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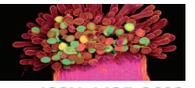
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## RESEARCH PAPER

# Patterns of pollen removal and deposition in *Polemonium brandegeei* (Polemoniaceae): the role of floral visitors, floral design and sexual interference

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Floral traits; herkogamy; pollen removal and deposition; pollinators; sexual interference.

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**ABSTRACT**

The arrangement, colour, shape and size of floral parts (collectively floral design) have evolved primarily to promote mating success *via* animal-mediated pollen transfer. Although numerous studies have examined variation in pollinator assemblages, relatively few have examined patterns of pollen removal and deposition in the presence of fluctuating pollinators and ineffective floral visitors; therefore, net pollen removal and deposition by entire visitor assemblages are unclear. We studied the timing (diurnal or nocturnal) and effects of floral traits on pollen removal and deposition under a dynamic visitor assemblage of *Polemonium brandegeei*. We quantified pollen grains remaining in anthers (pollen removal) and deposited on stigmas (pollen deposition) of plants visited during either the day (07:30–20:00 h) or night (20:30–07:30 h) in natural populations over two flowering seasons. Pollen removal and deposition occurred both diurnally and nocturnally during our study. Increased diurnal removal and deposition coincided with peak floral visitations in 2006. This increase in pollen removal and deposition may reflect increased visits by pollen consumers, effective hawkmoth pollinators and increased self-pollen deposition due to hot, dry weather. Nonlinear effects of style length significantly affected pollen removal, with less pollen remaining in flowers with intermediate style lengths. Pollen deposition was more complex, with herkogamy and anther height affecting deposition. Further, close proximity of stigmas and anthers increased the potential for sexual interference between pollen removal and deposition. Overall, flower visitations and pollen removal and deposition varied between years and populations, but sex organ placement consistently influenced the removal and deposition of pollen.

**INTRODUCTION**

The removal and deposition of pollen among individual plants is essential for sexual reproduction in outcrossing plants. Multiple factors influence successful pollination. First, in animal-pollinated species, attracting adequate numbers of visitors is necessary to ensure sufficient pollen removal and deposition for reproduction. Second, the efficiency of pollen movement varies among animal visitors. For example, some insect visitors actively collect and consume pollen at the expense of plant reproduction (Harder & Routley 2006; Hargreaves *et al.* 2012). Efficient pollen transport requires that pollen be placed on a region of the visiting animal's body where it can be deposited on the receptive stigmas of subsequently visited flowers (Inouye *et al.* 1994). Finally, deposition of self-pollen can also affect the reproductive output of flowering plants (Harder & Barrett 1995; Karron & Mitchell 2012). Self-pollen deposition can occur when pollinators transfer pollen among flowers on the same plant (geitonogamy), or within the same flower (facilitated autogamy). All the above components of pollen removal and deposition are also influenced by abiotic factors, making the efficiency of this process context-dependent (Anderson & Johnson 2007).

Many flowering plant species 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 populations. For example, wide variation in the rate of pollen deposition occurred across six populations and seven flowering seasons in *Ipomopsis aggregata* (Price *et al.* 2007). In addition, variation occurred within populations over a single flowering season due to changes in the relative abundances 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). Such variation in the efficiency of floral visitors can introduce variation in the removal and deposition of pollen (Adler & Irwin 2006), potentially leading to discordance between the amount of pollen removed and deposited (Wilson & Thomson 1991). However, other studies have found more consistent assemblages of pollinators, which may lead to more consistent patterns of pollen removal and deposition (Sahli & Conner 2007). Additional studies of pollinator assemblages and pollen removal and deposition are required for a complete understanding of pollinator–pollination dynamics. Further, the

influence of temporal and spatial variation in pollinator visitation and effectiveness on patterns of pollen removal and deposition necessitates multi-year and multi-population studies to determine whether some aspects of floral design are consistent in their effects on pollen removal and deposition.

Regardless of the timing of pollen movement, interference between female and male functions due to close sex organ proximity can limit reproductive success (Barrett 2002). For example, the process of pollen removal may impede pollen deposition by the close placement of the stigma relative to the anthers. Further, deposition of outcross pollen may be blocked by the presence of self-deposited pollen (Webb & Lloyd 1986). This sexual interference can be facilitated *via* floral visitors or abiotic factors (e.g. wind). Therefore, both biotic and abiotic factors should be considered during the process of pollination.

In the present study, we examined the effects of variation in floral and inflorescence traits on the removal and deposition of pollen over two consecutive flowering seasons, in natural populations of the sub-alpine perennial *Polemonium brandegeei*. Previous work determined that among a variable and diverse floral visiting fauna, hummingbirds and hawkmoths are important pollinators (Kulbaba & Worley 2008). Further, the floral traits of *P. brandegeei* display a large degree of variation that relates to hummingbird and hawkmoth pollination, particularly the spatial separation of sex organs (herkogamy). The flowers of *P. brandegeei* display a continuous range of sex organ arrangement, with plants exhibiting flowers with stigmas exerted above the anthers (approach herkogamy), to flowers with stigmas recessed below anthers (reverse herkogamy; Kulbaba & Worley 2008).

