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FLORAL DESIGN IN *POLEMONIUM BRANDEGEI* (POLEMONIACEAE): GENETIC AND PHENOTYPIC VARIATION UNDER HAWKMOTH AND HUMMINGBIRD POLLINATION

Mason W. Kulbaba[1] and Anne C. Worley

Department of Botany, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada

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. We examined continuous variation in anther position, style length, herkogamy, and flower shape in the self-sterile perennial herb *Polemonium brandegei*. We 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 *Hyles 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, independent 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 ($h^2 = 0.851$; $\mathrm{CV_a} = 36.88$; $\mathrm{CV_a}$ is the coefficient of additive genetic variation). Temporal fluctuations in pollinator frequency likely maintain the variation in herkogamy by imposing heterogeneous selection on floral traits.

Keywords: additive genetic correlations, heritability, herkogamy, Polemoniaceae, hummingbird and hawkmoth pollination.

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 is 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 1992*a*). Both heterostyly and stigma height polymorphisms are controlled by one or two Mendelian loci (Lloyd and Webb 1992*b*). Although stylar polymorphisms have been extensively studied, the majority of angiosperms display a unimodal distribution of style length (Barrett et al. 2000).

Although stylar polymorphisms exhibit Mendelian inheritance (reviewed by 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.

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[1] Author for correspondence; e-mail: umkulbam@cc.umanitoba.ca.

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Third, stylar variation might 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 0. In this case, the left-hand tail of the herkogamy distribution may cross 0, 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 color, 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 associated not only with reverse herkogamy but also with white or cream-colored 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 Sterling 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, we investigate pollination and floral variation in *Polemonium brandegei*. *Polemonium brandegei* is a subalpine 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 odor. 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 (A. C. Worley, unpublished data). Preliminary observations of floral visitors by A. C. Worley indicated hawkmoth (*Hyles lineata*) pollination. Also, both approach- and reverse-herkogamous plants were present in the same breeding populations. *Polemonium brandegei*'s closest relative, *Polemonium viscosum*, is pollinated by bumblebees and large flies (Galen 1989), has blue, approach-herkogamous flowers, and is very similar to *P. brandegei* genetically (A. C. Worley, unpublished manuscript). Thus, changes in pollinators appear to have coincided with recent floral divergence between these sister species.

As a first step toward understanding floral evolution in *P. brandegei*, our general objectives were to identify potential pollinators and to characterize phenotypic and genetic variation in floral traits. This study included three specific objectives. (1) We documented floral visitors to *P. brandegei* 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. C. Worley, personal observation). (2) Our second objective was to characterize phenotypic variation in floral design in three natural populations. Our measurements allowed us to determine whether variation in floral design, particularly stigma-anther separation, of *P. brandegei* was continuous or polymorphic and whether herkogamy was correlated with corolla dimensions. We also compared floral dimensions in *P. brandegei* with those of other species pollinated by hummingbirds and hawkmoths. (3) Our third objective was to determine the heritability of and genetic correlations among *P. brandegei*'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.

Material and Methods

Study Populations

This study included a total of five field populations of *Polemonium brandegei*, visited from 2000 to 2006. These populations are located near the center of the species range and include a range of elevation, moisture, and soil types (see table 1). The three primary sites were Taylor Canyon and Deer Mountain in Colorado and Vedauwoo recreational area in Wyoming. Geographic locations (latitude and longitude), approximate elevations, dates each field population was visited, and associated species are presented in table 1.

The southernmost site, Taylor Canyon, was located ca. 25 km north of Gunnison, Colorado. Individuals of *P. brandegei* were found in the crevices of rock faces and at the base of trees. Taylor Canyon was relatively dry and warm and had

Table 1

Name, Location (Latitude, Longitude, Elevation), Approximate Number of Individuals, and Dates Visited of *Polemonium brandegei* Field Sites Used in This Study

Population	Latitude, longitude (elevation)	*N*	Associated species	Dates visited
Taylor Canyon, CO[a]	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[a]	40°46′57″N, 105°53′01″W (2500 m)	200	*P. contorta*, *Ribes cereum*	May 28–June 7, 2004; June 1–11, 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[a]	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; June 12–17, 2006
Lone Tree Gulch, CO	38°30′17″N, 107°11′26″W (2743 m)	100		August 2000

[a] Primary field site.

little available soil. Deer Mountain was located within Rocky Mountain National Park, along Deer Ridge trail. This population was situated on a slope (ca. 30°) with a moderate layer of soil. Twin Sisters Mountain was also located in Rocky Mountain National Park. We visited Twin Sisters to observe pollinators in 2006 because it was the first site where hawkmoths (*Hyles lineata* Fab.) were observed visiting *P. brandegei* (A. C. Worley, personal observation). The northernmost site, Vedauwoo recreational area, was located ca. 65 km west of Cheyenne, Wyoming. Vedauwoo is moist and has a thick soil layer compared to the other sites.

We collected seeds for the greenhouse experiments in August 2000 from plants located in Lone Tree Gulch, ca. 15 km west of Saguache, Colorado. This site was geographically close to Taylor Canyon and had similar habitat conditions. Between 2001 and 2004, a wildfire destroyed the population at Lone Tree Gulch. Therefore, field measurements and floral visitor observations were not collected for this population.

Floral Visitors

During peak flowering periods of 2004–2006, we observed floral visitors at Taylor Canyon, Deer Mountain, Vedauwoo, and Twin Sisters (table 1). We documented floral visitors for 5–11 d per population, three times per day (7:30 a.m., 12:30 p.m., and 7:30 p.m.) for a minimum of 30 min. We also made observations past dusk (8:00–10:30 p.m.) at the Vedauwoo population. These evening observations were not done for the other populations because of limited accessibility after dusk. Finally, we made casual observations throughout the day. We spent a total of 340.5 h documenting floral visitors to *P. brandegei* 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. We identified hummingbirds with the aid of binoculars and captured insects. In addition, we noted whether the visitors contacted the stigma and anthers because effective pollination requires consistent physical contact between the visitor and both sex organs. Finally, we 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 *P. brandegei* is self-sterile, pollen transfer between individual plants is required for successful pollination. Thus, our index captured both the frequency of foraging bouts and the potential that each bout contributed to reproductive success.

