

LIMITS TO FEMALE REPRODUCTIVE SUCCESS IN THE BOTTLE GENTIAN

PNEUMONANTHE BIGELOVII (GENTIANACEAE)

by

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Limits to female reproductive success in the bottle gentian *Pneumonanthe bigelovii*

(Gentianaceae)

Thesis directed by Associate Professor Thomas A. Ranker

Female reproductive success in plants is often thought to be limited by the availability of either pollen or resources. The pollination and reproductive biology of two populations of *Pneumonanthe bigelovii* (Gray) Greene (Gentianaceae) near Eldorado Springs, Colorado, were studied in an attempt to identify its pollinators, mating system, and factors limiting reproductive success. Few visitors were observed, all of which were queen bumblebees of the species *Bombus huntii* Greene and *B. griseocollis* Degeer. *Pneumonanthe bigelovii* is self-compatible and capable of spontaneous selfing. Open-pollinated control plants appeared to experience both selfing and outcrossing. Excluding plants from pollinators decreased seed production. Hand-selfed plants showed signs of inbreeding depression. Seed production appeared to be at least partly pollen-limited. Fruit set was unaffected by pollination treatment but was affected by site. Fruit set was negatively correlated with number of flowers per plant, suggesting that flowers compete for limited resources in order to produce fruits. Mean seed mass was higher in plants excluded from pollinators compared to hand-outcrossed plants, but the causes of this difference

are unclear. This study suggests that pollen and resources limited different aspects of female reproductive success in these populations of *P. bigelovii* in the year studied.

ACKNOWLEDGMENTS

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Beth Mendoza strained their eyes and necks helping me count seeds. Lynn Riedel, Plant Ecologist for the City of Boulder Open Space and Real Estate Department, pointed me to the study sites, allowed me to use them, and remained calm when citizens questioned why Home Depot flags were littering the mesas. Chavo was an excellent field partner.

Finally, my husband, Alex, helped with most of the things mentioned above and then some.

During the day,
you're my energy for working. At night,
you're my deepest sleep."

-Rumi

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CHAPTER 1

INTRODUCTION

Two models are often invoked to attempt to identify the factors that limit female reproductive success in plants. The first model suggests that pollen is most often the limiting factor (Fischer and Matthies 1997; Windus and Snow 1993), while the alternate model theorizes that resource availability plays a greater role (Stephenson 1981; Barnes and Rust 1994; Luijten et al. 1998). However, sexual reproduction in plants requires both pollen and resources, and evidence has been found for both pollen limitation and resource limitation in the same population (Spira and Pollak 1986; Kwak and Jennersten 1991; Campbell and Halama 1993; McCall and Primack 1985).

Pollen limitation can occur as the result of one or more of the following: inability to self-pollinate, lack of pollinators, ineffective pollinators, and receipt of poor quality pollen. Plants that are unable to spontaneously self-pollinate due to herkogamy (spatial separation of the anthers and stigma) or dichogamy (temporal separation of pollen maturity and stigma receptivity) require the assistance of a

pollinator (Luijten et al. 1998). Even if these barriers are overcome, the plant may still be an obligate outcrosser due to self-incompatibility. Plants like these must rely on pollinators for sexual reproduction.

Pollinator abundance and attractability affect female reproductive success.

Very early in the growing season, pollinators may not be active, and early blooming species may lack visitors. The first individuals or flowers to bloom in a given species may be especially pollen-limited (Dudash 1993) because they may be unable to attract the attention of naive pollinators or those that are already cued in to other species. The last flowering individuals of a species or flowers on an inflorescence may also be neglected by pollinators that have already moved on to a more abundant pollen or nectar crop or have become inactive for the season (Petanidou et al. 1995; Kwak and Jennersten 1991; McCall and Primack 1985). Pollen limitation may also be more pronounced in small populations due to difficulties in attracting pollinators (Fischer and Matthies 1997; Luijten et al. 1998; Oostermeijer et al. 1993). Plants may compete for pollinators, and inflorescences that are smaller (Dudash 1993), have less showy flowers, or offer fewer rewards may lack visitors (Kwak and Jennersten 1991).

Not all visitors are effective pollinators. Nectar-robbing visitors often chew through corollas to access nectar rewards without contacting reproductive structures. Other visitors may form a poor fit with specialized plant reproductive structures so

that pollen is not delivered effectively. Inconstant pollinators visit many species on the same foraging trip and may not deliver any conspecific pollen (Kwak and Jennersten 1991). Thus, even plants that experience high levels of visitation may be pollen limited if they lack effective pollinators.

When conspecific pollen is delivered, it still may be of poor quality. Pollen from the same individual or a close relative can result in inbreeding depression through the expression of recessive lethals and/or a reduction in heterosis (Fischer and Matthies 1997; Wagner and Mitterhofer 1998; Luijten et al. 1998; Oostermeijer et al. 1995). Inbred plants may be less able to adapt to stochastic events because they lack genetic diversity. Conversely, pollen from a distant plant (e.g., a different population) can result in loss of fitness due to outbreeding depression (Fischer and Matthies 1997; Waser and Price 1994). Introducing pollen from another population may disrupt coadapted gene complexes. The progeny of an interpopulational cross may not be adapted to the environment of either of its parents; instead, it may be adapted to an intermediate environment that simply does not exist (Oostermeijer et al. 1995).

Even if a plant is visited by effective pollinators bearing enough high quality pollen for every ovule of every flower to develop into a seed, the plant may not be able to access the resources required to do this. Plants must partition resources to roots, shoots, and reproductive structures such as flowers, fruits, ovules, and seeds

(Spira and Pollak 1986). Abortion due to lack of resources can happen to any of these reproductive structures, as plants will shift resource allocation in order to ensure survival (Oostermeijer et al. 1995). An inverse relationship between measures of reproductive success and number of reproductive structures is often indicative of resource limitation (Luijten et al. 1998; Gorchov 1988).

Pollen limitation can be difficult to demonstrate, partly due to possible reallocation of resources. Usually, when plants with hand-supplemented pollination show greater reproductive success than open-pollinated controls, pollen limitation is invoked (Young and Young 1992; Bierzychudek 1981). However, these results need to be interpreted with caution. If only selected flowers on a plant receive supplemental pollen, the maternal plant may invest more resources in flowers that have received more or better quality pollen at the expense of unpollinated or underpollinated flowers on the same plant (Stephenson 1981). Therefore, it is best to apply the same pollination treatment to every flower on a plant (Dudash 1993; Corbet 1998; Campbell and Halama 1993; McCall and Primack 1985). Even then, a single season's data may be misleading. Plants may invest so much energy in reproduction after receiving supplemental pollen that the following year they show a dramatic decrease in number of reproductive structures since they must provide more resources to roots and shoots that were "short-changed" the previous year (Corbet 1998; Campbell and Halama 1993). If hand-pollinated plants do not show increased

reproductive success when compared to open-pollinated controls, one may infer that pollen limitation is not present. However, Young and Young (1992) presented evidence that hand-pollinated plants may have decreased reproductive success due to increased competition among pollen tubes, increased attraction of pollen thieves, damage to female reproductive parts, decreased donor diversity, adverse effects of bagging, missed stigma receptivity, use of inviable pollen, or delivery of an insufficient quantity of pollen.

Pneumonanthe bigelovii (Gray) Greene (Gentianaceae), bottle gentian, possesses many qualities that make it useful for pollination studies. The tubular corollas are approximately 3 cm long, making it relatively easy to manipulate the five stamens and bilobed stigma inside (Fig. 1). Flowers are protandrous (anthers mature before the stigma becomes receptive) like most other gentians (Dudash 1993; Barnes and Rust 1994; Petanidou et al. 1995; Windus and Snow 1993; Luijten et al. 1998) and bloom for about 2.5 months, from the beginning of September to the middle of November. Some degree of herkogamy is present; the stigma usually elongates past the anthers before becoming receptive. The inside of the deep blue corolla is extensively patterned with nectar guides, typical of many bee-pollinated species. Indeed, several other gentian species have been shown to be primarily visited by bumblebees (Windus and Snow 1993; Luijten et al. 1998; Oostermeijer et al. 1995), pollinators that are easy to observe



Fig. 1. Hand-pollination of *P. bigelovii*.