In experimental populations of *P. brandegeei*, plants displaying flowers with reverse herkogamy experienced more pollen deposition under hawkmoth pollination, and plants displaying flowers with relatively narrow corolla tubes had more pollen removed (Kulbaba & Worley 2012). Under only hummingbird pollination, plants displaying approach herkogamy experienced more deposition of outcross pollen, and plants with longer corolla tubes had more pollen removed (Kulbaba & Worley 2013). Examining patterns of pollen removal and deposition by whole floral visitor assemblages allows a more complete view of pollination dynamics in natural populations. Our objectives were to determine the timing (day *versus* night) of pollen removal and deposition, and examine the effects of floral traits on pollen removal and deposition under natural floral visitor assemblages. We further examined the potential role of variable female and male sex organ proximity to the removal and deposition of pollen grains.

We predicted that (i) when daytime pollinators (particularly hummingbirds) were abundant, pollen removal and deposition would primarily be diurnal. (ii) In contrast, when nocturnal or crepuscular pollinators (hawkmoths) were more abundant, pollen removal and deposition would occur during the evening/night. Based on the pollination biology literature and our previous experiments measuring selection by hawkmoths and hummingbirds, (iii) we predicted more pollen would be removed from flowers with long-corolla tubes, and more pollen would be deposited on flowers with approach herkogamy when hummingbirds were abundant. When hawkmoths were common, we predicted flowers displaying reverse herkogamy would exhibit higher pollen deposition. (iv) When pollen-consuming visitors were common, pollen removal was predicted

to increase but not necessarily pollen deposition due to pollen theft. Finally, we predicted (v) that when female and male sex organs were in close proximity, pollen removal and deposition would increase in both visited and unvisited control plants.

## MATERIAL AND METHODS

### Experimental design and data collection

We quantified pollen removal and deposition among individuals over the 2005 and 2006 flowering seasons of *P. brandegeei*. To determine the timing of pollen removal and deposition, we manipulated the time of day that plants could be visited by pollinators in three natural populations near the centre of the range of *P. brandegeei*. We then quantified pollen removal and deposition from plants exposed to pollinators at different times of the day. Patterns of pollen removal and deposition were then compared with variation in floral visitation rates. Both study seasons coincided with the floral measurements and observations of flower visitations described in Kulbaba & Worley (2008).

We performed a series of pollinator exclusion experiments to determine the timing of pollen removal and deposition (Table 1). These experiments included three manipulative treatments of individual plants that differed in when the plants were exposed to pollinators: *Day* plants were exposed to day-flying floral visitors from 07:30 h to ~20:00 h (daylight hours), while for the remaining time, plants were enclosed within tightly woven mesh tents to prevent visitation. *Night* plants were exposed to potential pollinators from ~20:00 h to 07:30 h (included dusk and dawn), and covered for the remaining hours. *Control* plants remained covered throughout the entire course of the exclusion trial, and provided data on self-pollen deposition due to mechanical contact between stigma and anthers. Each exclusion trial lasted 48 h; therefore each *Day* and each *Night* treatment plant was covered and exposed twice.

In 2005, exclusion trials were performed in Taylor Canyon and Deer Mountain, Colorado, and Vedauwoo, Wyoming. In 2006, we performed exclusion trials in the Deer Mountain and Vedauwoo populations. We ensured that a balance between approach and reverse herkogamous plants was included in each experimental treatment type to compare the patterns of pollen removal and deposition among these floral designs.

Two flowers on each experimental plant were designated as 'focal flowers' and marked on the calyx with acrylic paint just

**Table 1.** Sample sizes for pollinator exclusion experiment for 2005 and 2006 field seasons. Data from the 2005 field season are from all three field populations (Taylor Canyon, Deer Mountain and Vedauwoo), while only two populations (Deer Mountain and Vedauwoo) were visited in 2006.

	2005		2006	
	approach	reverse	approach	reverse
herkogamy				
treatment				
control	71	12	19	9
day	66	52	14	14
night	74	37	16	12

Intermediate (–0.5 to 0.5 mm) stigma–anther separations are included in the Approach category.

prior to opening. After the 48-h period of the exclusion trials, all five anthers from each focal flower were stored in 70% ethanol and the stigmas mounted in fuschin-stained jelly on a microscope slide (Beattie 1971) for later quantification. Pollen remaining in anthers was used as an indicator of pollen removal. Plants with less pollen in their anthers were assumed to have experienced higher removal. The numbers of grains remaining in anthers (pollen removal) and deposited on stigmas were averaged from both focal flowers for analysis. To determine the total number of pollen grains produced at the population level, unvisited anthers from one flower on seven plants were collected from each population.