Floral Morphology

To examine corolla shape and sex organ position in *P. brandegei*, we measured floral characters on randomly selected plants ($n = 50$–100) in the three primary field populations. Measurements included flower length and width and corolla tube length and width, as well as the height, exsertion, and relative positions of stigmas and anthers (fig. 1). The flowers are protandrous, so a "male" flower (before the stigma lobes reflexed and became receptive) and a "female" flower (after the stigma became receptive) were measured on each plant to determine whether herkogamy changed with flower age. Finally, we compared the distribution of tube lengths in *P. brandegei* with the bill length for the hummingbird *Selasphorus platycercus* (from Waser 1978) and the proboscis length for the hawkmoth *H. lineata* (from Grant 1983).

To determine the narrow-sense heritabilities and genetic correlations among *P. brandegei*'s floral traits, we used a greenhouse crossing experiment with extreme and intermediate phenotypes. The parental generation consisted of 121 *P. brandegei*

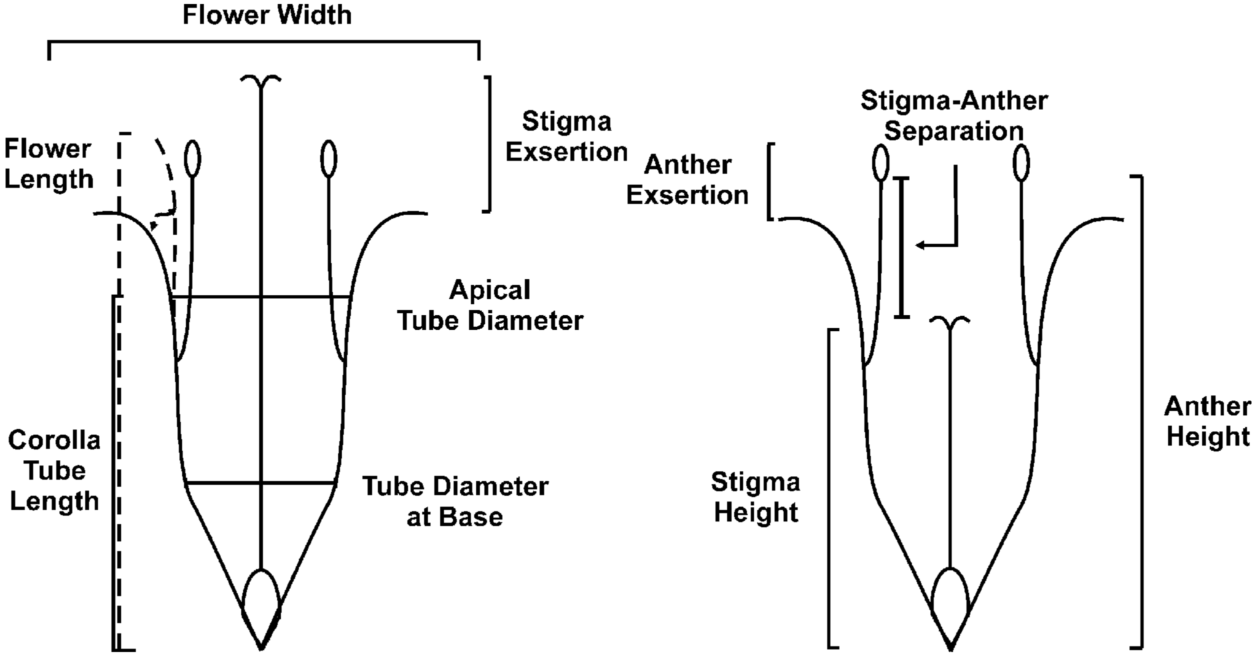


Fig. 1 Diagrams of approach- (left) and reverse- (right) herkogamous flowers, with precise locations of floral measurements.

plants, representing 22 maternal families, from seed collected at Lone Tree Gulch. We measured two flowers from the first two inflorescences produced by each plant. All measurements were identical to those made in field populations. We 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 and −1.50 mm were classed as "intermediates"; these plants had an average stigma-anther separation of 0.75 mm. We performed a total of 36 reciprocal crosses that were roughly evenly distributed among stigma height categories, comprising both assortative and disassortative crosses (table 2). This crossing strategy has been shown to increase both the power and the precision of narrow-sense heritability estimates (Falconer and Mackay 1996; Lynch and Walsh 1998).

We planted eight seeds from each cross, 344 seeds in total. We chose the number of offspring to plant by using power estimation curves (Lynch and Walsh 1998) to cover a wide range of possible heritabilities and an anticipated 75% germination rate. Because of a lower-than-expected germination rate (ca. 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 generations. Seeds were stratified at 4°C for 2–3 wk in an incubator, planted in plug trays, and moved to the greenhouse. Natural lighting was supplemented with sodium lamps for a photoperiod of ca. 14 h; plants received 75–125 μmol of light. The temperature typically ranged from 22° to 30°C but did reach higher than 35°C. We raised seedlings in a mixture of peat and Osmocote Plus slow-release fertilizer (10 : 10 : 10) in conical Deepots (800-mL conical pot), and we bottom watered by suspending the Deepots in water-holding trays. This allowed optimal water availability for the plants. The Deepots containing the plants remained in the same greenhouse and conditions as the plug trays.

Statistical Analysis of Floral Morphology

Before analysis, we tested all morphological characters for departures from normality with the Kolmogorov-Smirnoff test in SAS 9.1.2 (SAS Institute 2004). Morphological data were 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.

Table 2

Summary of 36 Reciprocal Crosses Made Based on Stigma Position Relative to the Anthers, or Stigma-Anther Separation

Stigma-anther separation (SA)	Recessed (≤−1.50 mm)	Intermediate (±~.75 mm)	Exserted (≥1.50 mm)
Recessed (≥−1.50 mm)	6 (5)		
Intermediate (±~.75 mm)	6 (4)	6 (4)	
Exserted (≥1.50 mm)	7 (6)	4 (3)	7 (5)

Note. 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.