Fig. 2. Lindsay Mesa. *Pinus ponderosa* forest typical of *P. bigelovii* habitat.

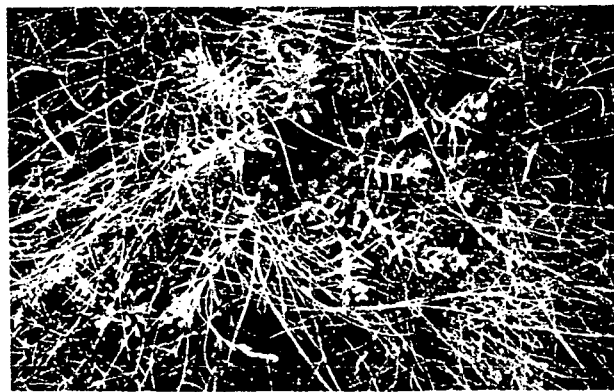


Fig. 3. A single *P. bigelovii* plant.

Much of the work concerning gentian pollination biology that has been done to date has involved European species that have become rare as a result of changing land-use patterns, especially changes in grazing and mowing regimes. *Pneumonanthe bigelovii*, on the other hand, is relatively common in the foothills of Colorado and New Mexico below 2438 m (Weber and Wittmann 1996). On the mesas around Boulder, Colorado, it may be found in open *Pinus ponderosa* (Pinaceae) parks, and is sometimes associated with *Andropogon gerardii* (Poaceae), big bluestem (Fig. 2). Populations of a few hundred individuals, delimited by the mesa's sides, are not uncommon. Each individual produces from one to over forty decumbent stems, and a single plant may bear over 150 flowers (Fig. 3). An average plant bears six stems and just over twenty flowers (personal observations). The two fused carpels contain an average of 280 ovules total, and the resulting capsules may contain over 400 seeds (personal observations).

I undertook this study of the pollination and reproductive biology of *P. bigelovii* to address the following questions:

Who are its pollinators?

What mating system or systems appear to be operating in the study populations?

Does this species exhibit signs of pollen limitation, resource limitation, or both?

CHAPTER 2

METHODS

STUDY SITES

Two study sites were selected. Both were located on land owned and managed by City of Boulder Open Space near the town of Eldorado Springs, Colorado. The sites will be referred to as Lindsay Mesa and Towhee Mesa (Fig 4).

Lindsay Mesa (T1S R70W SEC31 N1/2; UTM Z13 N4419500 E477000), elevation 1853 m, was approximately 1 km south of Eldorado Springs Drive (State Route 170) and 0.5 km east of County Road 67. Spring Brook marked the mesa's western edge. The Lindsay Open Space property appeared to receive relatively low human visitation compared to other Open Space properties in the area, possibly because its only developed trail was a gravel road (the extension of County Road 67), bicycles were not permitted, and dogs were only permitted in the northern section. Mule deer (*Odocoileus hemionus* Cervidae) were abundant. Most of the *P. bigelovii* plants at this site were found under *P. ponderosa* trees on the mesa top, although some plants occurred on the grassland to the east. All of the plants in the population,

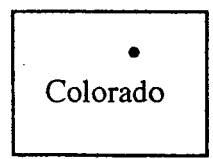
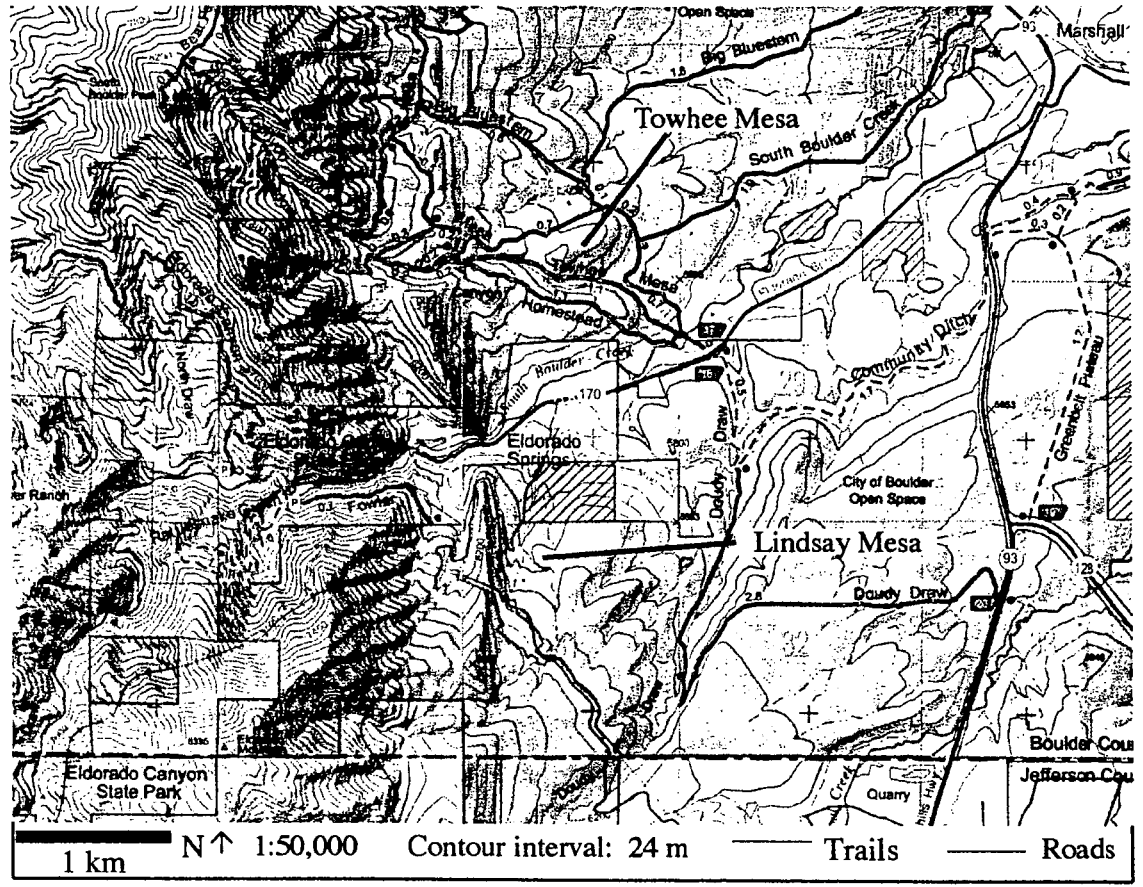


Fig. 4. Site locations (adapted from City of Boulder 1999).

about 200-300 individuals, were found within an area 170 m long from north to south and 120 m wide from east to west (Fig. 5).

Towhee Mesa (T1S R70W SEC19 S1/2; UTM Z12 N4421500 E477000), elevation 1840 m, was located approximately 2 km due north of Lindsay Mesa, about 1.25 km northwest of the South Mesa trailhead on Eldorado Springs Drive (State Route 170). The base of the mesa was surrounded by trails. The Mesa Trail followed its northeast and northwest sides, while the Towhee Trail followed the southern edge, as did a tributary of South Boulder Creek that flowed down through this area from Shadow Canyon. This area experienced heavy human visitation (personal observation). Most visitors remained on the trail, however, and I encountered other people on the mesa top only twice during the two and a half months during which the study was conducted. Mule deer were common, but less abundant than at Lindsay Mesa (personal observation). *Pneumonanthe bigelovii* plants were common in the *P. ponderosa* forest on the mesa top and in the grassland on the mesa's northwestern slope. This population was larger than the Lindsay Mesa population, and included approximately 500 individuals in an area 410 m long from southwest to northeast and 120 m wide from northwest to southeast (Fig. 5).