To examine how variation in floral traits affected pollen removal and deposition, we measured floral dimensions in both female phase (stigma open and receptive) and male phase flowers (anthers dehisced) on the same inflorescences as the focal flowers. We measured flower width (distance across opposite petal lobes), total flower length (distance from the base of the corolla to the tip of an upturned petal), corolla tube length (distance from the base of the corolla to the tube opening), tube width at the base and apex of the flower, herkogamy (distance between the stigma and closest anther), height of sex organ presentation relative to the base of the flower, and flower mass (dry weight of corolla, calyx, androecium and gynoecium). We also measured several aspects of floral display, including: number of inflorescences per plant, inflorescence height, number of open flowers per inflorescence, and number of flowers in female and male phase.

Pollen grains were dislodged from the anthers by placing them in a sonicator (Branson, Danbury, CT, USA) for 5 min. The amount of pollen remaining in anthers was determined with a Multisizer III particle counter (Beckman Coulter, Mississauga, Ontario, Canada). Anthers from each focal flower were suspended in 25 ml of electrolyte solution (Isotone II<sup>®</sup>, Beckman Coulter, Mississauga, Ontario, Canada) and three 500- $\mu$ l subsamples were averaged for the final estimate. Pollen deposition was quantified by manually counting fuschin-stained pollen under light magnification with the aid of image capture software (Image-Pro express, version 4.5.1, Beckman Coulter, Mississauga, Ontario, Canada). 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, our quantification of pollen deposition reflected both self and out-cross pollen.

Abiotic factors may affect pollen removal and deposition directly, or through effects on pollinator activity. We calculated the average high and low temperatures and average wind speed for the three study populations during our treatments. Temperatures and average wind speeds for Taylor Canyon and Vedauwoo were obtained from the Weather Underground database ([www.weatherunderground.com](http://www.weatherunderground.com), accessed 17 January 2012) and the Almanac database for Deer Mountain ([www.almanac.com](http://www.almanac.com), accessed 17 January 2012).

### Floral visitor observations

We observed floral visitors at all populations, in both years of study, at three standardised times: 07:30, 12:30 and 19:30 h. Each observation period lasted 30 min, with additional observations occurring in the evening when weather permitted (20:00–22:30 h). Informal observations were also made

throughout the day to supplement the standardised observation periods. The floral visitor observations coincided with those described in Kulbaba & Worley (2008).

### Statistical analyses

Pollen remaining in anthers and pollen deposited on stigmas was analysed using general linear models (Proc GLM) in SAS version 9.2.2 (SAS Institute, Cary, NC, USA) and with generalised linear models (Proc GENMOD) that uses a maximum likelihood approach. Both techniques gave identical results; therefore, results from the general linear models are presented.

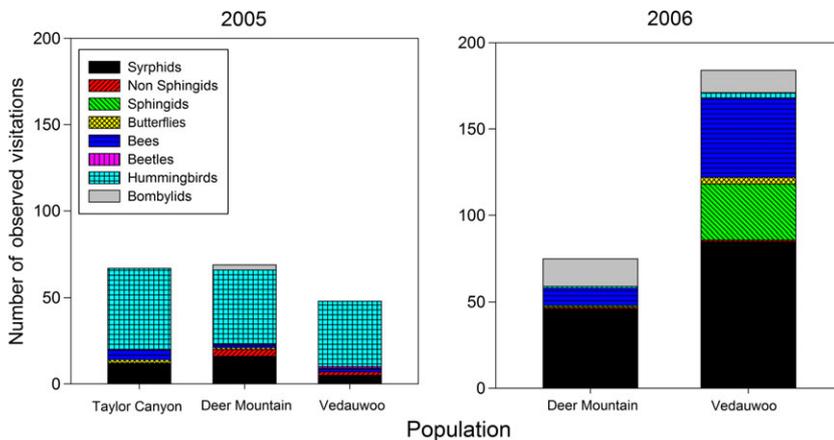
General linear models were analysed with pollen remaining in anthers and pollen deposited on stigmas as dependent variables. Treatment type (Control, Day, Night) was a categorical fixed effect, and population (Deer Mountain, Taylor Canyon, Vedauwoo) was a categorical random effect. Separate analyses were conducted for each year of the study because the Taylor Canyon population could not be examined in 2006. To determine if differences among treatments in pollen remaining in anthers were influenced by variation in total pollen production, we also estimated pollen removal. We examined the average amount of pollen removed, as the difference between the average number of pollen grains produced (as determined from seven unvisited anthers at each population) and the number of pollen grains remaining in anthers for each experimental treatment. Both linear and quadratic terms for floral and inflorescence characters were included in the model. We explored the impact of all two-way interactions among covariates, and of class variables and covariates on the dependent variables. A manual reverse-stepwise elimination process was employed to remove non-significant interaction terms and covariates (Sokal & Rohlf 1994). Finally, least-squared means were calculated and compared *via t*-tests to examine differences among treatment and population effects. To meet assumptions of normality and stable 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 remaining in anthers and deposited on stigmas, we obtained slope estimates of the relationships between significant covariates and dependent variables. 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, we adjusted the data in our figures to account for significant effects of specific floral traits. 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.