Phenotypic variation. Female and male flowers were compared with a Pearson's product moment correlation. We 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"), we averaged their measurements for subsequent analyses.

We used principal components analysis (PCA) to summarize variation in floral design. We analyzed 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). We performed the ordinations in CANOCO 4.52, developed by ter Braak and Schaffers (2004).

We reduced the original nine floral traits to five on the basis of correlations among traits, the weighting of eigenvectors in preliminary analyses, and the probable function of strongly correlated traits in pollination. The elimination of redundant, highly correlated variables from the ordination allowed us to examine the traits that explained the maximum amount of variation. Composite variables of floral traits were not used, because we sought to determine the influence of actual floral characters on overall floral variation. The traits we 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. We 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, we retained corolla tube length over other measures of total corolla length because the fit between the length of the corolla tube and the length of a visitor's mouth parts has been hypothesized to be functionally important (see Grant and Grant 1968). Finally, we retained the measures of sex organ length over sex organ exsertion because style length and anther height are direct measurements of organ size.

We initially performed two separate ordinations to determine whether 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 whether floral morphology differed significantly among the Taylor Canyon, Deer Mountain, and Vedauwoo populations, a multivariate ANOVA (MANOVA) was conducted. When the MANOVA revealed significant differences in floral traits, we 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).

Genetic variation. We 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://ftp.tzv.fal.de). We performed this analysis on the traits that explained a large amount of floral variation, as determined by the PCA. The program VCE REML estimates variance components by 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., ANOVA) 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).

We accounted for variation in flower size by including the dry weight (mass) of flowers as a covariate (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. We assessed the significance of the heritabilities and the genetic correlations with one- and two-tailed one-sample *t*-tests, respectively, with the standard errors produced by VCE REML. Significance thresholds were determined with the sequential Bonferroni correction factor to control for tablewise Type I error (Rice 1989).

We also calculated the coefficient of additive genetic variation (evolvability) for each floral trait as $\mathrm{CV_a} = 100 \times \left[(V_a)^{1/2}/\bar{x}\right]$, where V_a is the additive genetic variance and $\bar{x}$ is the trait mean (Houle 1992). Houle (1992) has described $\mathrm{CV_a}$ 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 0 is possible, which would result in an infinitely large $\mathrm{CV_a}$. Therefore, we 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 0 and resulted in a conservative measure of $\mathrm{CV_a}$.

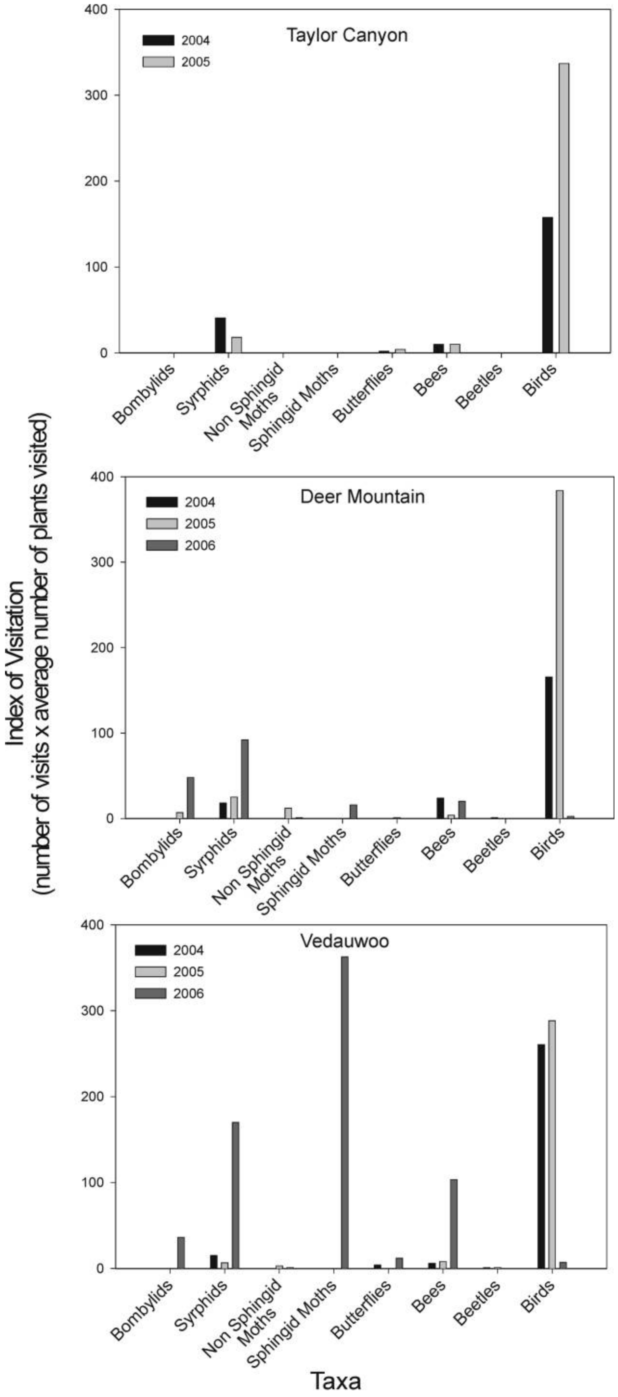


Fig. 2 Visitation indices (number of observed visitations × average number of plants visited per foraging bout) by eight major groups of visitors. Indices are presented across three flowering seasons and three field populations (Deer Mountain, Taylor Canyon, and Vedauwoo). We did not conduct floral visitor observations at Taylor Canyon in 2006.