Pneumonanthe bigelovii voucher specimens from both sites were deposited at the University of Colorado Museum Herbarium (COLO) in Boulder, Colorado.

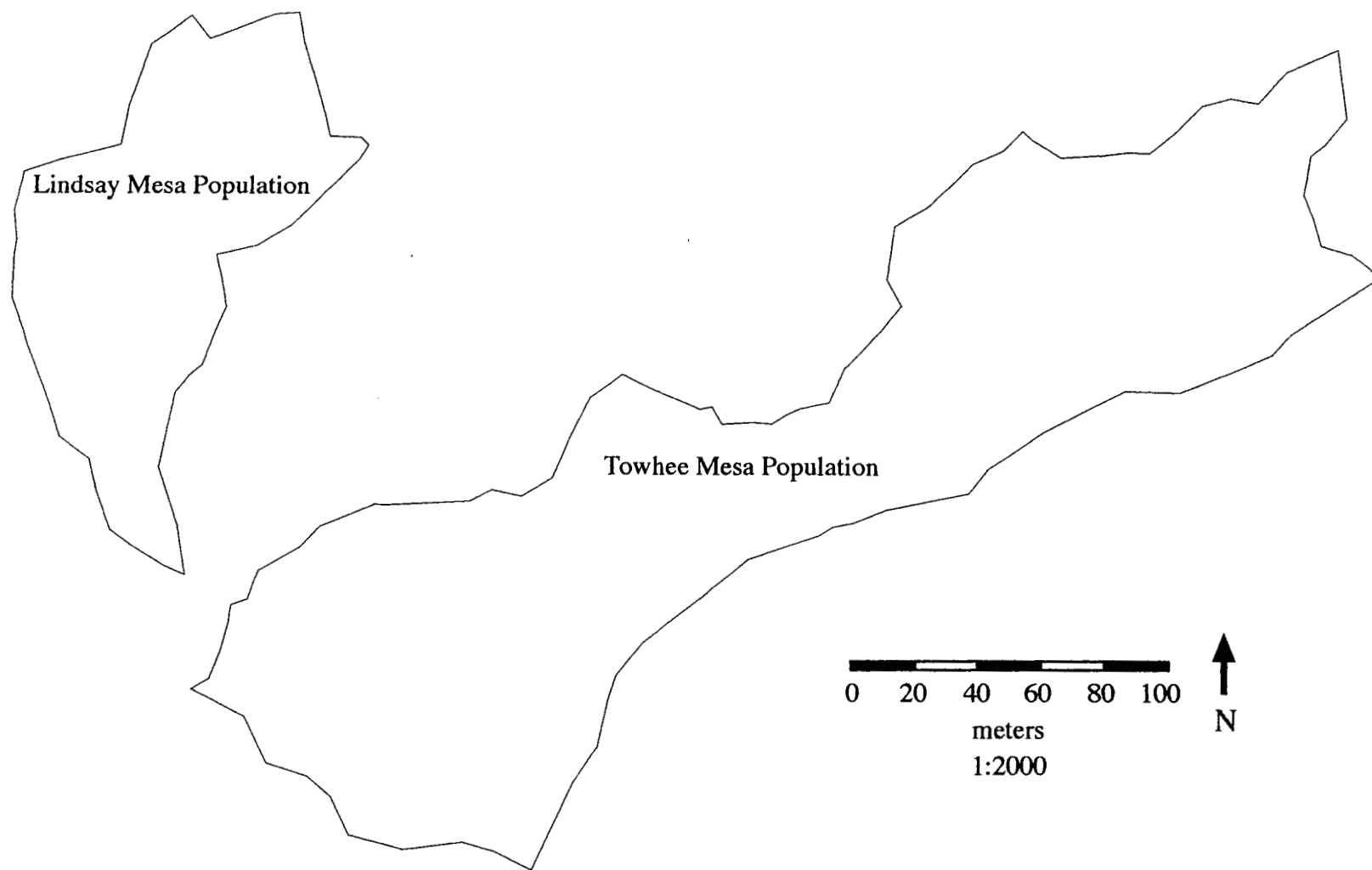


Fig. 5. Area occupied by the *P. bigelovii* populations at Lindsay and Towhee Mesas. See Fig. 4 for the locations of the mesas. Relative positions of mesas to each other are not to scale.

POLLINATION TREATMENTS

Four pollination treatments were conducted: exclusion from pollinators (X), hand-selfed pollination (S), hand-outcrossed pollination (H), and open-pollinated controls (O). Some plants at both sites exhibited signs of infestation by insect larvae. An attempt was made to exclude infested plants from all of the treatment groups.

Between 8 September and 11 September 1999, 40 *P. bigelovii* plants at each site that did not yet have any receptive stigmas were chosen randomly and bagged by surrounding the entire plant with bridal veil netting and then staking the netting to the ground (Fig. 6). A receptive stigma was considered to be one in which lobes or papillae were detected. Twenty of these bagged plants at each site were randomly assigned to the hand-selfed treatment, while the remaining 20 plants at each site experienced no further manipulation and formed the pollinator exclusion treatment group.

Each flower on the hand-selfed plants was pollinated once with pollen from another flower on the same plant. If only one unpollinated flower remained on a plant, it was pollinated with its own pollen, but usually the last hand-pollination event involved more than one flower and pollen was still donated by a different flower on the same plant. A stamen was removed by grasping the filament with forceps and twisting until it snapped free. The anther was then rubbed on one to two receptive



Fig. 6. A *P. bigelovii* plant bagged with bridal veil netting.



Fig. 7. A *P. bigelovii* plant that has experienced herbivory.

stigmas. The corollas of pollinated flowers were marked with a permanent marker pen. Bags were removed only during pollination.

Between 18 September and 2 October 1999, 20 plants at each site that had stigmas that were all either not yet receptive or still receptive were chosen randomly and marked with flags for hand-outcrossing. Stigmas were considered still receptive if the papillae looked sticky and whitish-green rather than brown, the stigma lobes had not recurved more than 180° , and there were no signs of fruit development (i.e., brown, withered corolla, enlarged ovary, style elongated past corolla). Each flower on the hand-outcrossed plants was pollinated once using pollen from two different donor plants, each located at least 1 m away from the recipient. Pollination was effected in the same manner as the hand-selfed plants: a stamen was removed from the donor plant and its anther was rubbed on one to two receptive stigmas. This process was then repeated using pollen from a second donor. Pollinated flowers were marked using a permanent marker pen. After losing several plants to herbivory (Fig. 7), presumably by mule deer, all hand-outcrossed plants were bagged in the same manner as the exclusion and hand-selfed plants. Bagging was performed between 13 October and 22 October 1999.

Twenty open-pollinated plants per site were randomly selected at the time of fruit collection. The first 20 plants that had at least three fruits on one stem that I

encountered when walking transects at each site formed the open-pollinated control group.

DATA COLLECTION

Because few *P. bigelovii* visitors were detected, no attempt was made to measure visitation rates. All pollinator observations were made informally, while completing other tasks at the study sites, but an effort was made to identify all observed visitors. Voucher specimens of observed pollinators were identified by Virginia Scott, Collections Manager of the Entomology Section of the University of Colorado Museum in Boulder, Colorado, and were deposited in that collection.

Between 10 November and 16 November the following data were collected for each of the 161 plants in the study: total number of stems, number of stems that had flowers, number of stems that remained vegetative, and number of stems that experienced herbivory (which made it impossible to say whether they had flowered). The total number of flowers and the number of resulting fruits were recorded for each flowering stem. One flowering stem with at least three fruits was chosen from each plant for fruit collection. When there was more than one stem to choose from, stems were assigned numbers and then a random number table was used to select the stem for sampling. However, stems with fruits that had obviously been infested by insect larvae were avoided. Fruit set for each plant was calculated by dividing the number

of fruits on the plant by the number of flowers on the plant. Fruit set was also calculated separately for the stem from which fruits were collected.