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 effect on the relationship between anther height and pollen deposition. The removal of this single data point changed the slope of the relationship from positive to negative. As this value was well outside the distribution of anther height from all three populations, over 2 years of

**Table 2.** Average maximum and minimum temperature and average wind speed for dates visited for three study populations of *Polemonium brandegeei*.

population (latitude and longitude)	dates visited	average maximum temperature (°C)	average minimum temperature (°C)	average wind speed (km·h <sup>-1</sup> )
Deer Mountain, CO	June 1–11, 2005	24.9	7.8	11.9
(40°46'57"N, 105°53'01"W)	June 6–12, 2006	34.4	13.4	13.0
Taylor Canyon, CO	May 25–31, 2005	20.9	1.6	6.3
(39°34'33"N, 104°22'26"W)				
Vedauwoo, WY	June 12–19, 2005	21.8	6.3	19.1
(44°29'39"N, 116°18'50"W)	June 12–17, 2006	28.2	8.4	21.2

**Fig. 1.** Average diversity and abundance of floral visitors in the 2005 (three populations) and 2006 (two populations) flowering seasons. The number of colours in each bar indicates the diversity of the floral visitor assemblage, and the height of each colour bar indicates the number of visits by a given taxon.

measurements, we present analyses and results without the extreme value of anther height. Similarly, an individual with 23 inflorescences from the Vedauwoo population was removed that was well beyond the maximum of 15 inflorescences found across all individuals. Finally, one individual with an unusually large number of flowers (39) in Deer Mountain was removed. These outliers individually affected the resulting analyses and were well beyond the range of observed traits; therefore, we removed these data prior to analysis.

## RESULTS

### Environmental conditions and pollinator activity

The dates on which each population was visited and the environmental data over those periods are presented in Table 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.

The abundance and diversity of floral visitors varied across populations and flowering seasons. The largest abundance of floral visitors occurred in 2006 (Fig. 1), in the Vedauwoo population, while visitors and observed visitations were fewest in Taylor Canyon in 2005. Further, the diversity of floral visitors was lowest at Taylor Canyon in 2005 and highest at Vedauwoo in 2006 (Kulbaba & Worley 2008). The highest number of pollen-collecting floral visitors (e.g. syrphids, bees, etc.) occurred in Vedauwoo during the 2006 flowering season (Kulbaba & Worley 2008; Fig. 1).

### Pollen remaining in anthers

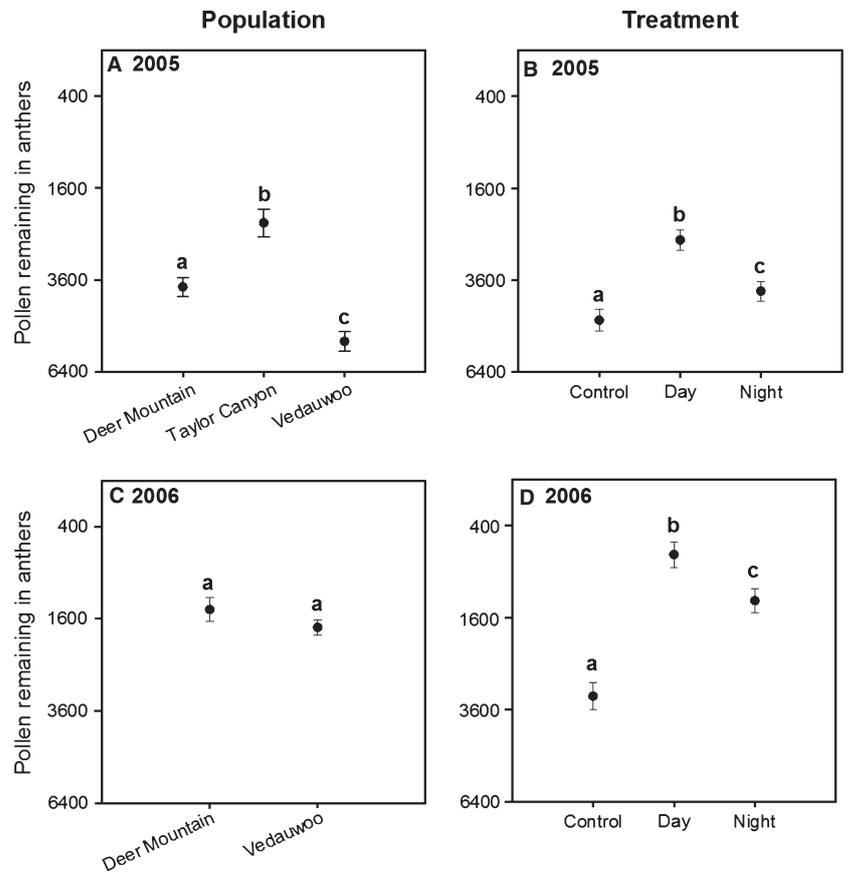
Similar within-population patterns of pollen remaining in anthers were found across all three treatment types in 2005 and 2006 (Table 3). In both years, significant differences occurred among all three treatments, with least pollen remaining in the day and most in unvisited control plants (for all pair-wise comparisons,  $t > 2.5$ ,  $P < 0.01$ ; Fig. 1). Overall, less pollen remained in 2006 compared to 2005, particularly in the day and night treatments (Fig. 2).

