollinator assemblage of *Calathea ovandensis* contributed to variation in corolla length (Schemske and Horvitz 1989). Alternatively, parallel selection on the same floral trait by multiple pollinators may result in additively stronger overall selection, and therefore reduced variation. However, the prevalence of parallel versus contrasting selection by pollinators is unknown (Sahli and Conner 2011).

Even in the presence of a single pollinator species or functional group, the response of heritable traits to selection may be constrained by gender conflict or genetic correlations (Roff 1997; Kellermann et al. 2009). First, contrasting selection through female and male functions has been predicted to be common in hermaphrodites (Morgan 1992). However, few studies have compared gender-specific selection on floral traits because male fitness (seeds sired) is difficult to assess and therefore seldom directly measured. Studies comparing gender specific selection on floral traits by multiple pollinators are even more rare. Finally, correlations among floral traits can also constrain the response to selection, irrespective of gender function or differing targets of selection among pollinators (Delph et al. 2004).

Hummingbirds are an important functional group of new world pollinators, and have influenced the floral design of many North American plant species. A common feature of plant taxa pollinated by hummingbirds is a tubular corolla, of similar length to the pollinator's bill (Grant and Grant 1968). The correspondence between tube and bill length enforces contact between plant sex-organs and the feathered regions of the bird's head where pollen is carried (Lertzman and Gass 1983). The typical exsertion of sex-organs beyond the opening of the corolla tube further reinforces contact between sex-organs and the hummingbird. In contrast, plants pollinated by hawkmoths display flowers with narrow corolla-tubes, and sex-organs recessed within the tube (Alexandersson and Johnson 2002). Finally, plants pollinated by hummingbirds often produce large volumes of dilute nectar (Baker 1972). However, the production of dilute nectar may have evolved more as a deterrent to bee visitation and overly vigorous bird visitation, rather than as a bird attractant (Cronk and Ojeda 2008).

The sub-alpine perennial *Polemonium brandegeei* displays floral traits associated with hummingbird and hawkmoth pollination, and both animals are confirmed pollinators. Overall, *P. brandegeei* flowers exhibit tubular corollas of dimensions that are typical of hummingbird-pollinated taxa (Chapter 2; Kulbaba and Worley 2008). However, the cream-white colouration of the corolla, and the strong, heavy sweet smell emitted by the flowers are traits associated with hawkmoth pollination (Grant 1983; Grant 1985). The flowers of *P. brandegeei* display exceptional continuous and heritable variation in style length, resulting in a range of approach (exserted stigmas) to reverse (recessed stigmas) herkogamy. Here I explore selection by hummingbirds through direct measurements of female and male selection on floral traits. I then compare these results

to my study examining selection by hawkmoths on *P. brandegeei* floral design (Chapter 5).

I explored the behaviour of and selection imposed by hummingbirds on the floral design of *P. brandegeei*. Specifically, (1) I predicted that increased flower size, or nectar reward (volume, sugar concentration), would result in more visits from hummingbirds and/or increased handling time of flowers. In contrast to floral rewards, I did not expect herkogamy or corolla dimensions other than flower size to affect hummingbird behaviour, but I did expect these traits to influence female and male fitness through their effects on pollen removal and deposition, respectively. In particular, (2) I expected plants displaying flowers with approach herkogamy to receive more outcrossed pollen, and therefore set more seeds. Finally, (3) I predicted that plants displaying flowers with longer corolla-tubes would have more pollen removed from their anthers, and sire more seeds under hummingbird pollination because the corolla tubes of *P. brandegeei* flowers are generally shorter than the hummingbird's bill (Chapter 2).

6.2 MATERIALS AND METHODS

6.2.1 General design

I used a similar approach to that described in Chapter 5, where I measured pollen movement and selection by hawkmoths (*Hyles gallii*), with all differences presented below. While the Ruby-throated Hummingbird (*Archilochus colubris*) is not a confirmed pollinator of *P. brandegeei*, it is similar in behaviour and morphology (most importantly bill length; 13.4 to 19.0 mm) to the confirmed bird pollinator, Broad-tailed Hummingbird, *Selasphorus platycercus* (16.0 to 20.3 mm; Pyle 2001).

I grew individuals of *P. brandegeei* from seed collected at Taylor Canyon, Colorado (N 39° 34' 33'' W 104° 22' 26'') under similar greenhouse conditions to those described in Chapter 5. I chose individual plants to include in experimental populations, hereafter arrays, containing 12-14 plants based on their floral phenotypes. As one of my major goals was to examine how hummingbirds selected on stigma-anther separation (herkogamy), I ensured an equal number of approach and reverse herkogamous plants in each array. As with the hawkmoth selection experiment, the range of herkogamy and corolla trait variation in my experimental arrays was similar to the variation observed in natural populations (Appendix 6.1).

I conducted the experiments in two rural Manitoba locations (approximately 174 km separation) to ensure different birds, and therefore potential variation in bird-behaviours, were involved in the pollination of my experimental plants. The first location was in an oak/aspen woodland north of Anola, Manitoba (N 49° 56.355' W 96° 36.734'). My second location was west of Overton, Manitoba (N 51° 00.719' W 98° 46.664'), in another oak/aspen clearing. In May of 2010, I presented returning migrant

Ruby-throated Hummingbirds with feeders consisting of 30% sucrose solution, to entice hummingbirds to our study sites. Feeders remained at the sites for the duration of the experimental period, but were emptied and thoroughly washed before arrays were assembled.

Hummingbirds visited each array for a total of two days. I presented the arrays to hummingbirds between July 18, 2010 and August 27, 2010 when weather and plant phenotype availability permitted. The experimental arrays were arranged as in Figure 5.2 in Chapter 5. The empty and cleaned feeders remained in the centre of the arrays, because the hummingbirds had been habituated to the feeder. Hummingbirds were permitted to visit each experimental population during the daylight hours (approximately 7:00 a.m. to 8:30 p.m.) for two consecutive days. An observer was present for the entire duration of each experiment, to discourage any non-bird visitors from foraging on *P. brandegeei* flowers. After dusk, the experimental plants were placed indoors to prevent night-flying visitors from foraging.

6.2.2 Hummingbird behaviour

All hummingbird foraging bouts were recorded with a video camera to document foraging behaviour on *P. brandegeei* flowers. The total number of flowers visited, number of plants visited, and number of flowers per plant visited was recorded for each foraging bout. I calculated handling time for visits to individual flowers by dividing the number of frames the visit lasted by the video frame rate (29 frames/second). Mean handling times were calculated for each plant, and used in the analyses described below.