The bottom, middle, and top fruits on the selected stem were collected to attempt to control for variation in reproductive success based on a flower's position in an inflorescence (Diggle 1995, Stephenson 1981).

Each fruit was dissected and the number of seeds and aborted ovules were counted using a dissecting microscope (Fig. 8). No attempt was made to distinguish whether the ovules referred to as aborted had truly aborted or had never been fertilized. In any case, they had clearly not developed into seeds. Aborted ovules were much smaller and flatter than seeds (Fig. 9). A few immature fruits were collected where ovules graded into seeds (Fig. 10). No seed data were collected for these fruits, or for fruits infested by insect larvae, in which case the seeds and aborted ovules were reduced to an amorphous mass (Fig. 11). The number of seeds and the number of aborted ovules were added together to arrive at a proxy for the original number of ovules present in each fruit, under the assumption that unfertilized ovules were not resorbed. Seed set was then calculated by dividing the number of seeds in a fruit by the original number of ovules in the fruit.

All of the seeds from a fruit were weighed together. The measured mass was then divided by the number of seeds in the fruit to arrive at mean seed mass. Some

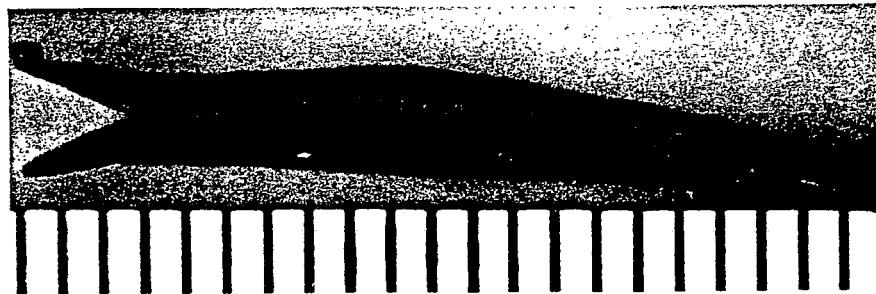


Fig. 8. A mature *P. bigelovii* capsule (the corolla has been removed). Scale in mm.



Fig. 9. *Pneumonanthe bigelovii* seeds and aborted ovules. Scale in mm.



Fig. 10. Cross-section of an immature *P. bigelovii* capsule. Scale in mm.



Fig. 11. An infested *P. bigelovii* capsule. Scale in mm.

fruits contained seeds that became moldy while awaiting analysis. Seeds from these fruits were not weighed.

DATA ANALYSIS

All analyses were performed using SAS version 8 (SAS Institute, Inc. 1999).

The dependent measures were subjected to Type III SS factorial ANOVAs for unequal sample sizes. The effects of site, pollination treatment, and their interaction were investigated. Because the sites were not chosen at random (i.e., their proximity to each other, the ease of access to each site, and the relatively large *P. bigelovii* populations at both sites led to their selection), site was treated as a fixed effect. The differences between the least squares means of all pollination treatment pairs were compared using the Tukey-Kramer alpha adjustment for multiple comparisons.

ANOVAs were conducted for the following dependent measures: number of stems per plant, number of flowers per plant, number of fruits per plant, percent of stems experiencing herbivory, fruit set, seed set, number of seeds per fruit, mean seed mass, and original number of ovules per fruit. Data collected at the fruit level (seed set, number of seeds per fruit, mean seed mass, and original number of ovules per fruit) were averaged for each plant. Twelve fruits containing fewer than 75 ovules were excluded from all analyses based on the fact that the fruits had begun to dehisce at the time of collection and on the assumption that they had already begun to

disperse at the time of collection. Two sets of analyses were conducted using the fruit-level data: the first set included all observations, while the second set was restricted to those plants from which data from all three sampled fruits were collected (i.e., plants in which none of the three collected fruits were immature or infested).

Methods suggested in Judd and McClelland (1989) were used to evaluate whether the data met the assumptions of the analysis of variance. Because outliers can overinfluence the ANOVA model, an attempt was made to identify them by examining the studentized deleted residuals generated during each ANOVA.

Observations with a studentized deleted residual value of greater than the absolute value of 2.5 were excluded from the analysis and the ANOVA was repeated without them. This resulted in the deletion of no more than three observations (less than two percent of the data) per analysis. The results presented are those of the ANOVAs conducted after deleting outliers.

The analysis of variance also assumes a near-normal error distribution, although, according to the central limit theorem, normality becomes less critical as sample size increases (Sokal and Rohlf 1981). Ratio data were arcsine-transformed and continuous data were log- and square-root-transformed in an effort to normalize the data. After completing an ANOVA, the residuals were examined by plotting histograms and normal quantile-quantile plots. The transformation that generated the

residuals that best met the assumptions required of the analysis of variance was used to obtain the results presented.

The specific transformations selected were as follows. Number of stems per plant was log-transformed. Number of flowers per plant, number of fruits per plant, number of seeds per fruit, and original number of ovules per fruit were square-root-transformed. Fruit set and percent of stems experiencing herbivory were arcsine-transformed. Untransformed data yielded the most normal quantile-quantile plots for seed set and mean seed mass. The same transformations were used in the analyses involving only observations based on data from three fruits with one exception: untransformed data were used for number of seeds per fruit in these analyses.

Pearson r correlations were employed to determine if there was a significant relationship between reproductive success and number of reproductive structures or extent of herbivory. The effects of site, pollination treatment, and their interaction were partialled out of the analysis. Fruit set, seed set, and mean seed mass served as measures of reproductive success, and were tested against number of flowers per plant, number of fruits per plant, number of flowers on the sampled stem, number of fruits on the sampled stem, number of seeds per fruit, and percent of stems experiencing herbivory. Both plant-level and stem-level flower and fruit production were included in the analysis because factors at both levels may have influenced patterns of resource allocation (Stephenson 1981). The transformations listed above

for the ANOVAs were used. Number of flowers on the sampled stem was square-root-transformed, while number of fruits on the sampled stem was log-transformed.

CHAPTER 3

RESULTS

POLLINATORS

Queen bumblebees were the only observed visitors to *P. bigelovii* aside from ants found inside the corollas early in the study. At Lindsay Mesa, only one *P. bigelovii* visitor, a *Bombus huntii* Greene queen, was observed over the course of the study (20 August-16 November 1999). The pollen that she carried in her corbiculae was analyzed by Michael Kerwin of the Institute of Arctic and Alpine Research in Boulder, Colorado. Only pollen from *P. bigelovii* and an undetermined Asteraceae species was found. *Pneumonanthe bigelovii* pollen grains far outnumbered Asteraceae grains (263 *P. bigelovii* grains to six Asteraceae grains after one slide transect, M. Kerwin, personal communication).

Two *B. huntii* queens were observed visiting *P. bigelovii* flowers at Towhee Mesa. One was clearly carrying pollen. I also observed four *Bombus griseocollis* Degeer queens visiting the study species at this site. Two of them visited *Liatris punctata* (Asteraceae) flowers on the same foraging trip. Four of the six Towhee

Mesa visitors (two individuals from each species) were observed on the same day, 11 September 1999.

SITE EFFECTS

Plants at the two sites differed in some characteristics and were similar in others. Towhee Mesa plants had a significantly greater number of stems, flowers, and fruits than those at Lindsay Mesa (Fig. 12, Table 1).

Plants at Lindsay Mesa had a significantly greater number of stems experiencing herbivory and significantly higher fruit set when compared to plants at Towhee Mesa (Fig. 12, Fig. 13, Table 2).

Site had no significant effect on seed set, number of seeds per fruit, or mean seed mass (Fig. 13, Table 2).

EFFECTS OF POLLINATION TREATMENT

The number of ovules per fruit was not significantly affected by site, pollination treatment, or their interaction (Tables 3 and 4).