The amount of pollen remaining in anthers differed among populations only in 2005 (Table 3). The fewest pollen grains remaining were found in Taylor Canyon, intermediate in Deer Mountain, and the most pollen remaining occurred in plants from Vedauwoo (for all pair-wise comparisons,  $t > 3.2$ ,  $P < 0.001$ ; Fig. 2). Mean floral trait measurements and standard errors from all populations are reported in Appendix S1.

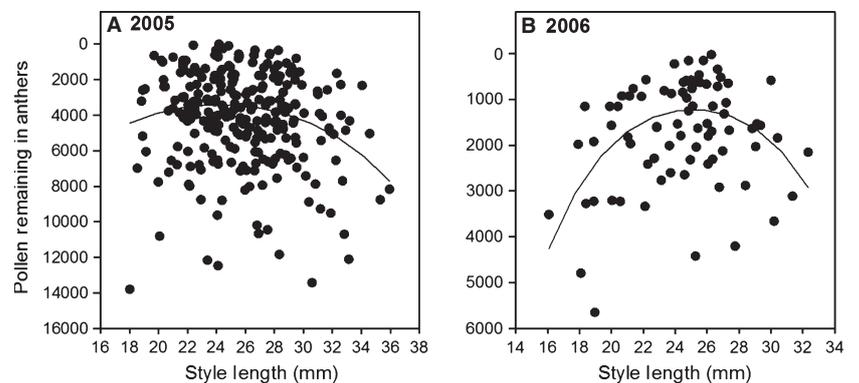
**Table 3.** General linear model describing the effects of population, treatment and floral traits on pollen removal (pollen remaining in anthers) in *Polemonium brandegeei* during the 2005 and 2006 field seasons.

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) <sup>2</sup>	$F_{1, 301} = 6.29^*$	$F_{1, 75} = 11.49^{**}$
$R^2$	0.30	0.61

\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$ .



**Fig. 2.** Adjusted back-transformed, square-root transformed least square mean ( $\pm$ SE) pollen remaining in anthers of *Polemonium brandegeei* across populations (left panels) and treatments (right panels) for 2005 (A and B) and 2006 (C and D) flowering seasons. Significant differences between the factor levels (at  $\alpha = 0.05$ ) are denoted by differences in the letters above the symbols. Note inverted axes describing pollen remaining in anthers.



**Fig. 3.** 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  $b \pm$  SE for 2005: style:  $-7.49 \pm 4.24$ ,  $\text{style}^2$ :  $0.16 \pm 0.07$ . Estimate of  $b \pm$  SE for 2006: style:  $-18.86 \pm 4.69$ ,  $\text{style}^2$ :  $0.38 \pm 0.09$ . Note difference in scale between pollen remaining in anthers from 2005 and 2006 and inverted axis of pollen remaining in anthers.

Pollen from unvisited anthers was examined in each population to determine if variation in overall pollen production contributed to differences among populations in pollen remaining in anthers. Total pollen production varied across populations and generally corresponded with our estimate of flower size (dry mass). Unvisited anthers from Taylor Canyon produced the fewest pollen grains (2005 mean  $\pm$  SE:  $6646 \pm 659$  grains) and the smallest flowers (2005:  $5.95 \pm 0.16$  mg). Plants from Deer Mountain produced an intermediate number of pollen grains (2005 and 2006:  $8689 \pm 403$  grains), and intermediate flower mass (2005 and 2006:  $7.11 \pm 0.19$  mg), and finally, Vedauwoo plants produced the most pollen grains (2005 and 2006:  $10690 \pm 1457$  grains) and largest flowers (2005 and 2006:  $7.53 \pm 0.22$  mg). Once total pollen production was taken into account, no significant

differences in pollen removal were detected among populations (results not shown).

In both years, style length was the only floral or inflorescence trait that was significantly associated with the amount of pollen remaining in anthers. Both linear and quadratic terms of style length were significant, and the effect did not differ significantly among populations or treatments. Finally, we found that plants displaying intermediate values of style length consistently had the least amount of pollen remaining in anthers, and therefore more pollen removed (Fig. 3).