6.2.3 Floral traits, pollen movement and fitness

I measured floral dimensions and nectar properties (volume and sugar concentration) prior to birds visiting each replicate population. Measured traits included: corolla tube-length, corolla tube-width at the base and apex of the flower, height of sex-organ presentation relative to the base of the flower, flower mass (dry weight of corolla, calyx, androecium and gynoecium; see Methods of Chapter 5). I measured nectar sugar concentration with the aid of a calibrated handheld refractometer (Fisher Scientific), with a temperature correction. I corrected our values with the conversion table (Table 5.2, page 172) in Kearns and Inouye (1993) because small volumes of nectar can introduce error when estimating the concentration of sucrose equivalents. Finally, 11 plants were reused in 3 replicate arrays, but flowers on different inflorescences were used. Therefore, I resampled the nectar properties to determine if nectar volume or sugar concentration changed, and obtained new floral measurements. Further, I performed a repeated measures analysis of variance on the number of seeds set, to explore the consistency of female fitness in these reused plants.

Pollen movement and female and male fitness were measured in the same manner as the hawkmoth selection experiment (Chapter 5). Pollen removal was quantified as the number of pollen grains remaining in anthers after hummingbird visitation, and pollen deposition was the number of pollen grains deposited on stigmas. As I was unable to distinguish between self and outcross pollen, pollen deposition measures reflect both. Female fitness was the number of seeds set, and male fitness was the number of seeds sired.

I examined statistical associations between floral traits and (1) pollinator behaviour, (2) pollen removal and deposition, and (3) female and male fitness. I used the same general analysis of covariance (ANCOVA; Proc GLM, Proc GENMOD) approach used in the hawkmoth selection experiments (see *Statistical Analyses* in Chapter 5). Pollen remaining in anthers and deposited on stigmas, and the number of seeds set (female fitness) and seeds sired (male fitness) were analyzed as dependant variables, with floral traits and visitation behaviour as explanatory covariates. Hummingbird visitation behaviour was analyzed with handling time, number of flowers visited per plant, and number of times a plant was visited ("plant visits") as dependent variables, with floral traits as explanatory covariates. Array number and site were included as fixed effects. All analyses were performed in SAS 9.1.2 (SAS institute, 2004).

To test for potential multivariate or correlated selection, I analysed nonlinear multidimensional fitness surfaces. I used the projected pursuit regression described in Schluter and Nychka (1994) to fit spline curves to our fitness data without making assumptions about the shape of the fitness function. Fitness surfaces were analysed for relative female, male, and total fitness. Total relative fitness was calculated as the average of relative female and relative male fitness. I used relative female and male fitness to prevent the larger values of seed set from dominating variation in total fitness.

6.3 RESULTS

My analyses provided the opportunity to detect both linear and nonlinear selection though analysis of covariance, and multivariate projected pursuit regression. All interaction and quadratic terms in the ANCOVA models were non-significant, and removed from the models. These results were similar in the projected pursuit regressin analysis. Therefore, the effects of covariates in the present analyses were linear, and independent of other covariates. Further, the effects of different sites (Overton and Anola, Manitoba) were nonsignificant. Analyses presented below do not include site effects.

6.3.1 Pollinator foraging behaviour

Ruby-throated Hummingbirds visited an average of 5.8 plants, 3.3 flowers per plant, and an average total of 17 flowers per foraging bout. Visits to individual flowers were very brief, with a mean handling time of 0.74 seconds (range: 0.34 to 1.33 seconds). All array plants were visited in each of the replicate arrays. I observed a total of 1042 movements between flowers, within an inflorescence. A total of 489 were movements that occurred in an upward direction, while the remaining 553 proceeded in a downward direction. I did not detect a significant difference in movements within an inflorescence by hummingbirds (χ^2 = 2.48 for expectation of equal number of upwards and downwards movements P = 0.214).

The volume of nectar produced by *P. brandegeei* flowers affected two aspects of hummingbird foraging behaviour. First, hummingbirds visited more total flowers per plant on plants that produced smaller volumes of nectar. Secondly, hummingbirds spent longer handling times on plants that produced larger volumes of nectar (Table 6.1). The

effect of array was significant in both analyses, and likely resulted from an unequal number of hummingbird visits across arrays. Across all arrays, I observed an average of 16.4 foraging bouts, but the values ranged from 12 (Array 2) to 28 (Array 5) observed foraging bouts.

Table 6.1. Analyses of *Archilochus colubris* handling time and the number of flowers visited per plant on arrays of *Polemonium brandegeei*. Handling time was log-transformed and the number of flowers visited per plant was square-root transformed prior to analysis for slope estimates b (SE) are from analyses of transformed data. Initial models included all floral measurements and non-significant covariates were deleted using backwards elimination.

Effect	Handling Time	Number of Flowers Visited per Plant
Array	$F_{4,53} = 9.54$***	$F_{4, 53} = 10.85$***
Nectar Volume	$F_{1, 53} = 3.78^{\dagger}$	$F_{1, 53} = 6.07$**
b (SE)	**0.012 (0.01)**	**-0.337 (0.14)**
R^2	0.46	0.47

**$P < 0.001$, ***$P < 0.0001$, $^{\dagger}P = 0.057$

Hummingbirds did not respond to variation in corolla dimensions or sex organ positioning. The sequence of plants visited by hummingbirds, and the total number of times an individual plant was visited did not vary with floral design or nectar rewards.

6.3.2 Female function – pollen deposition and seeds set

Basal-tube diameter (hereafter tube diameter) affected pollen deposition. Plants displaying flowers with relatively wide tubes had more pollen deposited on their stigmas

after hummingbird visitation. However, the relationship between seed set and corolla-tube diameter was marginally nonsignificant ($P = 0.074$, Table 6.2). Regardless, the number of pollen grains deposited on stigmas was significantly correlated with seed set ($r = 0.266$, $P < 0.05$). Also, the plants that were reused in multiple arrays did not set significantly different numbers of seeds across the arrays, as determined through repeated measures analysis of variance ($F_{2,32} = 0.67$, $P = 0.523$).

Table 6.2. Analyses of pollen deposition and seed set in experimental populations of *Polemonium brandegeei* after pollination by *Archilochus colubris*. Slope estimates, b (SE), are in bold and are from untransformed analyses. Selection gradients, β (SE), are in normal typeface and are based on untransformed, but standardized data. Initial models included all floral measurements and visits per plant. Non-significant covariates were deleted using backwards elimination.