Pollination treatment also had no significant effect on fruit set, but there was a significant site x treatment interaction (Table 2, Fig. 14).

Seed set was significantly affected by pollination treatment (Table 2). Hand-outcrossed plants had significantly higher seed set than hand-selfed plants and

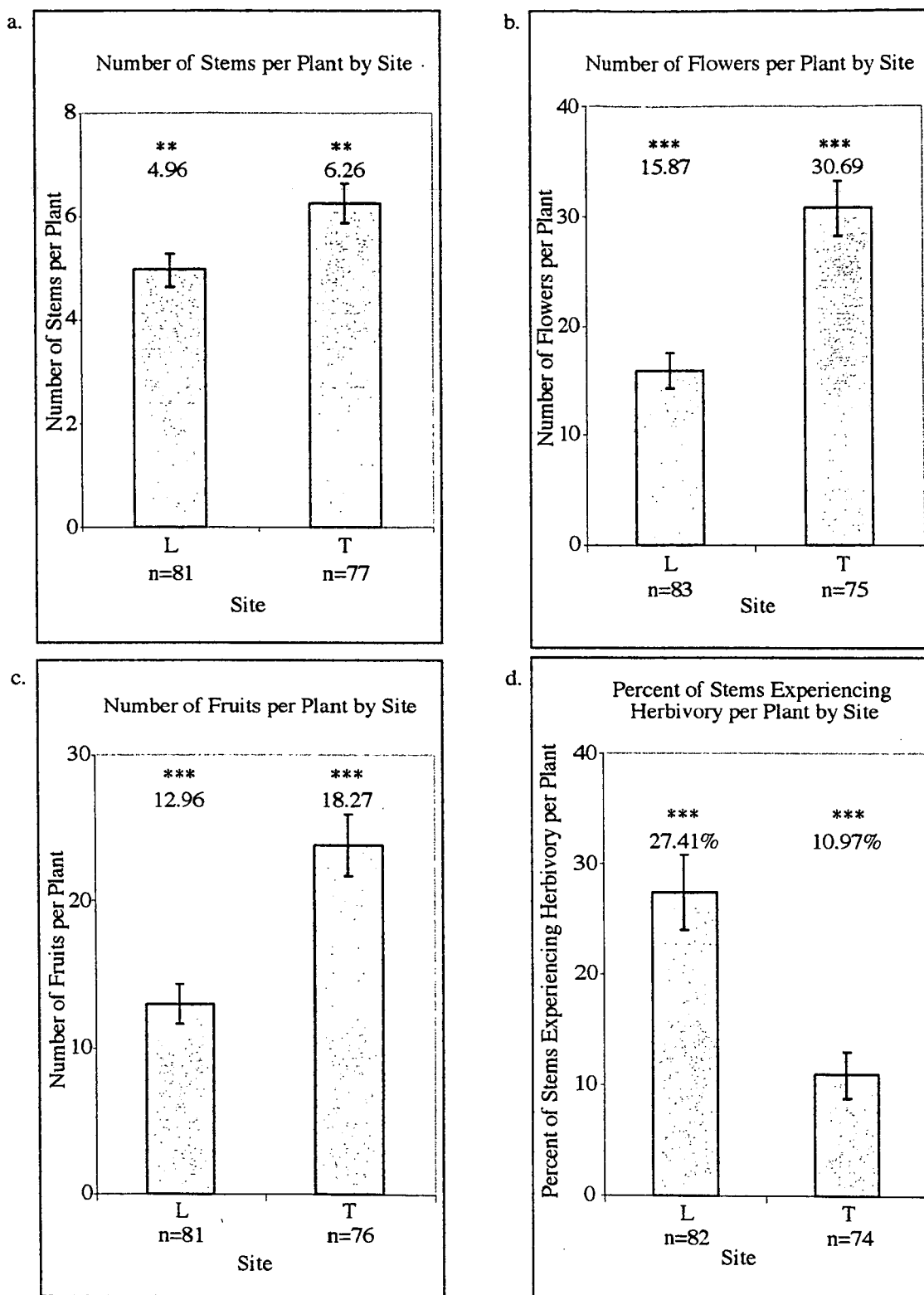


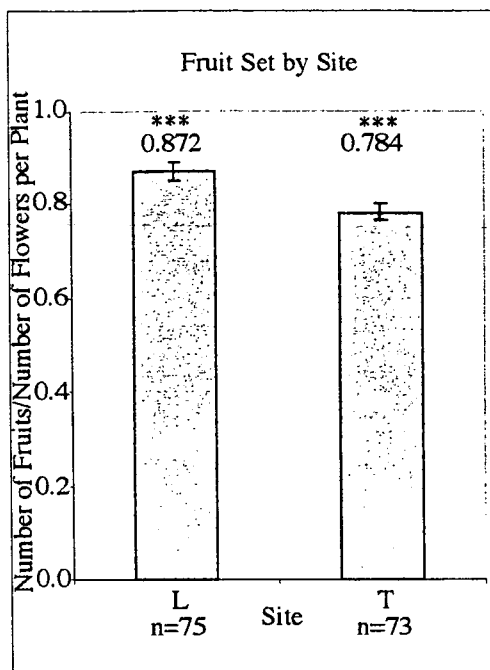
Fig. 12. Effects of site on a. number of stems per plant, b. number of flowers per plant, c. number of fruits per plant, and d. percent of stems experiencing herbivory per plant. L=Lindsay Mesa. T=Towhee Mesa. Means are given above bars. Error bars are plus or minus one standard error. Sample size is given below bars. See Table 1 for ANOVA results. **= $p < 0.01$; ***= $p < 0.001$.

Table 1. Effects of site on measures of plant size or herbivory. See Fig. 12. for means and sample sizes. P-values of <0.05 are in bold.

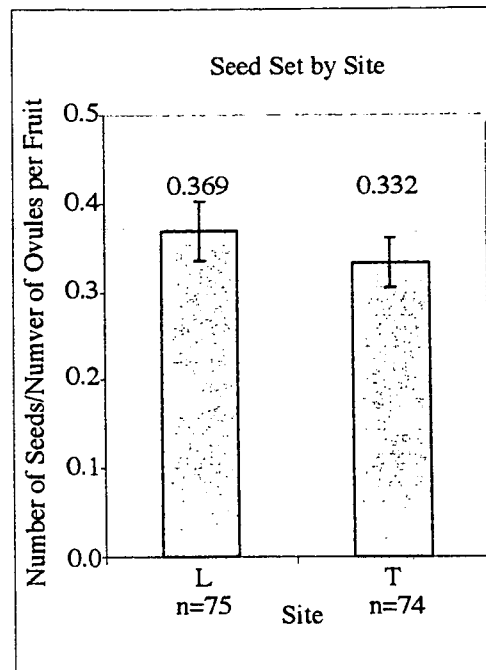
Dependent Measure	df	F	P
Number of Stems per Plant	1	7.16	0.0083
Number of Flowers per Plant	1	28.90	<0.0001
Number of Fruits per Plant	1	22.90	<0.0001
Percent of Stems Experiencing Herbivory per Plant	1	12.86	0.0005

Table 2. Effects of site, treatment, and their interaction on measures of reproductive success. P-values of <0.05 are in bold. See Figure 13 for site means and sample sizes. See Table 5 for comparisons of treatment pairs. See Fig. 14.