Results

Floral Visitors

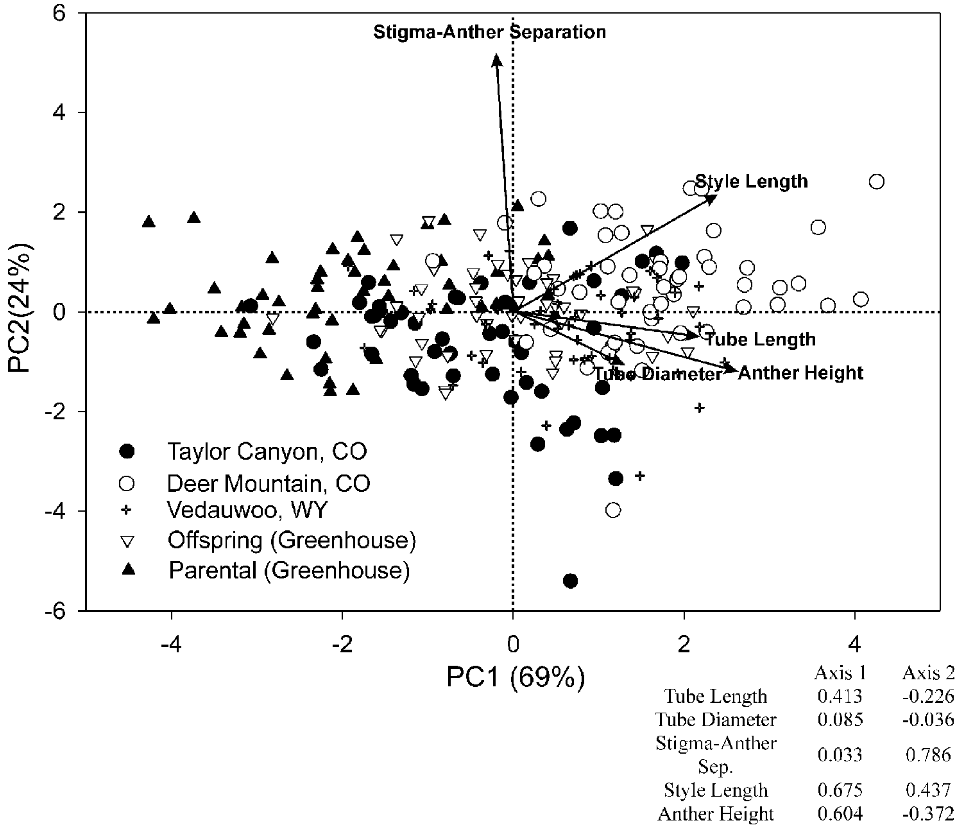
The visitation indices for each of the three primary populations varied with respect to year of observation and visiting taxa (fig. 2). The two components of this visitation index, the total number of observed visits and the average number of plants visited per visiting bout, for each population are in appendix A. We observed consistently high visitation rates by syrphids (Diptera) over three years of observations (app. A). 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 (fig. 2). In addition, syrphids collected pollen by hovering above the flowers, rarely contacting the stigma. This visitation behavior would further reduce the potential for pollen movement by syrphids. Bees (Hymenoptera) were also relatively frequent visitors but usually visited only two to four plants. Beetles (Coleoptera), non-sphingid moths, and butterflies (Lepidoptera) were infrequent visitors and contacted few flowers or plants per visit.

Hummingbirds (*Selasphorus platycercus* Swainson; Trochiliformes) and hawkmoths (*Hyles lineata* and *Hyles gallii*; Sphingidae) appear to be important pollinators of *Polemonium brandegei* (latter labeled "sphingid moths" in fig. 2). Hummingbirds were observed visiting multiple plants of *P. brandegei* in 2004 and 2005 in all three of the primary populations. However, we observed considerably fewer visits by hummingbirds in 2006 (fig. 2). Hawkmoths had the highest visitation index in 2006 at Vedauwoo (fig. 2). At Deer Mountain, we also observed a single hawkmoth, 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 (app. A), a pattern that provides ample opportunity for pollen transport. Our visual observations indicated that hummingbirds and hawkmoths consistently contacted both sex organs, making them likely vectors of pollen transport.

Phenotypic Variation in Floral Morphology

The stigma-anther separations of female and male flowers were strongly correlated with each other ($r = 0.910$, $P < 0.001$, $n = 83$) and displayed a mean difference of only 0.26 mm (SE = 0.011). By contrast, stigma-anther separation ranged from −2.77 to 5.80 mm in this group of plants. Therefore, we averaged the female and male flowers for each individual plant for subsequent analyses.

The final five measures of floral shape explained a large proportion of total floral variation in the multivariate analysis (fig. 3). The first two eigenvalues (axes) extracted from the analysis accounted for 93% of the total variation (69% and 24% for the first and second, respectively). Style length and anther height were most closely associated with the first axis of variation, $r = 0.675$ and 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 (fig. 3). To confirm that PC1 reflected variation in size, we 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 weightings similar to those in the previous ordination. There were no groupings of discrete phenotypes, indicating that herkogamy and the other traits comprising *P. brandegei*'s



	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

Fig. 3 Ordination diagram from the first two principal components of 50 individuals of *Polemonium brandegei* from each field site (Deer Mountain, Taylor Canyon, and Vedauwoo) and both parental and offspring generations of greenhouse plants. The nine original 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 and second principal components accounted for 69% and 24% (cumulatively, 93%) of the total variation, respectively. Loadings of eigenvectors on both axes are also indicated in the bottom right.

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 (fig. 3).

Stigma-anther separation (herkogamy) displayed continuous variation that 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 ($r = 0.786$; fig. 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 (fig. 4). This resulted from the fact that most anthers were positioned slightly below the opening of the corolla tube. Ranked plots of stigma-anther separation for the three primary field populations and greenhouse plants indicate that extreme values for approach and reverse herkogamy were roughly evenly distributed around 0 (fig. 5). However, there were ca. 80 approach-herkogamous for every 20 reverse-herkogamous plants in each population (fig. 5). Means and standard errors for all floral traits in the three field populations are in appendix B.

Floral traits differed significantly among populations of *P. brandegei*, as determined through MANOVA (Wilks's $\lambda = 0.927$; $F_{16,940} = 2.28$, $P = 0.003$). Therefore, we conducted a canonical discriminant functions analysis to determine which variables are best at distinguishing among the three analyzed field populations (Taylor Canyon, Deer Mountain, and Vedauwoo). The discriminant analysis maximizes among-population variance along the first discriminant axis, which in this 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 (fig. 5).