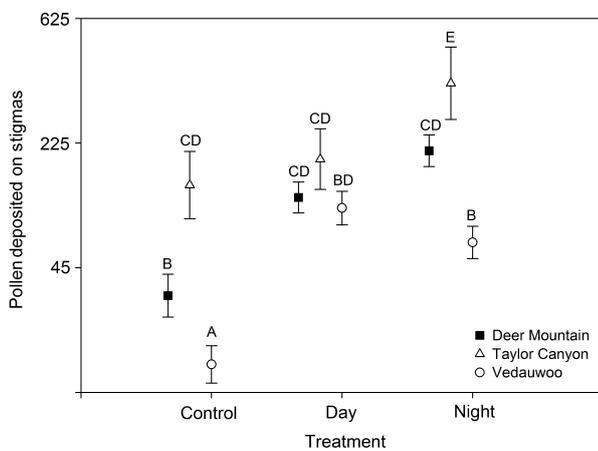
#### Pollen deposition

The factors that affected pollen deposition differed in each year (Table 4). In 2005, the amount of pollen deposited in the

**Table 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 $\times$ 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) <sup>2</sup>	$F_{1, 301} = 15.62^{***}$	–
anther height	$F_{1, 301} = 0.86$	–
anther height $\times$ population	$F_{2, 301} = 8.04^{**}$	–
(stigma–anther separation) <sup>2</sup> $\times$ treatment	$F_{1, 301} = 5.65^{**}$	–
$R^2$	0.41	0.15

\* $P < 0.05$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0001$ .



**Fig. 4.** Least square mean comparison of pollen deposition across populations and treatments in 2005. Data points are least-squares means, error bars represent  $\pm 1$  SE. Data points denoted by different letters are significantly different at  $\alpha = 0.05$ .

different treatments varied among populations (treatment  $\times$  population interaction). Herkogamy and anther height also influenced pollen deposition in 2005. Although overall levels of pollen deposition were comparable between years, pollen deposition in 2006 was only influenced by herkogamy, with recessed stigmas exhibiting higher pollen deposition. However, the overall model was marginally non-significant ( $P = 0.056$ ; Table 4).

In 2005, across all treatment types, we observed the highest pollen deposition in Taylor Canyon; Deer Mountain had intermediate pollen deposition and Vedauwoo exhibited the lowest amount of pollen deposition. However, all three populations displayed comparable stigma loads on day-treatment flowers (all  $t > -1.35$ ,  $P > 0.17$ ; Fig. 4). 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 from controls in Taylor Canyon ( $t > -3.04$ ,  $P < 0.04$ ). In 2006, we did not detect any significant differences among populations or treatments with respect to pollen deposition.

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 the day and night treatments and linear in the control treatment. However, in 2006, the effect of herkogamy was linear in all three treatments (Fig. 5). Therefore, in 2005, slightly recessed stigmas received the highest pollen loads, while in 2006 more recessed stigmas received more pollen.

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 populations (Fig. 6). All slope estimates were significant, and significantly different from one another ( $t > 2.48$ ,  $P < 0.05$ ). However, the distribution of anther height differed across populations (Fig. 6, Appendix S1).

## DISCUSSION

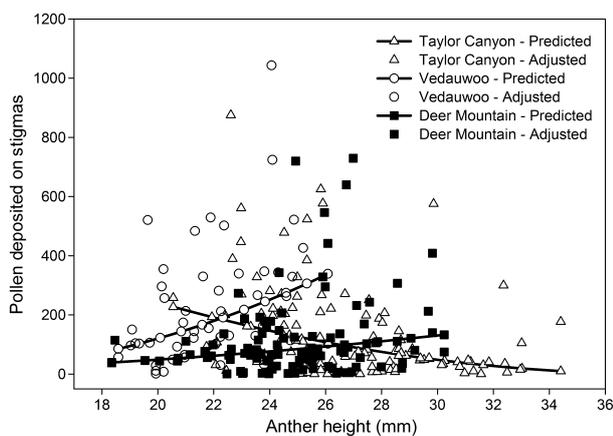
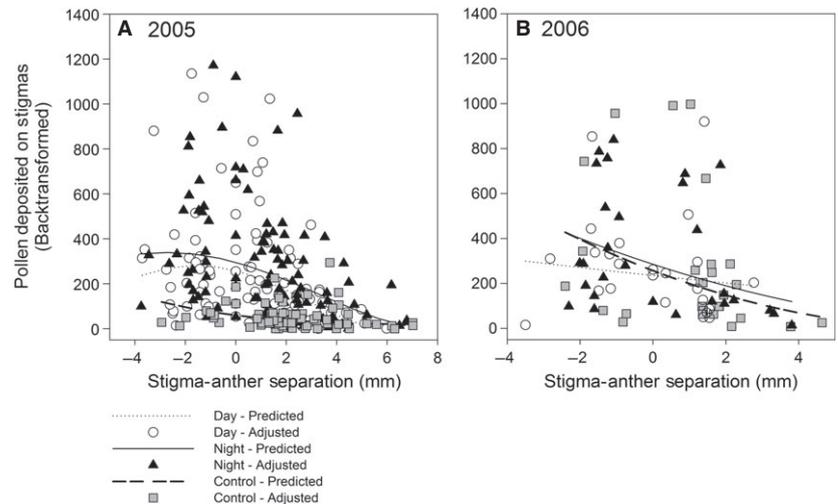
We measured pollen remaining in anthers of *P. brandegeei* as a proxy for pollen removal, and the number of pollen grains deposited on stigmas in plants visited by pollinators during the day, night or not visited at all. Quantifying pollen removal and deposition in unvisited plants allowed us to gauge the rate of mechanical self-pollen deposition within flowers, relative to plants visited during the day or night. Our detection of increased diurnal and nocturnal pollen deposition likely reflected pollen that contributed to reproductive success (out-cross pollen) as well as self-pollen. Further, we found that the close proximity of stigma and anthers increased both sexual interference through self-pollen deposition and pollen import, and that style length affected the amount of pollen remaining in anthers. Finally, we found that an increase in pollen-collecting insects resulted in less pollen remaining in anthers; however this effect might have been confounded by abiotic effects in 2006.