Effect	Pollen Deposition	Seeds Set
Array	$F_{4,\,52} = 0.17$	$F_{4,\,52} = 9.74$*
Tube Diameter	$F_{1,\,52} = 30.86$***	$F_{1,\,52} = 1.75^{\dagger}$
b (SE)	**1.148 (0.207)**	**9.891 (5.644)**
β (SE)	*1.223 (0.816)*	*0.858 (0.489)*
Herkogamy		$F_{1,\,52} = 29.42$***
b (SE)	n.s.	**11.029 (1.971)**
β (SE)		*0.913 (0.171)*
R^2 of Model	0.37	0.52

*P < 0.05, **P < 0.001, ***P < 0.0001, †P < 0.075

Herkogamy was an important aspect of floral design for female fitness. On average, plants displaying flowers with approach herkogamy and exserted stigmas set more seeds than did plants displaying flowers with reverse herkogamy (Table 6.2; Figure 6.1). This resulted in strong selection ($\beta = 0.913$) for approach herkogamy through female function, which supported my functional predictions of sex-organ arrangement

under hummingbird pollination. Curiously, I did not detect a significant effect of herkogamy on pollen deposition.

The number of flowers and plants visited, and the number of flowers per plant visited by hummingbirds did not affect pollen deposition or seed set in my arrays. This result contradicted my expectations, because pollinator visitation patterns typically influence pollen movement patterns.
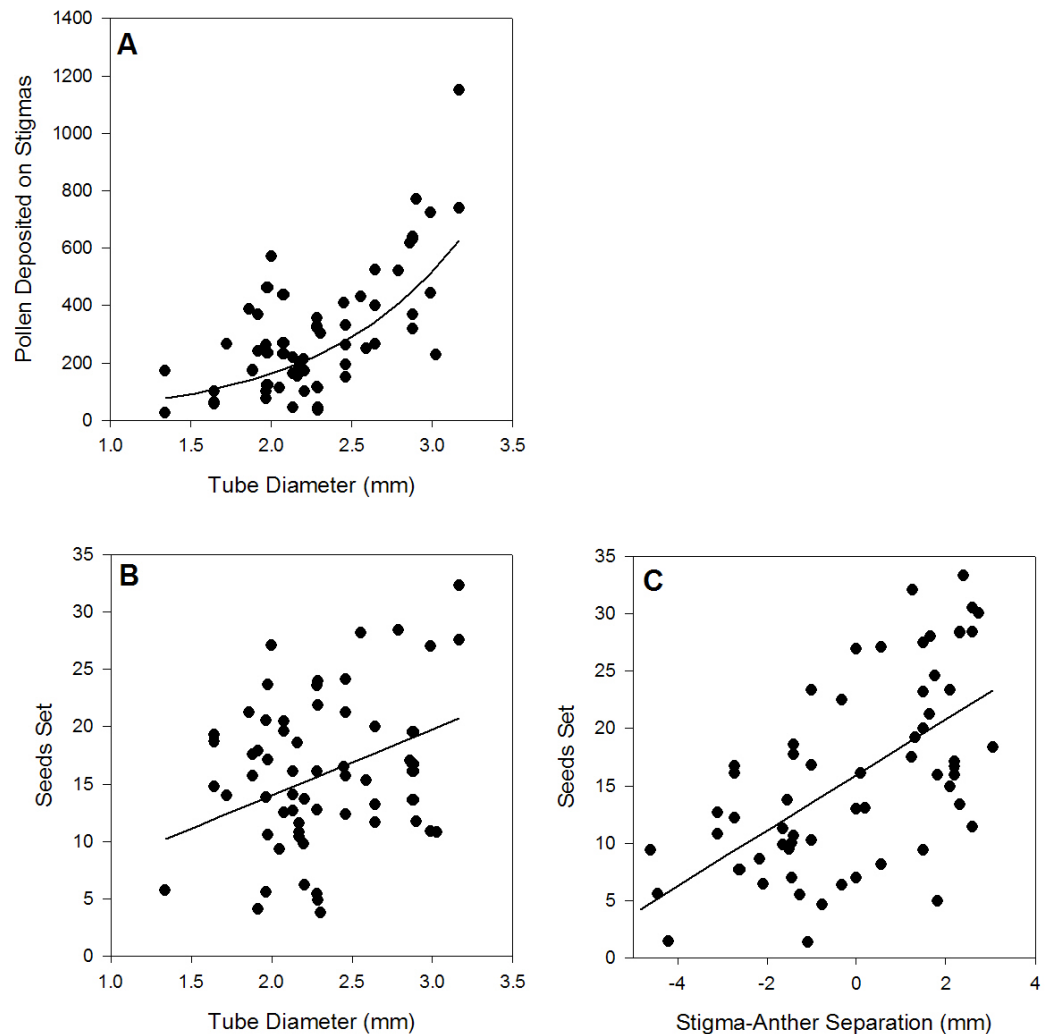
Figure 6.1. Effects of corolla-tube diameter (Panel A and B) and stigma-anther separation (Panel C) on the number of pollen grains deposited on stigmas (Panel A) and number of seeds set (Panel B and C) after pollination by *Archilochus colubris*. Points are adjusted values to account for the effects of other significant variable in the model. See Methods of Chapter 5 for further details.

6.3.3 Male function – pollen removal and seeds sired

I genetically screened 310 offspring across the 5 replicate arrays, and successfully assigned paternity to 272 individuals (ca. 88%), at the minimum confidence level of 0.80. I was unable to determine parentage for the remaining 38 offspring, and therefore removed them from the subsequent analyses. This resulted in paternity being assigned for an average of 4.3 offspring per maternal plant.

Corolla tube length but not hummingbird visitation patterns affected pollen removal in the arrays. Plants displaying flowers with relatively long corolla tubes had less pollen remaining in their anthers, and therefore more pollen removed after hummingbird visits (Table 6.3; Figure 6.2). Further, the increased pollen removal translated into more seeds sired. Therefore, I detected selection for longer corolla tubes through male function (Table 6.3; $\beta = 0.124$), which supported my prediction of a functional relationship between corolla design and male function (Figure 6.2).

Table 6.3 Analyses of pollen remaining in anthers (Pollen Removal) and seeds sired in experimental populations of *Polemonium brandegeei* after pollination by *Archilochus colubris*. Slope estimates, b (SE), are in bold and are from transformed data, while selection gradients, β (SE), are in normal typeface and are from untransformed but standardized analyses. Initial models included all floral measurements and visits per plant. Non-significant covariates were deleted using backwards elimination.