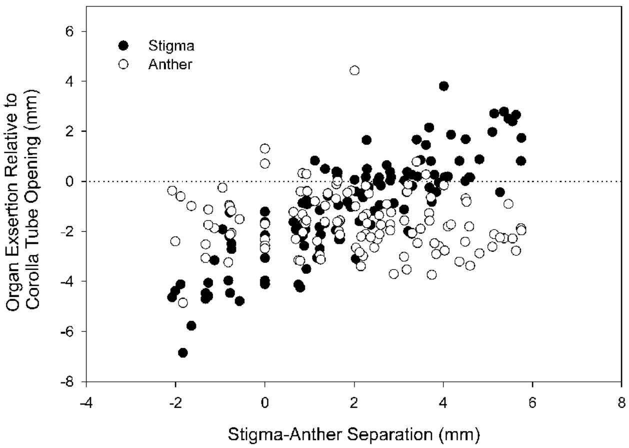


Fig. 4 Variation in stigma and anther exsertion across the range of stigma-anther separations in the parental greenhouse generation. An identical pattern was observed in the offspring generation and the field data (not shown). The stigma-anther separation (SA) ranges from reverse (negative SA values) to approach (positive SA values). Stigmas within ±0.25 mm of anthers were approximated to 0.

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 = 17.85 mm; Waser 1978). In contrast, the mean *H. lineata* proboscis length (38 mm; Grant 1983) was well beyond the distribution of corolla tube lengths in *P. brandegei* (fig. 6).

Genetic Variation in Floral Morphology

All floral traits of *P. brandegei* exhibited heritable variation (table 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 4). The largest additive variance was attributed to stigma-anther separation ($V_a = 4.636$), which coincided with a coefficient of variation four to 12 times 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: $CV_a = 3.39$).

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 3). Style length showed moderate positive correlations with measures of corolla size ($r_a \approx 0.3$ for both), but anther height was not correlated with either corolla dimension (table 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 Bonferroni correction.

Interestingly, stigma-anther separation was positively correlated with style length but negatively correlated with anther height (table 3). This association indicates 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 (cf. fig. 3 and table 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 brandegei* displayed continuous variation in both type and degree of herkogamy (stigma-anther separation). A ratio of ca. 80 : 20 approach- to reverse-herkogamous individuals occurred in all three study populations. Herkogamy in *P. brandegei* was highly heritable, was largely independent of flower age, and was primarily

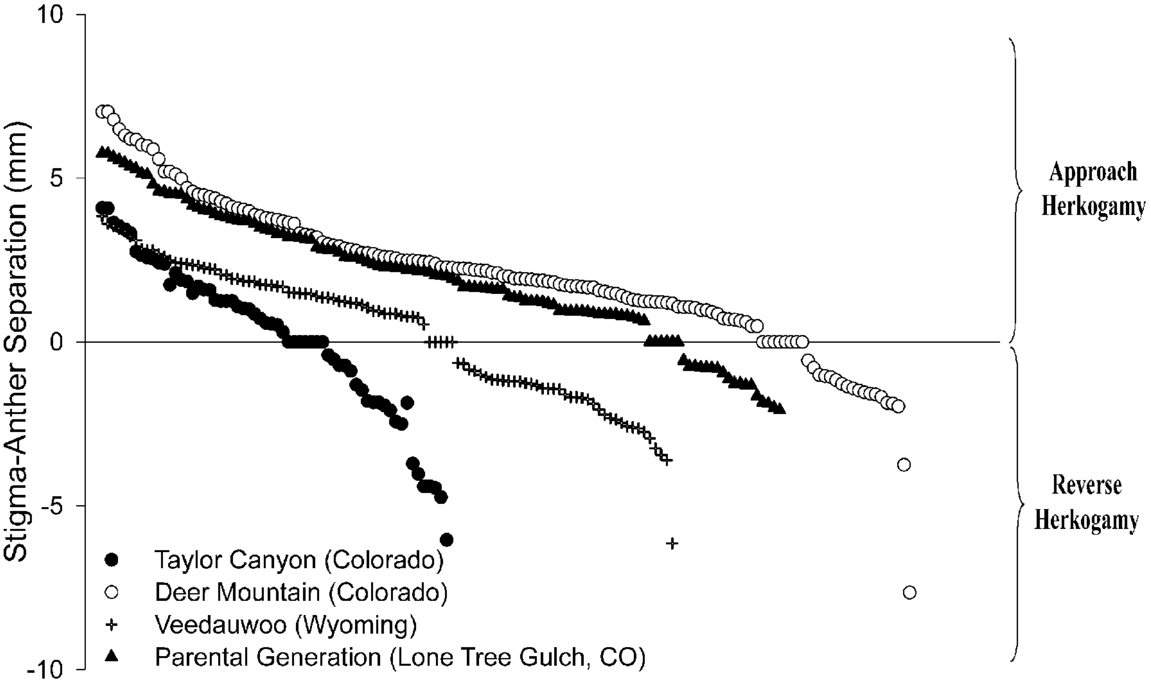


Fig. 5 Ranked stigma-anther separation of three field populations and the parental generation of greenhouse-grown *Polemonium brandegei*. Stigma-anther separation (distance from top of the anthers to the bottom of the stigma) is ranked from highest to lowest. Highest-ranking individuals are on the left of the horizontal axis, while the lowest-ranking ones are on the right. Measurements above the horizontal line are from approach-herkogamous flowers, while those below the line are from reverse-herkogamous flowers.

determined by variation in style exsertion. Based on visitation rates and behavior, hummingbirds and hawkmoths were likely important pollinators of *P. brandegei*. These pollinators displayed marked annual fluctuations in their relative abundances over our 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.