### Temporal and spatial variation in pollen remaining in anthers and deposited on stigmas

Rates of pollen removal and deposition generally increase with visitation rate (Sahlí & Conner 2011). Differences in pollinator activity partially explain variation in the removal and deposition of pollen in our study of *P. brandegeei*. Overall, fewer pollen grains remained in the anthers of day-treatment plants. This pattern was consistently observed across populations and in both flowering seasons. As predicted, this pattern of removal corresponded with our observation of the highest floral visitor activity during daylight hours (Kulbaba & Worley 2008). 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 (Fig. 2). Our predictions of less pollen remaining in flowers displaying reverse herkogamy when hawkmoth pollinators were common, and of less pollen in flowers with long corolla tubes when hummingbirds were common were not confirmed.

In contrast to pollen removal, increased visitor activity in 2006 did not translate into increased pollen deposition, perhaps due to the high frequency of pollen-collecting insects. Pollen-collecting bees and syrphid flies can remove large amounts of pollen, which is then consumed (Hargreaves *et al.* 2009). As this pollen is not transferred to stigmas on subsequent visits, these ‘pollen thieves’ do not positively contribute to plant reproduction (Wilson & Thomson 1991) because the fraction

**Fig. 5.** Effects of stigma–anther separation of *Polemonium brandegeei* on pollen deposition during 2005 (quadratic relation) (panel A) and 2006 (panel B) flowering seasons (linear relation). Estimates of linear ( $\pm$ SE) and quadratic ( $\pm$ SE) terms for herkogamy (stigma–anther separation) in 2005: Control:  $-0.52 \pm 0.08$  and  $-0.14 \pm 0.06$ ; Day:  $-0.29 \pm 0.11$  and  $-0.23 \pm 0.05$ ; Night:  $-0.18 \pm 0.07$  and  $-0.20 \pm 0.07$ . Estimates of linear terms ( $\pm$ SE) for herkogamy in 2006: Control:  $-1.93 \pm 0.76$ , Day:  $-0.56 \pm 0.22$ , Night:  $-1.56 \pm 0.77$ . Negative values of herkogamy represent stigmas recessed below anthers, and positive values of herkogamy represent stigmas exerted beyond the anthers. Lines are predicted functions and points represent adjusted values that account for all three treatments (Control, Day and Night).



**Fig. 6.** Predicted linear functions (solid lines with open circles) and adjusted values (points) of the effects of anther height of *Polemonium brandegeei* on pollen deposition. Data are from the 2005 flowering season, for three populations, to highlight the interaction between anther height and population. Estimates of  $b$  ( $\pm$ SE) for anther height: Deer Mountain:  $-0.82 \pm 0.18$ , Taylor Canyon:  $1.03 \pm 0.41$ , Vedaauwo:  $0.45 \pm 0.20$ . Note: estimate for Vedaauwo was marginally significant ( $P = 0.044$ ).

of pollen deposited on conspecific stigmas can be very small (Thomson *et al.* 2000; Wolf *et al.* 2003). Therefore, the amount of pollen remaining in anthers may overestimate pollen export. Consumption of pollen without pollination service reduces reproductive output and potentially fitness in flowering plants (Hargreaves *et al.* 2010). However, the consumption of pollen without efficient pollination service may be beneficial in some instances by enhancing the outcrossing rates for some pollinators (Brunet & Holmquist 2009).

Although pollen removal and deposition 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 removal and deposition raises the possibility of unobserved crepuscular or nocturnal visitors; however, nocturnal hawkmoths were common in 2006 and likely contributed to pollen movement (Kulbaba & Worley 2008).

In addition to insect activity, abiotic factors can greatly influence pollen movement (Strauss & Whittall 2006). In *P. brandegeei*, changes in temperature and wind speed may have contributed to variation in pollen removal and deposition between years and populations. The flowering season in 2006 was characterised by warmer temperatures and moderately more wind than in 2005 (Table 1), resulting in dried, loosely aggregated pollen that was easily dislodged. Agitation by floral visitors and wind may explain why plants in the 2006 control treatments had less pollen remaining in anthers than control treatment plants in 2005, and why deposition in 2006 was similar in control plants and plants exposed to pollinators. An additional (non-exclusive) explanation may lie in the reduced sample size in 2006 compared to 2005.