Effect	Pollen Removal	Seeds Sired
Array	$F_{4, 52} = 68.53$**	Wald's $\chi^2 = 4.52$*
Tube Length	$F_{1, 52} = 111.63$***	Wald's $\chi^2 = 42.31$***
b (SE)	**-0.162 (0.052)**	**0.126 (0.019)**
β (SE)	*-0.145 (0.020)*	*0.124 (0.040)*
Plant Visits		Wald's $\chi^2 = 6.57$*
b (SE)	n.s.	**0.301 (0.099)**
β (SE)		*0.069 (0.009)*
R^2 of Model	0.76	--

*P < 0.05, **P < 0.001, ***P < 0.0001
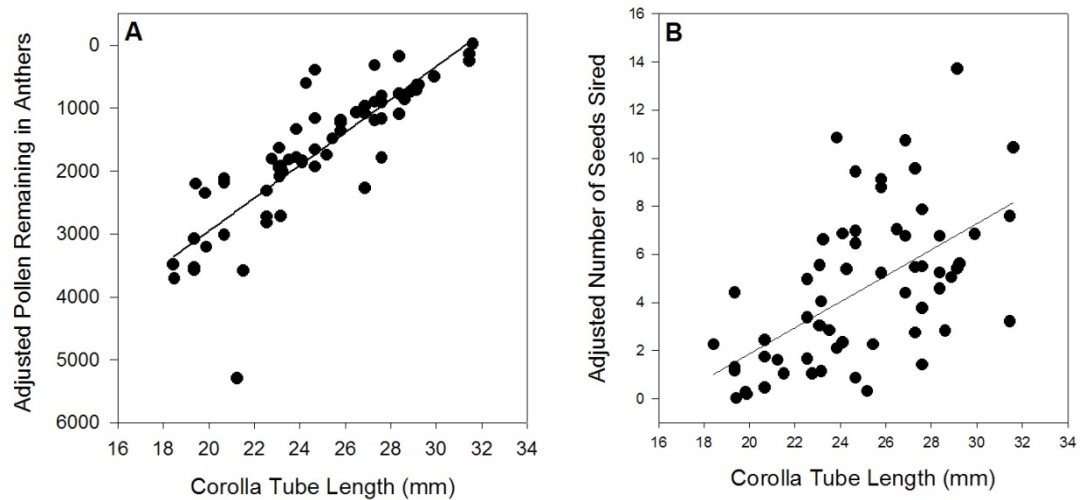
Figure 6.2 Effect of corolla tube-length on pollen remaining in anthers (Panel A) and the number of seeds sired (Panel B) after pollination by *Archilochus colubris*. Points are adjusted values to account for the effects of other significant variable in the model. See Methods of Chapter 5 for further details.

Plants that received relatively more hummingbird visits sired more offspring. I detected weak but significant selection on the number of visits to plants through male function ($\beta = 0.069$). Although the number of hummingbird visits did not affect the amount of pollen remaining in anthers, interestingly, this measure of pollen removal was correlated with seeds sired ($r = -0.568$, $P < 0.0001$) and seeds set ($r = -0.258$, $P < 0.05$).

6.3.4 Fitness surfaces

Projected pursuit regression indicated that selection by hummingbirds on floral traits was primarily linear. This result supported my initial ANCOVA models. Primarily linear functions (average effective number of parameters 2.004) described relative female, male, and total fitness. Further, the floral traits identified by analysis of covariance were also important in the projected pursuit regression. Specifically, relative female fitness was largely affected by positive selection on herkogamy, and male fitness largely varied with positive selection on corolla-tube length. Total relative fitness varied positively with herkogamy and corolla-tube diameter (Figure 6.3). Tube diameter was not significant in the multivariate analysis of female fitness but did affect female fitness in the analysis of covariance models. Finally, flower mass had moderate effects on all three measures of fitness, but was not significant in the ANCOVA models or the projected pursuit regression analysis.

Tube Length	0.09		Tube Length	-0.11		Tube Length	0.71 *
Tube Diameter	0.39 *		Tube Diameter	0.05		Tube Diameter	0.01
Herkogamy	0.59 *		Herkogamy	0.62 *		Herkogamy	-0.11
Mass	0.51		Mass	0.51		Mass	0.31
Nectar Volume	-0.02		Nectar Volume	-0.01		Nectar Volume	0.12
Sugar Content	-0.02		Sugar Content	-0.02		Sugar Content	-0.01
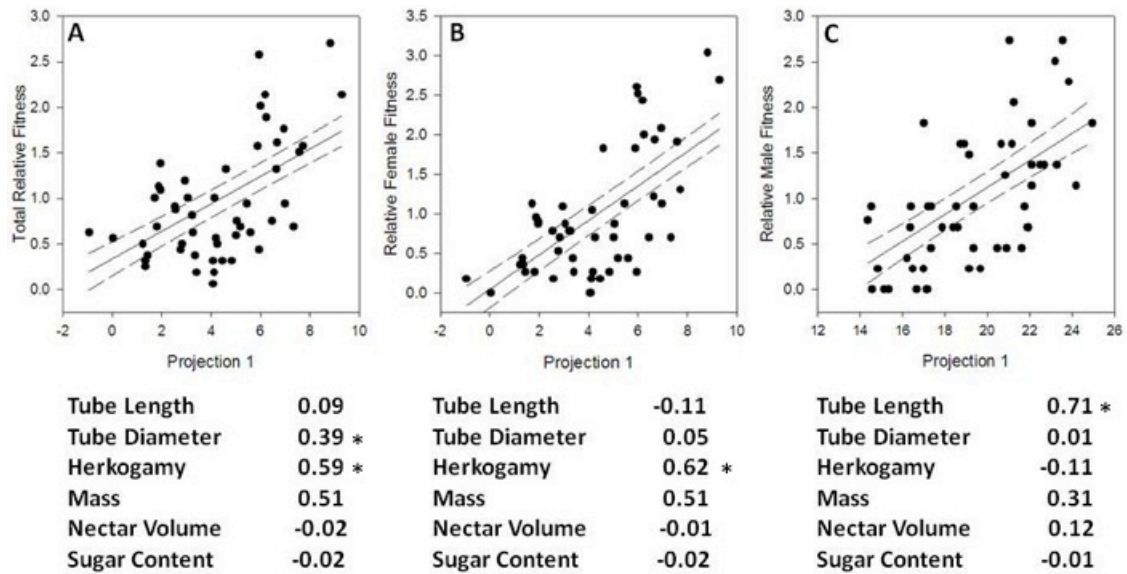
Figure 6.3. Relative total fitness (Panel A), female fitness (Panel B), and male fitness (Panel C) along the first fitted projection from projected pursuit regression analysis. Solid lines represent the mean bootstrap predictive line, and dashed lines represent a 95% confidence interval. Smoothing parameters (λ) values were 6, 6, and 9 for A, B, and C, respectively. Values were chosen based on minimized GCV scores. Values below each panel represent mean bootstrap estimates of variable (floral trait) coefficients. Confidence intervals were generated from 1000 bootstraps.

6.4 DISCUSSION

The selection imposed by pollinators on floral traits can have a major influence on floral evolution. I examined selection on *Polemonium brandegeei* floral traits under hummingbird pollinators in controlled conditions. I was particularly interested in the role that a highly variable stigma-anther separation (herkogamy) played in female and male fitness, among other aspects of floral design. These results are then compared to patterns of selection by hawkmoths (*Hyles gallii*) determined in a previous study (Chapter 5). This allowed me to document selection on floral traits by multiple pollinators.