Floral Visitors

The flowers of *P. brandegei* were visited by a morphologically diverse fauna. Although several types of visitors may transfer pollen among individuals of *P. brandegei*, morphological and behavioral characteristics of both the animals and flowers likely reduced pollen transfer by flies, butterflies, and beetles. Bees and syrphids visited on numerous occasions (especially at 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 behavior, number of visitations, and plants visited indicate that hummingbirds (*Selasphorus platycercus*) and hawkmoths (*Hyles lineata*, *Hyles gallii*) may be important pollinators of *P. brandegei*. This is reflected in the calculated visitation index, which accounts for the frequency and duration of visits by each taxon (fig. 2). Both hummingbirds and hawkmoths seem likely to have imposed selection on *P. brandegei*'s floral morphology.

Hummingbirds were abundant in 2004 and 2005 and uncommon in 2006, whereas hawkmoths were observed only in 2006, mostly at Vedauwoo but also at Deer Mountain and Twin Sisters. The slightly later observation periods in 2006 (table 1) may explain the reduction in hummingbird observations during that year (fig. 2; app. A). However, our 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 (*H. 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. brandegei*, as occurs in several other species (reviewed in Fenster et al. 2004).

Phenotypic Variation in Floral Morphology

Polemonium brandegei did not exhibit polymorphic variation in stigma height but rather exhibited 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

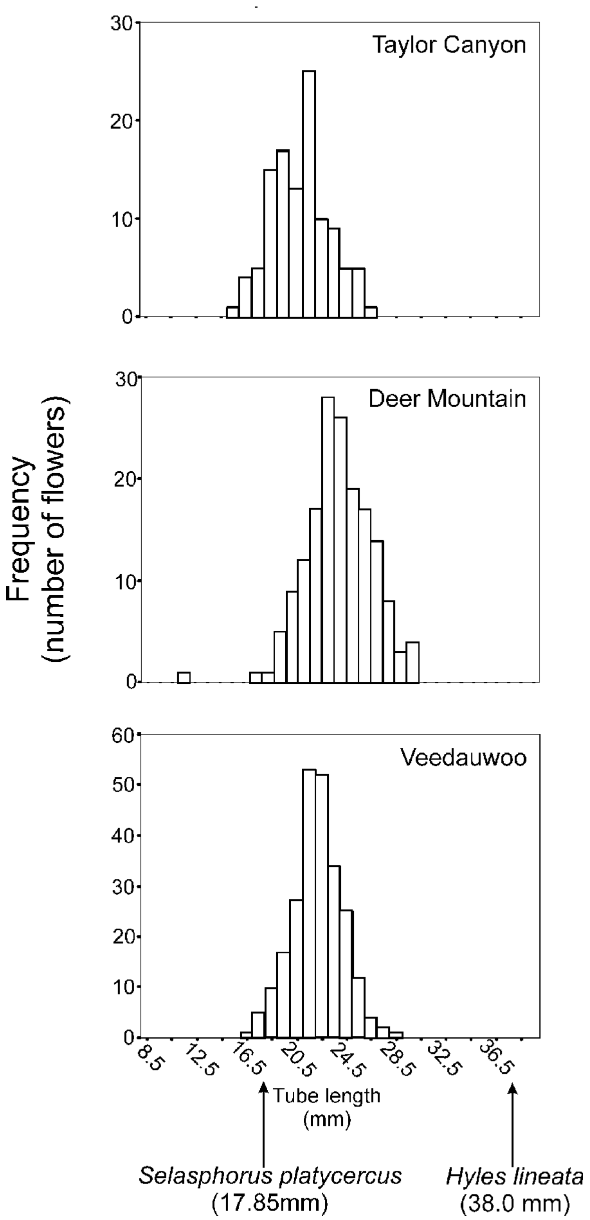


Fig. 6 Frequency distribution of corolla tube length from three field populations of *Polemonium brandegei*. Bill length of *Selasphorus platycercus* (Waser 1978) was averaged between two means for male and female birds (17.0 and 18.7 mm, respectively). *Hyles lineata* proboscis length is from Grant (1983).

their foreheads (Grant and Grant 1965, 1968). In *P. brandegei*, 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 *P. brandegei* and other species with similar pollinators conform to the above expectations, we compiled a survey of published floral dimensions (table 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 5). In *P. brandegei*, 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 favored by hummingbirds. Similarly, reverse herkogamy is required to separate the stigma and anthers and to display the inserted stigma favored 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 that sex organs ranged from exserted to inserted, as we observed in *P. brandegei* (Baker 1964; Grant and Grant 1983; Boyd 2002, 2004; Wolff et al. 2003).

In contrast to the sex organs, the corolla tube in *P. brandegei* was more comparable to tubes in other species pollinated by hummingbirds than to tubes in species pollinated by hawkmoths (table 5). Most *P. brandegei* flowers had corolla tubes longer than *S. platycercus*'s bill length (fig. 6). Therefore, hummingbirds pushed their heads deep into the flowers, thus increasing contact between the sex organs and the forehead (Grant and Grant 1965, 1968; Lertzman and Gass 1983). The proboscis of *H. lineata* is considerably longer than *P. brandegei*'s corolla tube (fig. 6) and is more likely to transport pollen than the forehead or thorax (see Grant and Grant 1965). Therefore, *P. brandegei*'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 Populations" for details). Similarly, *Achillea millefolium*, *Hypochaeris radicata*,

Table 3

Narrow-Sense Heritabilities (Diagonal) and Additive Genetic Correlations (above Diagonal) of Floral Traits in *Polemonium brandegei* with the Effects of Mass (Flower Size) Removed as a Covariate

	Tube length	Tube diameter	Style length	Anther height	Stigma-anther separation
Tube length	.125**	.997***	.309*	−.040	−.177***
Tube diameter		.044**	.314*	.005	−.211^{a}
Style length			.293***	.246	.442***
Anther height				.238**	−.653**
Stigma-anther separation					.851***

Note. Underlined values differed significantly from 0 after a sequential Bonferroni correction was applied.

[a] Value was significant before sequential Bonferroni correction.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

Table 4

Mean Trait Values and Standard Errors (mm) for Offspring Generation, Additive Genetic Variances (V_a), and Evolvabilities (CV_a) for the Offspring Generation

	Mean (SE)	V_a	CV_a
Tube length	21.14 (.16)	.513	3.39
Tube diameter	3.45 (.04)	.011	3.04
Style length	21.80 (.27)	2.757	7.62
Anther height	21.36 (.27)	2.899	7.97
Stigma-anther separation	5.84 (.14)[a]	4.639	36.88

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

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).