Pollinator assemblages can vary in relative abundance or even presence and absence across years and populations (Herrera *et al.* 2006). Consequently, spatial and/or temporal variation in pollinators can result in a matching variation of pollen removal and deposition (Waites & Agren 2004). For example, in 2005, we frequently observed hummingbirds visiting *P. brandegeei* flowers in all three populations, and they were most frequent in the Vedaauwo population (Kulbaba & Worley 2008). Surprisingly, we did not observe proportionally higher rates of deposition in Vedaauwo, although hummingbirds likely contributed to diurnal pollen deposition.

#### Effects of floral design on pollen remaining in anthers

The amount of pollen removed from flowers depends in part on the interaction between floral visitors and floral traits. In both flowering seasons, style length was an important floral trait affecting pollen removal. We predicted that plants with shorter styles would have more pollen removed from anthers relative to plants with longer styles, regardless of the pollinator type, because a recessed stigma imposes less interference to pollen removal by a floral visitor than an exerted stigma (Sahli & 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). Our prediction was partially confirmed, but *P. brandegeei* flowers with intermediate style lengths had the least amount of pollen remaining in all treatment types. In

contrast, controlled pollination experiments with only hawkmoth or hummingbird pollinators did not detect any effect of style on pollen removal (Kulbaba & Worley 2012, 2013). The effect of style length on pollen removal in natural populations may, therefore, partly reflect mechanical interference between sex organs, combined with removal by smaller pollen-collecting visitors.

Corolla dimensions can play a large role in the attraction and orientation of pollinators during floral visitation. Interestingly, we did not find that corolla dimensions significantly affected pollen removal. This contrasted with a previous study that found more pollen removal and siring success of plants with relatively long corolla tubes under only hummingbird pollination (Kulbaba & Worley 2013). Whereas long corolla tubes have been shown to restrict or exclude ineffective visitors like bees and flies (Castellanos *et al.* 2004; Ford & Johnson 2008), the anthers of *P. brandegeei* are accessibly presented at or slightly exerted past the opening of the corolla tube (Kulbaba & Worley 2008). Therefore, pollen-consuming insects could remove pollen regardless of corolla tube length. This finding highlights the importance of considering relatively ineffective floral visitors when interpreting pollen removal and deposition.

### Effects of floral design on pollen deposition

Pollen deposition was a complex and variable process in *P. brandegeei*, with anther height and herkogamy influencing the number of grains deposited on stigmas in 2005. In contrast to anther height, herkogamy consistently affected pollen deposition in both study years, and likely influenced both sexual interference and pollen receipt. However, self-pollen deposition in 2006 due to dry and warm weather likely obscured patterns caused by the effective pollinators in this year.

The close proximity of female and male sex organs may lead to interference between gender functions, resulting in self-pollen deposition (Webb & Lloyd 1986; Barrett 2002). The spatial division of sex organs (herkogamy) separates the female and male organs, thus reducing the potential for sexual interference (Webb & Lloyd 1986). However, when pollinating animals are small (*e.g.* bees and flies), flowers must display reduced herkogamy to function both as pollen donors and recipients (Conner & Rush 1996). Therefore, a trade-off involving the benefits of imported and exported pollen *versus* the negative effects of self-pollen deposition may exist. In *P. brandegeei*, herkogamy is highly variable among individual plants. Individuals displaying flowers with stigmas recessed near or below anthers may receive more self-pollen than stigmas exerted beyond the anthers, as has

been observed in other species (Murcia 1990). In our study, unvisited control flowers with recessed stigmas (reverse herkogamy) exhibited more pollen deposition than controls with exerted stigmas, likely due to mechanical dislodgement of self-pollen (see solid lines in Fig. 5). However, in 2005, recessed stigmas to intermediate values of herkogamy also appeared to experience the highest pollen import, based on the differences between treatment and control plants (Fig. 6). Further work is needed to understand the extent to which the benefits from increased pollen import are offset by sexual interference.

The interaction effect between anther height and population on pollen deposition in 2005 is interesting and perplexing. Across populations, pollen deposition was highest in the short to intermediate values of anther height in *P. brandegeei* (Fig. 5). However, this pattern was apparently independent of herkogamy as determined in our analysis.

Small-bodied visitors such as bees and syrphids may function as both pollinators and pollen thieves. Previous studies have shown these insects are capable of consuming large amount of pollen (Wilson & Thomson 1991). Moreover, some legitimate and effective pollen movement likely occurs through these insects (Herrera 2005). Controlled experiments with hawkmoths (Kulbaba & Worley 2012) and hummingbirds (Kulbaba & Worley 2013) confirmed these animals as effective pollinators and agents of selection on floral traits. However, further studies are required to fully understand the influence of pollen-consuming visitors on the floral evolution of *P. brandegeei*.

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### SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Summary statistics for each of three study populations of *Polemonium brandegeei*, divided over the 2005 and 2006 flowering seasons. Values reported are: Mean ( $\pm$ SE), minimum value (Min.), maximum value (Max.) and coefficient of variation (CV).

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