Hummingbirds responded to floral rewards, and selected on the corolla dimensions and sex organ positioning of *P. brandegeei* flowers. Selection through female function (herkogamy and tube diameter) was generally stronger than selection through male function (tube length). However, selection on tube-length was consistently found through both pollen removal and seeds sired. Selection between hummingbirds and hawkmoths was similar in that both pollinators selected on herkogamy through female function. However, hummingbirds and hawkmoths selected in opposition on herkogamy. Similarly, selection for wider corollas by hummingbirds through female function contrasted with selection for narrower tubes by hawkmoths through male function. This may represent a potential constraint to the response of tube diameter. A further constraint on the response to selection may exist between when hawkmoths select for narrow corollas, and hummingbirds select for longer corollas. Since these traits are positively correlated, the response to selection may be impeded.

6.4.1. Hummingbird behaviour and foraging patterns

Hummingbirds did not alter their foraging behaviour in response to variation in *P. brandegeei* floral design, although I predicted that they would prefer larger flowers. This lack of preference may reflect the lack of a strong correlation between floral rewards (nectar volume or sugar) and floral dimensions (Appendix 6.1). Therefore, hummingbirds were unable to associate a visual cue with greater rewards, to develop a preference within *P. brandegeei* flowers. Indeed, selection on morphological traits (e.g. flower size) is often associated with a correlation between a trait and floral rewards (Mitchell 1993).

Hummingbirds did not visit more flowers on plants producing larger floral rewards, or visit these plants more often. This result contradicted my expectation that hummingbirds would prefer plants with larger nectar volumes and sugar content, and visit more flowers on these plants. For example, hummingbirds preferred plants of *Penstemon centranthifolium* with larger nectar rewards (Wilson and Jordan 2009). Further, hummingbirds are able to identify larger rewards in a very short period of time, and can specialize their foraging strategy on these large rewarding plants (Gass and Sutherland 1985). Therefore, it is unlikely that hummingbirds did not have sufficient time to identify plants with larger rewards in my artificial arrays. Although the number of times a plant was visited was not related to the floral traits I measured, plants receiving more hummingbird visits sired more seeds but did not set more seeds. Increased rates of visitation are often associated with greater pollen removal and siring success, but not necessarily female function (Stanton et al. 1986; Sahli and Conner 2011).

In my study, hummingbirds visited more flowers per plant when nectar rewards were small. This increase in the number of flowers per plant visited may reflect a short-term response to nectar rewards by hummingbirds, to maximize nectar intake from plants with small rewards. When a foraging hummingbird encounters a plant with smaller nectar volumes, the bird may have to visit multiple flowers to meet the high energetic demands of a hovering pollinator (Baker 1975; Hurly 2003). Alternatively, the exhaustive foraging behaviour observed by hummingbirds in my study may reflect the relatively small number of plants, and therefore resources available in my arrays. However, this alternative seems unlikely, because the hummingbirds were able to seek other food sources outside the arrays.

Hummingbirds probed flowers longer when nectar volumes were higher, although this result was marginally nonsignificant. A similar response was detected in hummingbird pollinated *Ipomopsis aggregata* (Mitchell and Waser 1992). However, in my study longer handling times of flowers did not affect pollen transfer, or the number of seeds set or sired.

Pollinator movements within inflorescences can have important consequences for the movement of self and outcross pollen within a plant. When multiple flowers per inflorescence are visited, the potential for the movement of self-pollen increases (Harder et al. 2004). Therefore, the segregation of functional genders within an inflorescence (e.g. female phase flowers below male phase flowers) may match the foraging patterns of pollinators within inflorescences (Harder et al. 2000; 2004). For example, bees typically move from lower to higher positions on an inflorescence, reinforcing the spatial separation of genders on an inflorescence. Hummingbirds did not exhibit any difference

between the number of upward and downward movements within an inflorescence.

Therefore, gender segregation within the inflorescence of *P. brandegeei* may not be

critical under hummingbird pollination, as has been demonstrated with artificial flowers

(Harder et al. 2004).

6.4.2. Selection by hummingbirds on floral design

I detected directional selection for wider corolla-tubes through female fitness, and

longer corolla-tubes by hummingbirds through male fitness. These patterns of selection

largely fit with my initial expectations, and other findings in the literature. Campbell et

al. (1996) detected similar selection by hummingbirds for wide corolla-tubes in

Ipomopsis aggregata. Wider corolla tubes allowed birds to fit more of their pollen-

bearing face into a flower, further enforcing contact with sex organs (Temels 1996).

Further, corolla tubes that match or are slightly longer than the hummingbird's bill

increase contact between sex-organs, and the pollen-bearing surfaces of hummingbirds

(Grant and Grant 1968; Grant and Temeles 1992; Temeles et al. 2009; de Waal 2010).

Although my study detected selection for longer corollas through male function, other

studies have found similar selection on corolla length through female function (Campbell

1989; Campbell et al. 1994).

After the initial attraction to and orientation of pollinators at a flower, the

positioning of sex-organs (herkogamy) influences pollen placement. At this stage of the

plant-pollinator interaction, the placement of pollen on a region of the animal where it

can be deposited on the stigmas of subsequently visited flowers is critical (Webb and

Lloyd 1986; Barrett 2002). As predicted, plants in my arrays that displayed exserted

stigmas (approach herkogamy) set more seeds under hummingbird pollination than those displaying recessed stigmas (reverse herkogamy). Stigmas exserted beyond the opening of the corolla tube make stronger contact with the face and chin regions of hummingbirds, where pollen is carried (Grand and Grant 1968). Other studies have detected selection for exserted stigmas in hummingbird-pollinated taxa through female function (Campbell 1989; Campbell 1996; but see Castellanos et al. 2004). For example, Reynolds et al. (2010) detected consistent long-term selection in 8 out of 9 years for exserted stigmas in *Silene virginica* by ruby-throated hummingbirds. My results indicate that hummingbirds exert similar selection on *P. brandegeei*.

Although my experimental arrays detected instances of strong floral selection by pollinators, natural field conditions may moderate these patterns. Visitation by pollen collecting insects and abiotic factors could change or obscure selection I detected by hummingbirds in isolation. In addition, an overlapping presence of other pollinators (hawkmoths) may alter the selection exserted by hummingbirds if selection is not additive (Sahli and Conner 2011). Finally, resource limitation of seed set may be more frequent in natural populations, which may affect the magnitude of selection through female function. My arrays likely did not experience resource limitation, which could have enhanced pollen limitation and therefore selection through female function. Nevertheless, pollen limitation of seed set may be common in natural populations (Ashman and Morgan 2004). In addition, my results provide a baseline view of how hummingbirds contribute to selection on floral design in *P. brandegei*.