Genetic Variation in Floral Morphology

Significant heritable variation in the floral traits of *P. brandegei* indicates a genetic basis to the phenotypic variation we measured. Developmental effects contribute to variation in stigma-anther separation (herkogamy) in some species (e.g., Lennartsson et al. 2000). However, our comparisons of flowers in female and male phase eliminated this possibility in *P. brandegei*. In fact, herkogamy showed the highest heritability we measured ($h^2 = 0.851$; $CV_a = 36.88$). This value is higher than most published heritabilities for herkogamy, which range from 0.30 to 0.85 (see Shore and Barrett 1990; Carr and Fenster 1994; Robertson et al. 1994; Motten and Stone 2000; but see Lennartsson et al. 2000; Caruso 2004). Similar evolvabilities (CV_a) were found by Carr and Fenster (1994) for stigma-anther separation in *Mimulus guttatus* and *Mimulus micranthus* ($CV_a = 27.7$ and 33.4, respectively). 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 *P. brandegei*'s closest relative, *P. viscosum*, has introduced additional variation in style length. This explanation seems unlikely because the populations analyzed in this study are distant from populations of *P. viscosum* (75–100 km, plus ca. 600–800 m elevation), with the possible exception of the Deer Mountain population (ca. 12–15 km, plus ca. 400 m elevation). However, flower morphology and variation in stigma height in the Deer Mountain population are very similar to those in the other two populations (figs. 3, 5). Furthermore, gene flow from *P. viscosum* would likely also affect other traits that differ between the species, such as tube diameter, flower color, and vegetative morphology.

High genetic correlations may reflect strong and consistent selection for the integration of floral traits (Conner 1997; Waitt and Levin 1998; Herrera et al. 2002) or constraints imposed by pleiotropy (Ashman and Majetic 2006). Our study revealed high genetic and phenotypic correlations between tube length and diameter, whereas other genetic correlations involving corolla measurements were low to moderate (table 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). The strong positive genetic 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, our field populations had slightly larger flowers than greenhouse plants. This could result from the greenhouse plants being derived from a population different from those used for field measurements. Alternatively, this size difference could be due to differences in environmental conditions 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). We cannot rule out this possibility from our study. However, levels of total phenotypic variation were comparable in the field and the greenhouse (figs. 3, 5; app. B), indicating that heritabilities may also be comparable. Similarly, genetic and phenotypic correlations within our study were consistent in magnitude and direction, as were the phenotypic correlations among greenhouse and field populations (cf. fig. 3 and table 3). Therefore, extreme bias in our 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).

Implications for Floral Evolution

Variation between approach and reverse herkogamy in *P. brandegei* (fig. 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 (figs. 4, 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 Martén-Rodríguez 2007 for a discussion regarding self-compatible species). The intermediates in *P. brandegei* may represent a compromise between two extreme phenotypes that 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 0. We plan to test these possibilities by quantifying selection imposed by hummingbirds and hawkmoths on *P. brandegei* flowers. We expect a negative correlation between fitness and herkogamy under hawkmoth pollination and a positive correlation between fitness and herkogamy under hummingbird pollination.

Table 5

Summary of 19 Studies That Reported at Least Two of Apical Corolla Diameter, Tube Length, Stigma-Anther Separation, and Sex Organ Exsertion

Taxa	Apical corolla diameter (mm)	Corolla tube length (mm)	Herkogamy (stigma-anther separation)	Sex organ exsertion/insertion	References
Polemonium brandegei	3.7–5.8 (mean = 4.90)	18–29 (mean = 22.48)	−7.5 to 8.3 mm (mean = 1.65)	Stigma: −5.39 to 7.82 mm; anther: −.85 to 3.72 mm	This study
Hummingbird pollinated:					
22 species	1.5–7.0 (mean = 3.85)	10–33 (mean = 22.08)	na	na	Grant and Grant 1968
Ipomopsis aggregata	3.61	25.01	Reverse	Stigma: −1.20 mm; anthers: .21 mm	Grant and Wilken 1988; Campbell 1996
Marginatocereus marginatus	6.0	22.4	14.86 mm[a]	Stigma: .50 mm;[a] anther: −14.28 mm[a]	Dar et al. 2006
Melocactus curvispinus[b,c]	2.76–3.17	24.48–25.89	.58–1.78 mm	Stigma slightly exserted; anther inserted	Nassar and Ramírez 2004
Mimulus cardinalis	3.90	25–30	6.0 mm[a]	Exserted	Grant and Grant 1968; Bradshaw et al. 1998
Nicotiana glauca[b]	6.93–8.55	35.17–42.59	Approach	Exserted	Schueller 2007
Penstemon barbatus[c]	6.0	37.0	Reverse	Exserted	Mitchell and Shaw 1993
Penstemon pinifolius[c]	4.0	37.0	Reverse	Exserted	Lange et al. 2000
Penstemon centranthifolius[c]	4.94	25.04	na	Inserted	Lange et al. 2000
Hawkmoth pollinated:					
28 species	na	10–170 (mean = 49.9)	na	na	Grant 1983
Datura stramonium	na	74.7–99.5	−4.04 to 3.28 mm[d]	Stigma inserted to exserted; anther inserted	Motten and Stone 2000
Diervilla lonicera	1.2	7.4	Reverse	At/near opening of corolla tube	Schoen 1977
Ipomopsis tenuituba	2.6–2.8	32.1–33.0	Approach	Inserted	Grant and Grant 1965; Campbell et al. 1997
Mirabilis longiflora	2.0	100–105	Approach	1.5–2.0 mm	Grant and Grant 1983
Phlox superba	na ("narrow")	26–33	Ca. .0 mm	Inserted	Grant and Grant 1965; Strakosh and Ferguson 2005
Hawkmoth and hummingbird pollinated:					
Istertia laevis	6.5	41.0	Ca. 9.0 mm[a]	Stigma slightly exserted; anthers inserted	Wolff et al. 2003
Macromeria viridiflora	7–12	45–77	Ca. .0 mm to slightly approach	Stigma inserted to exserted; anther inserted	Boyd 2002, 2004

Note. Also included are two additional surveys by Grant and Grant (1968) and Grant (1983). Studies with the appropriate data were selected from 196 articles that reported visitation/pollination by hummingbirds and/or *Hyles lineata* of western North American plant taxa. Values given are population means or ranges among populations. na = information not available.