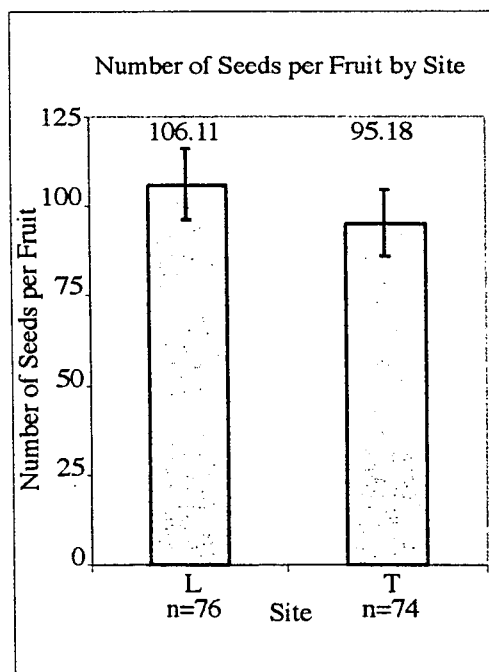
Effect	Fruit Set			Seed Set			Number of Seeds per Fruit			Mean Seed Mass		
	df	F	P	df	F	P	df	F	P	df	F	P
Site	1	19.36	<0.0001	1	0.45	0.5045	1	0.15	0.6957	1	0.13	0.7155
Treatment	3	2.13	0.0987	3	17.44	<0.0001	3	9.53	<0.0001	3	3.47	0.0182
Site x Treatment	3	3.69	0.0135	3	1.03	0.3798	3	0.89	0.4482	3	2.53	0.0601



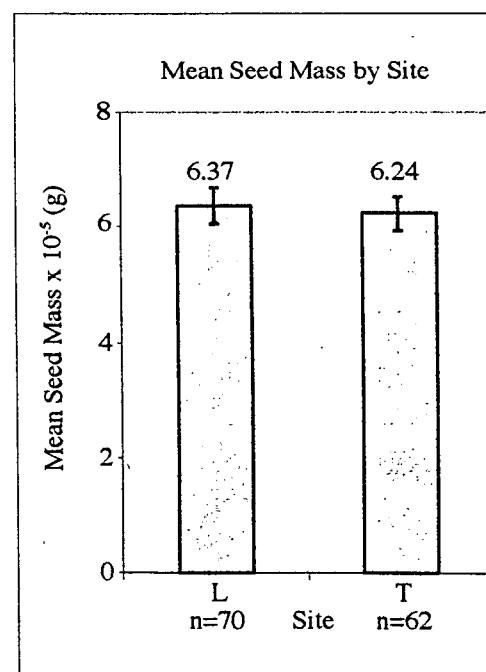
a.



b.



c.



d.

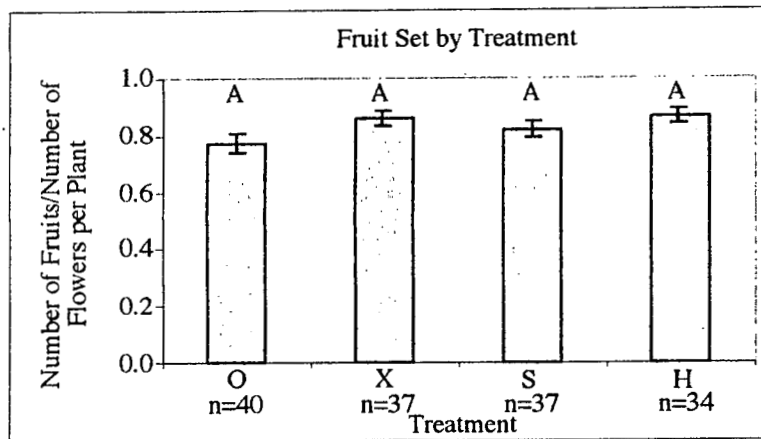
Fig. 13. Effects of site on a. fruit set, b. seed set, c. number of seeds per fruit, and d. mean seed mass. L=Lindsay Mesa. T=Towhee Mesa. Means are given above bars. Error bars are plus or minus one standard error. Sample size is given below bars. See Table 2 for ANOVA results. ***= $p < 0.001$.

Table 3. Number of ovules per fruit by site and by pollination treatment. See Table 4 for ANOVA results.

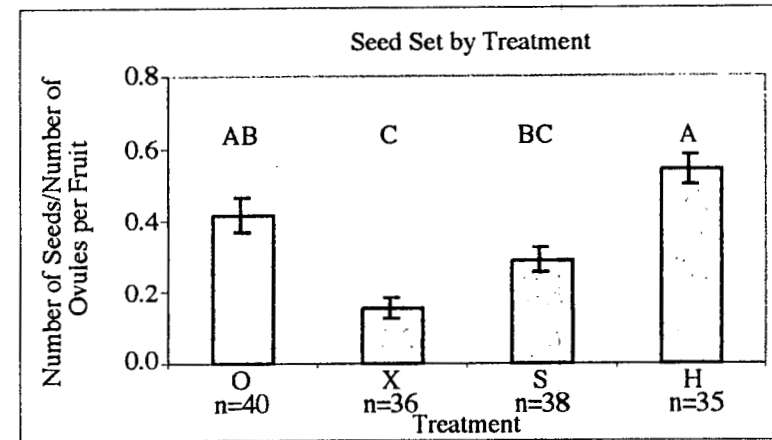
	Mean	Standard Error	n
Lindsay Mesa	299.52	9.76	76
Towhee Mesa	275.27	8.48	74
Open-Pollinated	269.50	10.59	40
Excluded	305.76	14.31	37
Hand-Selfed	300.37	12.38	38
Hand-Outcrossed	275.05	14.58	35

Table 4. Effects of site, treatment, and their interaction on number of ovules per fruit. See Table 3 for means and sample sizes.

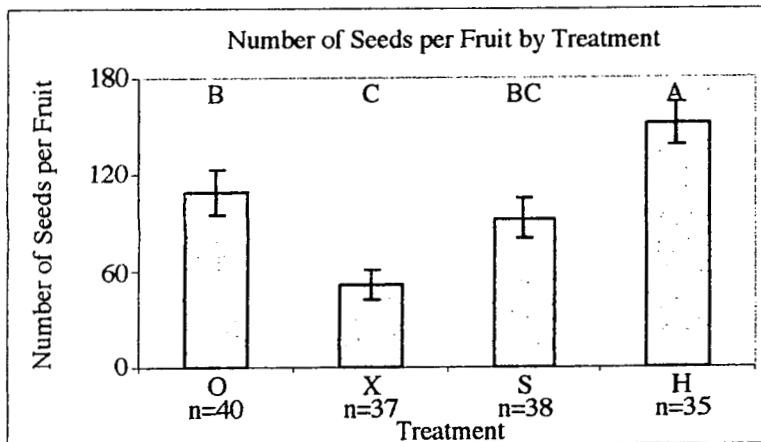
Effect	df	F	P
Site	1	3.66	0.0576
Treatment	3	2.01	0.1148
Site x Treatment	3	2.46	0.0654



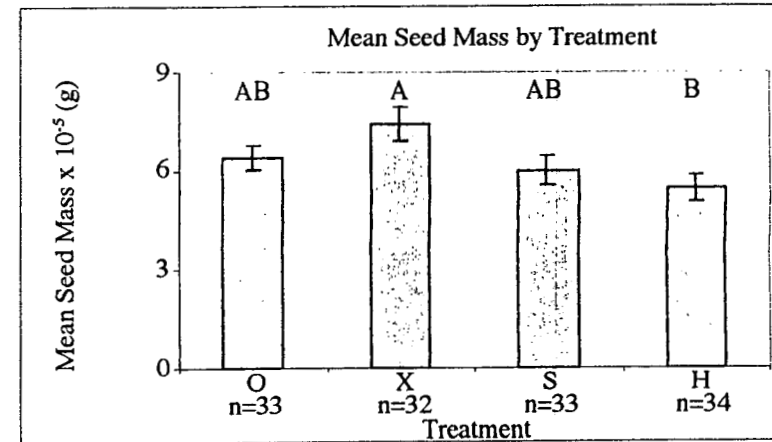
a.



b.



c.



d.