6.4.3. Selection by hawkmoths and hummingbirds- implications for floral evolution and trait variation

I measured selection on floral design by hummingbirds and hawkmoths under controlled conditions on experimental arrays of *P. brandegeei*. Selection was measured through female and male function, in the absence of less effective floral visitors and variable abiotic factors. This approach allowed me to gain a base-line estimate of selection by each of hummingbirds and hawkmoths, suitable for making direct comparisons of the type and targets of selection. Further, I was able to compare gender specific selection, and identify potential constraints to selection response within and between pollinators.

The selection gradients I observed (summary in Table 6.4) reflected differences both in the mechanics of pollen transport and in the range of phenotypes in the experimental arrays. First, hawkmoths transported pollen along a slender proboscis, and hummingbirds on their feathered forehead and chin regions (Kulbaba and Worley 2008; M. Kulbaba personal observation). Second, the extremes in the distribution of herkogamy in the arrays were at higher frequencies than in natural populations, whereas the remaining floral traits reflected natural variation. Therefore, this experimental design enhanced my power to detect selection on herkogamy by each pollinator. I was more likely to detect selection on other floral traits that were further from a hummingbird or hawkmoth optimum.

Table 6.4 Selection gradients (standard error) through female (seeds set) and male (seeds sired) function, and summary statistics of fitness measures of *Polemonium brandegeei* under hawkmoth and hummingbird pollination. Selection gradients are from two means of standardized estimates. β_1 are based on traits standardized to a mean of 0, and β_2 are based on traits standardized to a mean of 0 and variance of 1. All significant estimates ($P < 0.05$) appear in bold typeface.

Effect	Hawkmoth Pollination		Hummingbird Pollination	
	Female Fitness (Seeds Set)	Male Fitness (Seeds Sired)	Female Fitness (Seeds Set)	Male Fitness (Seeds Sired)
Nectar Sugar	$\beta_1 = -0.01$ (0.08)	$\boldsymbol{\beta_1 = 0.38\ (0.17)}$	$\beta_1 = -0.12$ (0.20)	$\beta_1 = 0.01$ (0.34)
	$\beta_2 = -0.01$ (0.09)	$\boldsymbol{\beta_2 = 0.30\ (0.10)}$	$\beta_2 = -0.09$ (0.09)	$\beta_2 = 0.01$ (0.15)
Tube Length	$\beta_1 = 0.01$ (0.10)	$\beta_1 = -0.04$ (0.13)	$\beta_1 = 0.19$ (0.61)	$\boldsymbol{\beta_1 = 0.19\ (0.05)}$
	$\beta_2 = 0.01$ (0.09)	$\beta_2 = -0.04$ (0.13)	$\beta_2 = 0.03$ (0.09)	$\boldsymbol{\beta_2 = 0.21\ (0.03)}$
Tube Diameter	$\beta_1 = -0.01$ (0.09)	$\boldsymbol{\beta_1 = -0.26\ (0.12)}$	$\beta_1 = 0.86$ (0.49)*	$\beta_1 = 0.08$ (0.84)
	$\beta_2 = -0.01$ (0.09)	$\boldsymbol{\beta_2 = -0.20\ (0.13)}$	$\boldsymbol{\beta_2 = 0.23\ (0.19)}$	$\beta_2 = 0.02$ (0.16)
Herkogamy	$\boldsymbol{\beta_1 = -1.05\ (0.43)}$	$\beta_1 = -0.14$ (0.12)	$\boldsymbol{\beta_1 = 0.91\ (0.17)}$	$\beta_1 = 0.01$ (0.19)
	$\boldsymbol{\beta_2 = -0.96\ (0.24)}$	$\beta_2 = -0.14$ (0.12)	$\boldsymbol{\beta_2 = 0.77\ (0.09)}$	$\beta_2 = 0.02$ (0.15)
Number of Visits to Plants	$\beta_1 = 0.40$ (0.31)	$\beta_1 = 0.45$ (0.24)	$\beta_1 = 0.13$ (0.18)	$\boldsymbol{\beta_1 = 0.07\ (0.01)}$
	$\beta_2 = 0.09$ (0.10)	$\beta_2 = 0.21$ (0.12)	$\beta_2 = 0.07$ (0.09)	$\boldsymbol{\beta_2 = 0.09\ (0.01)}$

	Mean	Minimum	Maximum	Mean	Minimum	Maximum
Pollen in Anthers	1871.73	232.39	5688.40	1801.95	114.04	8660
Pollen on Stigma	485.61	21	1406.0	302.13	25.6	1030
Seeds Set	14.63	0	45	11.52	0	35
Seeds Sired	2.47	0	7	4.39	1	12

*$P \cong 0.057$

Hummingbirds responded to different features of *P. brandegeei* flowers than did hawkmoths in my previous study (Chapter 5). Unexpectedly, hummingbirds did not respond to variation in *P. brandegeei* floral dimensions such as flower size. In contrast, hawkmoths initially visited larger flowers but finally visited more plants displaying

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reverse herkogamy (Chapter 5). Regardless of the lack of behavioural response by hummingbirds to *P. brandegeei* floral traits, plants that received more total visits generally sired more seeds. Under hawkmoth pollination, however, plants that were visited more by hawkmoths both set and sired more seeds. Therefore, increased visitation frequency directly enhanced both female and male function under hawkmoth pollination, but only male fitness under hummingbird pollination.

I detected contrasting selection by hawkmoths and hummingbirds on herkogamy in *P. brandegeei* through female function. Hummingbirds selected for exserted stigmas in approach herkogamy and hawkmoths selected for recessed stigmas in reverse herkogamy. Further, I previously documented temporal fluctuation in the relative abundances of these pollinators (Chapter 2). Such fluctuations in population size seem to be characteristic of hawkmoths (Campbell et al. 1997; Brunet 2009). These results may represent an example of how different pollinators may exert contrasting selection on the same target of floral design, resulting in an "intermediate" phenotype for that trait (cf. Sahli and Conner 2011). Indeed, most individuals of *P. brandegeei* display intermediate measures of herkogamy in natural populations (Chapter 2). Therefore, the combined results of my experiments with hummingbirds and hawkmoths support my hypothesis of contrasting selection maintaining variation in the separation of sex organs in *P. brandegeei*. Further, since hummingbirds were more frequently observed visiting *P. brandegeei* flowers, instances of selection for approach herkogamy likely outnumber instances of selection for reverse herkogamy by hawkmoths. Therefore, approach herkogamy would be predicted to be more common, as my observed ratio of 80:20 approach to reverse herkkogamous plants in natural populations confirm.