[a] Calculated from data given by cited study.

[b] Range of means reported from more than one population.

[c] Species where hummingbird/hawkmoth is not the sole pollinator but has been determined to be a primary pollinator.

[d] Variation related to selfing rate.

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Appendix A

Visitation Data Summary

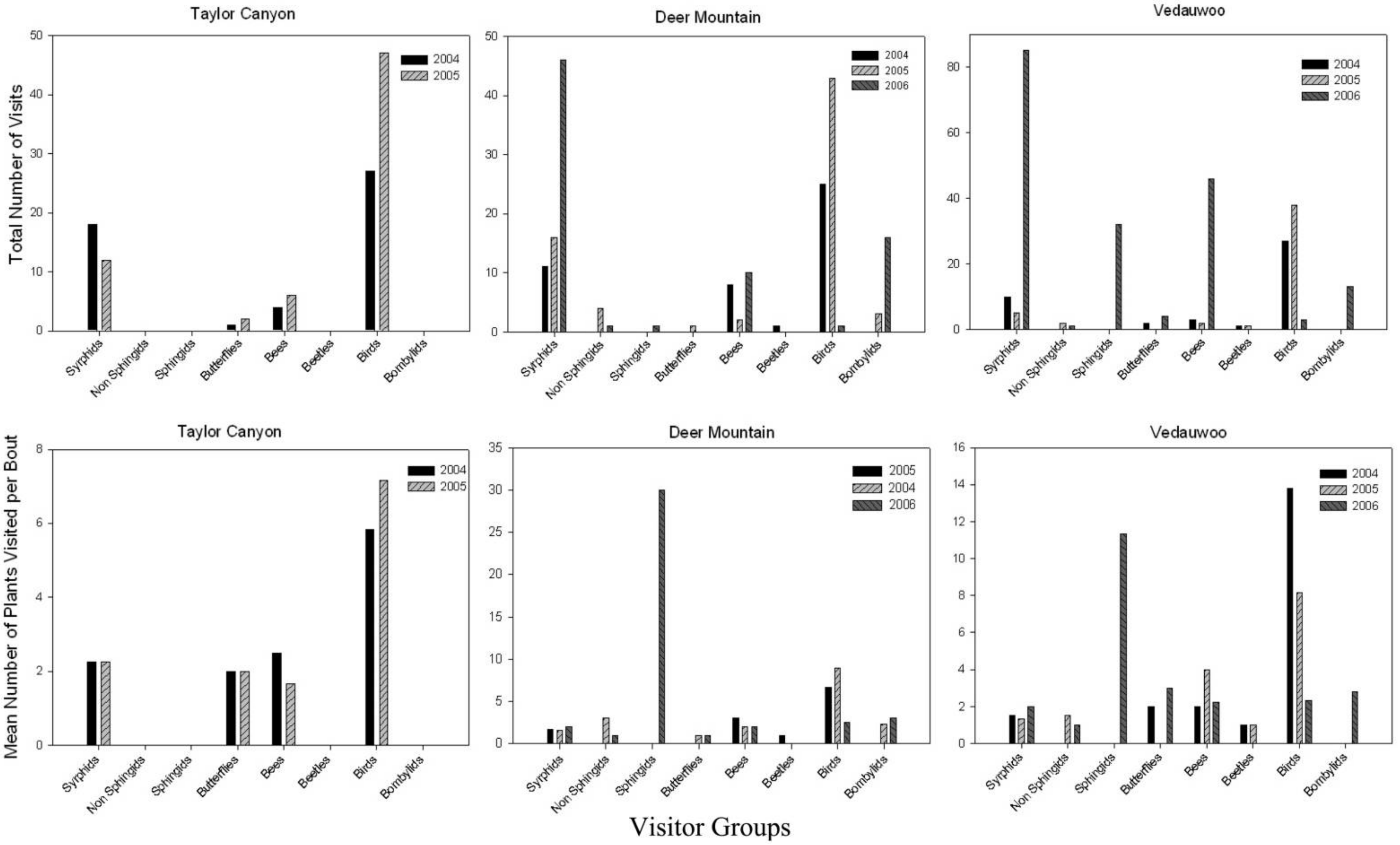

Fig. A1 Total number of observed visitations (top) and mean number of plants visited per foraging bout (bottom) of eight major visitor groups of *Polemonium brandegei*. Data are presented for the three analyzed field populations (Taylor Canyon, Deer Mountain, and Vedauwoo) for three flowering seasons (2004–2006). Observations were not made in Taylor Canyon during the 2006 season.

Appendix B

Floral Measurements by Population

Table B1

***Polemonium brandegei* Floral Measurements (mm) by Population**

Trait	Taylor Canyon ($N = 110$)	Deer Mountain ($N = 164$)	Vedauwoo ($N = 243$)
Flower width	14.25 (.20)	16.17 (.20)	17.80 (.17)
Flower length	27.66 (.28)	32.67 (.27)	30.60 (.17)
Tube length	20.85 (.22)	24.44 (.21)	22.16 (.13)
Basal tube diameter	2.36 (.11)	2.39 (.08)	2.92 (.03)
Apical tube diameter	4.65 (.14)	5.04 (.09)	5.02 (.05)
Stigma-anther separation	1.61 (.23)	2.05 (.18)	1.30 (.18)
Style length	22.32 (.33)	28.50 (.45)	24.98 (.22)
Anther height	22.27 (.30)	26.53 (.27)	25.67 (.15)

Note. Measurements are presented as means, with SE in parentheses. See figure 1 for precise location of measurements.

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