Fig. 14. Effects of pollination treatment on a. fruit set, b. seed set, c. number of seeds per fruit, and d. mean seed mass. O=open-pollinated. X=excluded from pollinators. S=hand-selfed. H=hand-outcrossed. Data are means. Error bars are plus or minus one standard error. Sample size is given below bars. See Table 2 for ANOVA results and Table 5 for pairwise comparisons. Within a graph, $p < 0.05$ for treatment pairs that do not share a letter.

exclusions (Table 5, Fig. 14). Seed set in exclusions was also significantly lower than in open-pollinated controls. Seed set was not significantly different in other treatment pairs.

The same patterns of significance were present when the number of seeds per fruit was considered, with one exception (Tables 2 and 5, Fig. 14). Hand-outcrossed plants produced more seeds per fruit than all other treatments, including open-pollinated controls (Table 5, Fig. 14).

Mean seed mass was also significantly affected by treatment (Tables 2 and 5, Fig. 14). Seeds from exclusions had the highest mean seed mass and hand-outcrossed seeds the lowest. These were the only treatments that were significantly different from each other (Table 5).

When only plants from which data were collected from all three sampled fruits were considered (i.e., plants in which none of the three collected fruits were immature or infested), the same patterns of significance were present, with the addition of the following significant results. A significant site x treatment interaction was detected in number of ovules per fruit (Table 6). Seed set was significantly higher in open-pollinated controls than in hand-selfed plants, and was higher in hand-selfed plants than in exclusions (Tables 7 and 8). Number of seeds was also significantly higher in hand-selfed plants than in exclusions. Mean seed mass was not significantly different between exclusions and hand-outcrossed plants (but

Table 5. P-values for least squares means comparisons of measures of reproductive success for all pollination treatment pairs after Tukey-Kramer alpha-adjustment for multiple comparisons. Note fruit set is not compared because no treatment effect was detected in the ANOVA. P-values of <0.05 are in bold. See Table 2 for ANOVA results. See Fig. 14.

Pollination Treatment Pair	Seed Set	Number of Seeds per Fruit	Mean Seed Mass
Open-Pollinated vs. Excluded	<0.0001	0.0248	0.4404
Open-Pollinated vs. Hand-Selfed	0.0972	0.9652	0.8753
Open-Pollinated vs. Hand-Outcrossed	0.0990	0.0485	0.3756
Excluded vs. Hand-Selfed	0.0776	0.0894	0.1154
Excluded vs. Hand-Outcrossed	<0.0001	<0.0001	0.0123
Hand-Selfed vs. Hand-Outcrossed	<0.0001	0.0151	0.8265

Table 6. Effects of site, treatment, and their interaction on ovule number, seed production and seed mass using only observations with data from three fruits. See Table 7 for site and treatment means and sample sizes. See Table 8 for comparisons of treatment pairs. P-values of <0.05 are in bold.

Effect	Number of Ovules per Fruit			Seed Set			Number of Seeds per Fruit			Mean Seed Mass		
	df	F	P	df	F	P	df	F	P	df	F	P
Site	1	1.64	0.2029	1	1.28	0.2603	1	0.39	0.5332	1	0.22	0.6423
Treatment	3	1.60	0.1940	3	21.54	<0.0001	3	14.46	<0.0001	3	4.57	0.0082
Site x Treatment	3	4.54	0.0049	3	0.41	0.7486	3	0.28	0.8388	3	3.58	0.0230

Table 7. Ovule and seed data by site and by pollination treatment using only observations with data from three fruits. Mean \pm 1 SE (n). See Table 6 for ANOVA results. See Table 8 for comparisons of treatment pairs.

	Number of Ovules per Fruit	Seed Set	Number of Seeds per Fruit	Mean Seed Mass $\times 10^{-5}$ (g)
Lindsay Mesa	297.83 \pm 11.00 (58)	0.3972 \pm 0.0363 (58)	107.32 \pm 10.43 (58)	7.041 \pm 0.429 (26)
Towhee Mesa	280.05 \pm 8.80 (60)	0.3454 \pm 0.0323 (60)	96.76 \pm 9.44 (59)	6.725 \pm 0.472 (18)
Open-Pollinated	277.94 \pm 11.68 (31)	0.4521 \pm 0.0548 (31)	108.66 \pm 13.96 (29)	6.158 \pm 0.587 (8)
Excluded	305.06 \pm 17.44 (29)	0.1524 \pm 0.0262 (29)	45.01 \pm 7.67 (29)	7.674 \pm 0.649 (11)
Hand-Selfed	301.16 \pm 13.73 (31)	0.3064 \pm 0.0281 (31)	97.14 \pm 11.48 (31)	7.943 \pm 0.561 (12)
Hand-Outcrossed	269.57 \pm 12.43 (27)	0.5865 \pm 0.0391 (27)	159.48 \pm 14.10 (28)	5.778 \pm 0.515 (13)

Table 8. P-values for least squares means comparisons of seed production and mass for all pollination treatment pairs after Tukey-Kramer alpha-adjustment for multiple comparisons. Only observations with data from three fruits were considered. P-values of <0.05 are in bold. See Table 6 for ANOVA results. See Table 7 for means and sample sizes.

Pollination Treatment Pair	Seed Set	Number of Seeds per Fruit	Mean Seed Mass
Open-Pollinated vs. Excluded	<0.0001	0.0020	0.4909
Open-Pollinated vs. Hand-Selfed	0.0387	0.8973	0.1862
Open-Pollinated vs. Hand-Outcrossed	0.0969	0.0234	0.8031
Excluded vs. Hand-Selfed	0.0320	0.0150	0.9041
Excluded vs. Hand-Outcrossed	<0.0001	<0.0001	0.0571
Hand-Selfed vs. Hand-Outcrossed	<0.0001	0.0024	0.0091

$p=0.0571$), but was significantly less in hand-outcrossed plants than in hand-selfed plants.

CORRELATIONS BETWEEN REPRODUCTIVE SUCCESS AND NUMBER OF REPRODUCTIVE STRUCTURES OR HERBIVORY

Only three out of 18 tested correlations proved to be significant when site, treatment, and their interaction were partialled out of the analysis (Table 9). Total number of flowers and number of flowers on the sampled stem were both negatively correlated with fruit set. Number of seeds was positively correlated with seed set.

Table 9. Pearson r correlation matrix with the effects of site, treatment, and their interaction partialled out of the analysis. Correlation coefficients are given above p-values. n=133. P-values of <0.05 are in bold.

	Number of Flowers per Plant	Number of Flowers per Sampled Stem	Number of Fruits per Plant	Number of Fruits per Sampled Stem	Number of Seeds per Fruit	Percent of Stems Experiencing Herbivory per Plant
Fruit Set	-0.30934 0.0004	-0.25718 0.0036	-0.04477 0.6186	0.01902 0.8326	0.09094 0.3112	-0.08390 0.3503
Seed Set	-0.10538 0.2402	0.01940 0.8293	-0.08204 0.3611	0.06771 0.4512	0.89130 <0.0001	-0.11641 0.1942
Mean	-0.00762	0.02314	0.07057	0.10128	0.03248	-0.11238
Seed Mass	0.9325	0.7970	0.4323	0.2591	0.7181	0.2103

CHAPTER 4

DISCUSSION

POLLINATORS

The paucity of observed pollinators raises the question: Are bumblebees truly the primary pollinators of *P. bigelovii*?

Ants were observed in some corollas at the beginning of the study. However, most ant-pollinated plants are prostrate, have flowers that are small and inconspicuous, and grow intertwined with other individuals of the same species (Proctor et al. 1996). None of these traits characterize *P. bigelovii*.

Pneumonanthe bigelovii flowers may have been visited by nocturnal pollinators; however, they are not typical of moth- or bat-pollinated species. They lack the pale color, deeply dissected petals (Proctor et al. 1996), strong scents, and corollas unmarked by nectar guides that are common in moth-pollinated flowers (Buchmann and Nabhan 1996). Bat-pollinated flowers are usually white or green, strongly-scented, bowl-shaped, and free of nectar guides (Buchmann and Nabhan 1996). Flowers of bat-pollinated plants usually bloom for one night only and often

display claw marks from their visitors (Proctor et al. 1996). More importantly, all 17 species of bats known to occur in Colorado are exclusively insectivorous (Armstrong et al. 1994).