Hummingbirds and hawkmoths selected on one common, and one different corolla dimension. Hummingbirds selected for longer corolla tubes through male function and for wider corolla tubes through female function. Hawkmoths selected for narrow corolla tubes through male function (chapter 5). The contrasting selection on corolla diameter differed from that on herkogamy in that the opposing selection occurred through different gender functions. Since the distribution of corolla dimensions was similar to natural populations in my arrays, corolla diameter may also be expected to assume an "intermediate" phenotype between the hummingbird and hawkmoth optima. However, the corolla dimensions of *P. brandegeei* generally resemble other taxa pollinated by hummingbirds. Therefore, hummingbirds may be more frequent pollinators, as observed in Chapter 2, and therefore provide more successive instances of selection on corolla diameter than hawkmoths.

Differing patterns of selection across gender functions within hermaphroditic plants have been hypothesized to be common (Morgan 1992; Morgan and Schoen 1997). However, limited empirical support has been found for this prediction. In an extensive study measuring selection by 4 pollinator groups through female and male function on *Raphanus raphanistrum*, Sahli and Conner (2011) detected significant differences between female and male selection on two floral traits (anther exsertion and flower number). While my results partially complement the findings of Sahli and Conner (2011), they differ in that I found selection through female function was stronger than through male function.

In addition to selection on corolla tube diameter, I also detected selection for long corolla tubes by hummingbirds through male function. Given the strong positive genetic

correlation between corolla length and width ($r_a = 0.997$, $P < 0.0001$; Chapter 2), response to selection by hawkmoths for narrow corollas may be subject to a correlational constraint. Such positive genetic correlations have been found to constrain selection, but typically across female and male genders (reviewed in Ashman and Majetic, 2006). However, selection by hummingbirds on corolla length in *P. brandegeei* was detected through male function alone. Nevertheless, selection by hawkmoths on tube diameter was stronger than selection by hummingbirds on tube length (Table 6.4). Weaker selection by hummingbirds may indicate that tube-length is closer to the optimum for hummingbird pollination than tube-diameter for hawkmoth pollination. Indeed, corolla dimensions (length and width) in *P. brandegeei* flowers are generally reflective of other taxa pollinated by hummingbirds (Chapter 2; Kulbaba and Worley 2008).

The combined results of my array experiments measuring selection by hummingbirds and hawkmoths on *P. brandegeei* floral design illustrate the potential of floral designs to display both intermediate phenotypes and a mosaic of apparently specialized phenotypes. Herkogamy likely represents an intermediate condition that reflects contrasting selection by both pollinators. Conversely, the corolla dimensions of *P. brandegeei* are either more important to one pollinator, or the corolla trait distributions are sufficiently functional over a wide range (Aigner 2001). My results add to the few studies examining selection by multiple pollinators. However, further work is required on a wider range of pollinators and plant taxa, to gain a more general view of pollinator-floral trait interactions.

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Appendix 6.1. Summary statistics of measured floral traits pooled across all 5 replicate arrays ($n = 62$). All measurements are in millimeters except mass (g), sugar (mg) nectar volume (µL), and number of visits (counts). As herkogamy contained negative values, I added the absolute value of the most negative value of herkogamy (6.81) to each value of herkogamy to shift the distribution to the right of zero.

Trait	Minimum	Maximum	Mean	Standard Error	Coefficient of Variation
Flower Width	14.09	22.52	16.77	0.22	10.39
Tube Length	18.43	31.62	24.77	0.44	13.90
Basal Tube Diameter	1.34	3.17	2.28	0.05	18.83
Apical Tube Diameter	2.69	4.81	4.10	0.06	11.31
Herkogamy	-6.81	3.05	-0.13	0.28	33.12
Style Length	21.08	30.19	26.14	0.31	9.40
Anther Height	20.53	33.13	26.90	0.39	11.32
Mass	3.95	14.20	8.62	0.26	24.11
Nectar Volume	3.10	14.80	7.67	0.40	41.48
Sugar	19.70	203.60	101.85	5.88	45.44
Flower Visits/Bout	4.00	44.00	16.81	1.23	57.62
Plant Visits/Bout	1.00	14.00	5.69	0.36	49.23

My thesis examined ecological and evolutionary parameters influencing the evolution of floral design in *P. brandegeei*. I documented floral visitors and variation in floral design in natural populations over consecutive flowering seasons (Chapter 2). These data identified hawkmoths and hummingbirds as important pollinators of *P. brandegeei* and provided a pollinator context to consider the measures of pollen removal and deposition that were obtained over the same flowering seasons (Chapter 3). To measure selection through male function, *P. brandegeei*-specific microsatellite markers were developed, and additionally I confirmed the use of these markers on *P. viscosum* (Chapter 4). The identification of *P. brandegeei*'s important pollinators led to two experiments that estimated selection by hawkmoths (Chapter 5) and hummingbirds (Chapter 6). This final chapter summarizes the major findings from my thesis, and presents a synthetic interpretation of the findings as a whole. Finally, I discuss future avenues of research identified from my work.

7.1 Floral visitors, pollinators, and floral trait variation

I documented multiple animals visiting the flowers of *P. brandegeei*. Based on an index of relative effectiveness, and whether or not sex organs were contacted, I classified these animals into floral visitors and pollinators. While pollinators visit multiple flowers per plant, and several plants per foraging bout, floral visitors visit relatively few flowers. In addition, effective pollinators contact both female and male sex organs, which is

critical for successful plant reproduction (Schemske and Horovitz 1984). Generally, small-bodied insects visited a small number flowers and plants. The relatively narrow corollas of *P. brandegeei* restrict the accessibility of nectar to animal visitors with long and narrow appendages. Bees and flies could typically only contact the anthers and were considered floral visitors. The restricted access to floral rewards likely resulted in fewer flower visits per plant by bees and flies. In contrast, hawkmoths and hummingbirds were able to access nectar, visited multiple flowers and plants, contacted both anthers and stigma within flowers, and were thus considered important pollinators. Therefore, my results indicated that not all floral visitors have the potential to equally select on floral traits; rather, most selection is imposed by a subset of floral visitors consisting of the most effective pollinators.

The identification of hawkmoths and hummingbirds as important pollinators matched the overall variation in floral traits of *P. brandegeei*. The floral design of *P. brandegeei* exhibits trait dimensions associated with both hawkmoth and hummingbird pollination. For example, the white-cream coloured corollas with narrow diameters, and stigmas recessed below the anthers (reverse herkogamy) suggested hawkmoth selection. In contrast, a corolla tube that is slightly longer than a bird's bill, and stigmas exserted beyond the anthers (approach herkogamy) are traits commonly associated with hummingbird selection. Further, I determined that the variation in floral traits of *P. brandegeei* is heritable, and could therefore respond to selection by hawkmoths and hummingbirds.

7.2 Pollen movement and floral trait selection

The removal and deposition of pollen occurred diurnally and nocturnally in natural populations over my study period. While the majority of pollinators were observed during the day, considerable nocturnal pollen movement occurred in both flowering seasons. Nocturnal hawkmoths were observed in 2006, and likely contributed to pollen removal and deposition at night. However, nocturnal pollen transfer may have occurred via unobserved pollinators or abiotic factors (e.g. wind).

Pollen removal and deposition in natural populations (Chapter 3) did not always correspond with that measured under controlled conditions (Chapters 5 and 6). Overall, pollen removal was greater in natural populations, relative to only hawkmoth or hummingbird pollination in experimental arrays. The removal of pollen collecting floral visitors, and a more stable environment in the arrays likely contributed to the differences. However, pollen deposition rates in natural populations were similar to deposition by only hawkmoths or hummingbirds under controlled conditions. Considerable interference between female and male functions was presumed to occur in natural populations. Indeed, even plants that were prevented from being visited exhibited considerable pollen deposition, indicating autonomous self-pollen deposition. Although hawkmoths and hummingbirds are effective pollinators of *P. brandegeei*, facilitated self-pollen deposition likely occurred even under the stable and controlled conditions of the array experiments. Therefore, pollen deposition in natural populations likely reflects the combined effects of effective pollinators and both autonomous and facilitated self-pollen deposition.

During my field study of pollen movement, flower sex organs were important for pollen removal and deposition (Chapter 3). Across both years of study, flowers displaying intermediate lengths of style had the least amount of pollen remaining in anthers. While my expectation of more pollen being removed from flowers with recessed stigmas was not confirmed, overall pollen removal was greater in 2006. Thus, my prediction of greater pollen removal during peak floral visitation was met. Herkogamy was an important floral trait for pollen deposition. In both years, intermediate to slightly recessed stigmas received more pollen deposition. Predictably, plants visited during the day and night received more pollen than plants that were prevented from being visited (Controls). However, I also predicted that pollen deposition would be greater during periods of peak floral visitation. Yet unlike pollen removal, greater pollen deposition did not coincide with increased floral visitation.

I also demonstrated herkogamy to be an important floral trait in my hawkmoth and hummingbird array experiments. As predicted under hawkmoth pollination, plants displaying reverse herkogamy received greater pollen deposition, and set more seeds. Under hummingbird pollination, I predicted more pollen deposition and more seeds set by plants displaying approach herkogamy. Interestingly, while I detected positive selection through female function on herkogamy, I did not find more pollen deposited on exserted stigmas. This may be a result from a balance between hummingbird-facilitated self-deposition on plants with recessed stigmas and imported pollen on plants displaying exserted stigmas. This scenario likely led to the nonsignificant effect of herkogamy on pollen deposition under hummingbird pollination. Overall, I detected greater pollen deposition on plants displaying reverse herkogamy, in the hawkmoth arrays. Otherwise,

hawkmoths and hummingbird pollination resulted in similar rates of pollen movement, and number of seeds set and sired (Table 6.4).

The influence of corolla dimensions on pollen movement and fitness largely supported the association between particular dimensions of floral traits and particular pollinators. For example, the corolla tubes of *P. brandegeei* are on average slightly longer than the length of hummingbird bills. Traditional interpretations of hummingbird pollinated flowers predicts that floral tubes that are slightly longer than the hummingbird bill will have more pollen removed (Grant and Grant 1968). I detected positive selection through male function (pollen removal seeds sired) on *P. brandegeei* corolla-tube length. I also detected positive selection on tube-diameter through female function, as was found in other species (e.g. Cambell 1996). In addition, narrow corollas are typically associated with hawkmoth pollination (Nilsson 1988). I detected negative selection on tube diameter by hawkmoths through male function. Again, this observation is consistent with the association of narrow corollas that constrict the opening of the flower, and enhances contact between the anthers and moth proboscis. While I detected contrasting selection on tube diameter that may suggest that the optimal diameter of *P. brandegeei* floral tubes differ between hawkmoths and hummingbirds, previous studies have shown both pollinators to effectively pollinate flowers of similar dimensions (Grant and Temmels 1992). Overall, my array experiments largely support the association between particular floral traits and specific pollinator types.

7.3 Selection on floral design by hawkmoths and hummingbirds

The response of floral traits to selection by multiple effective pollinators can result in a series of intermediate trait dimensions, or a mosaic of apparently specialized floral traits (Sahli and Conner 2011). Herkogamy likely represents one such intermediate trait that is likely result of contrasting selection by hawkmoth and hummingbird pollinators. In addition, an intermediate dimension of tube diameter may be displayed from the contrasting selection through different gender functions I detected from hawkmoths and hummingbirds. However, other traits exhibit dimensions that appear specialized to one pollinator. For example, the corolla length of *P. brandegeei* generally reflects other taxa pollinated by hummingbirds. In addition, while tube length did not influence pollen movement or fitness under hawkmoth pollination, I measured selection for longer tubes by hummingbirds. Therefore, corolla tube-length may be more important under pollination by hummingbirds than hawkmoths. My experimental arrays demonstrate that the traits comprising floral design of a given species can represent both intermediate and apparently specialized traits.

7.4 Future directions

My thesis research has provided valuable insights into factors influencing floral design. However, several aspects of plant-animal interactions in *P. brandegeei* that can further elucidate the evolution of floral design remain. For example, determining the influence of the less effective floral visitors on selection on *P. brandegeei* floral traits could give a more complete picture of selection on floral design. While these small bodied insects may not contribute to selection on floral design to the extent of

hummingbirds and hawkmoths, legitimate pollen transfer may occur among intermediate measures of herkogamy. Further, a reduced potential gamete pool that would result from the consumption of pollen can also reduce plant fitness. Therefore, examining the variation of both female and male fitness after visitation by pollen consumers would further clarify the role of floral visitors other than hawkmoths and hummingbirds.

The role of pollinator-facilitated self-pollination, on plant fitness is a relatively understudied field. The results from my exclusion experiments in natural populations, and array experiments under controlled conditions indicate that *P. brandegeei* experiences considerable sexual interference during animal visitation.   However, the inability to distinguish between self and import pollen in *P. brandegeei* precludes a clear distinction of the fraction of legitimate pollen import. Therefore, additional trials with emasculated flowers would likely indicate the fraction of imported pollen.

Finally, the role of abiotic factors in pollen transfer and fitness remains unclear. The large fluctuations in temperature observed in natural populations could have drastic effects on pollen transfer, and potentially physiology. Warm dry conditions may result in pollen grains that have not fully developed, and are unfit for germination and fertilization (Galen et al. 1999). Further, water and temperature stress may also affect ovule development. However, variation in the abiotic environment and in the frequency of pollinators may not be independent, but rather could covary. One potential avenue to explore the effect of environment is to examine the germination rates and pollen-tube growth rates of pollen grains grown under various stressful environments, under various frequencies of pollinator visitation.

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