The pleated petals of *P. bigelovii* keep the corolla tube nearly closed at all times, suggesting that anemophily is highly unlikely. Even in areas where gentians are common, gentian pollen is rarely found in sediments (M. Kerwin, personal communication). Petanidou et al. (1995) tested and rejected anemophily in *Gentiana pneumonanthe* L.

Many studies have documented that bumblebees are important gentian pollinators (Petanidou et al. 1995; Windus and Snow 1993; Barnes and Rust 1994; Luijten et al. 1998). Spira and Pollak (1996) even list *B. huntii*, one of the two species of visitors that I observed, as a pollinator of *Gentiana newberryi* Gray in California. Bumblebee abundances at the study sites may simply have been unusually low during the season studied. In 1991, Barnes and Rust (1994) typically found one to six bumblebees visiting *G. newberryi* flowers at their 2700 m² study site in Nevada at any time that the weather was calm and warm. In the following year of the same study, however, bumblebee visitors were rarely observed. Windus and Snow (1993) suspected that below-average precipitation adversely affected the bumblebee pollinators of *Gentiana saponaria* L. in one year of a five-year study.

Additional fieldwork could demonstrate if the number of visitors I observed was representative of normal pollinator activity at Lindsay and Towhee Mesas.

My observations of queens collecting pollen in September is consistent with the bumblebee life cycle. Unlike honeybees, bumblebee queens and workers perform many of the same tasks. Of all the bumblebee offspring produced in a season, only the new queens are capable of overwintering. In the fall, when the rest of the colony is declining, new queens may perform most of the foraging duties (Heinrich 1979)

Late-season queen visitation has been documented in other gentian studies. Petanidou et al. (1995) occasionally observed *Bombus pascuorum* Scopoli queens visiting *G. pneumonanthe*, another late-blooming gentian. Bauer (1983) found that *Gentiana algida* Pallas anthesis coincided with the emergence of new queens in the fall in Montana.

Seibert and Savidge (1991) presented another case where a plant species was pollinated almost exclusively by bumblebee queens, albeit by old queens in the spring. Twenty-three of 26 observed visitors to *Uvularia grandiflora* Sm. (Liliaceae) were *B. griseocollis* queens. This is the same bumblebee species that I observed at Towhee Mesa.

Pollinators are often less abundant in areas that have experienced habitat fragmentation (Jennersten 1988; Rathcke and Jules 1993; Kearns et al. 1998). In contrast, I observed more pollinators at Towhee Mesa, which appears to be part of a

more fragmented and disturbed landscape than Lindsay Mesa. The Towhee Mesa *P. bigelovii* population in general did not show any of the ill effects of habitat fragmentation when compared to Lindsay Mesa. The Towhee Mesa *P. bigelovii* population size was larger; the area populated was larger; plants had significantly more stems, flowers, and fruits; and seed set, number of seeds per fruit, and mean seed mass were unaffected by site. However, herbivory was significantly less common at Towhee Mesa than at Lindsay Mesa. Habitat fragmentation might help explain why deer were less abundant at Towhee Mesa (personal observation). Fruit set was also significantly lower at Towhee Mesa, but probably was unrelated to habitat fragmentation. Because fruit set was not affected by pollination treatment, and was negatively correlated with number of flowers per plant, this decrease in fruit set can be attributed to differences in flower production rather than differences in pollinator abundance or effectiveness between the two sites.

Plants with more flowers should be more attractive to pollinators (Gori 1989), as should populations with more individuals (Thomson 1981). These two factors could explain why more pollinators were observed at Towhee Mesa than at Lindsay Mesa. Of the measures of reproductive success studied, fruit set was the only factor affected by site, and, as was discussed above, was independent of pollination mode. In other words, the number of observed pollinators did not seem to affect *P. bigelovii* reproductive success.

MATING SYSTEMS

Seed set calculations were based on the assumption that the sum of the number of aborted ovules and the number of seeds would approximate the original number of ovules in a fruit. If unfertilized ovules were resorbed, this assumption would not be met, and one might expect that the original number of ovules per fruit would vary with treatment. Fischer and Matthies (1997) did not detect ovule resorption in their studies of *Gentianella germanica* (Willdenow) Boerner. However, Petanidou et al. (1995) found that fresh *G. pneumonanthe* flowers had significantly more ovules than was estimated from ripe fruits in two of three plots studied. I found the original number of ovules per fruit to be similar across treatments, which suggests that unfertilized ovules were not resorbed and seed set calculations were valid.

Pneumonantho bigelovii is capable of spontaneous selfing, but experienced greatly reduced seed production when pollinators were excluded. Many other species of gentians have also been shown to self spontaneously, yet have reduced reproductive success in the absence of pollinators (Spira and Pollak 1986; Petanidou et al. 1995; Fischer and Matthies 1997; Luijten et al. 1998). However, some gentians are either not capable of spontaneous selfing (Windus and Snow 1993; Spira and Pollak 1986), show high levels of spontaneous selfing (Wagner and Mitterhofer 1997), or do not show a reduction in reproductive success when pollinators are excluded (Spira and Pollak 1986).

Pollinators must have facilitated pollination in the open-pollinated *P. bigelovii* plants, given the significantly higher seed set and number of seeds per fruit in this pollination treatment compared to exclusions. Even though few visitors were observed, enough pollinators apparently were present to cause this difference between treatments. The bridal veil netting used to exclude pollinators should have had only minimal impacts on reproductive success beyond the actual exclusion of pollinators. Wyatt et al. (1992) found that temperature and humidity inside bags made of bridal veil netting were not significantly different from ambient measurements. They also detected only small and inconsistent differences in nectar volume and concentration in bagged inflorescences compared with controls. Hand-selfed and hand-outcrossed *P. bigelovii* plants, which were also subjected to bagging, showed increased reproductive success relative to exclusions, confirming that the observed decrease in seed set and number of seeds per fruit in exclusions was not solely due to the effects of bagging.

Pneumonanthe bigelovii plants exhibited signs of inbreeding depression, perhaps due to partial self-incompatibility. Seed set and number of seeds per fruit were significantly higher in hand-outcrossed plants compared to hand-selfed plants. While it is true that hand-outcrossed flowers received more pollen than hand-selfed flowers, the number of pollen grains applied to hand-selfed stigmas (one anther to one or two stigmas) should have far exceeded the number of ovules present (maximum

original number of ovules = 521). Inbreeding depression has also been detected in *G. newberryi* (Spira and Pollak 1986), *G. germanica* (Luijten et al. 1998; Fischer and Matthies 1997), and *G. pneumonanthe* (Oostermeijer et al. 1995).

Loss of fitness due to inbreeding depression may vary during different phases of a plant's life cycle (e.g., Schemske and Lande 1985; Waser and Price 1994). For example, inbreeding depression was not expressed in mean seed mass. This factor did not differ significantly in hand-outcrossed and hand-selfed seeds when all observations were considered, but was significantly lower in hand-outcrossed plants compared to exclusions. When only observations with data from three fruits were considered, mean seed mass was actually significantly lower in hand-outcrossed fruits than in hand-selfed fruits, but the difference between mean seed mass in hand-outcrossed plants and exclusions was not quite significant. In both analyses, only the two means at either end of the spectrum proved to be significantly different, which casts some doubt on the meaningfulness of the mean seed mass results. It is possible that hand-selfed seeds could experience lower germination rates or result in decreased plant size, reduced fecundity, and/or decreased life span. Additional studies would be required to test for inbreeding depression in selfed offspring at other life stages.

Pneumonanthe bigelovii seeds in open-pollinated controls were most likely produced through a mixture of selfing and outcrossing. Mean seed set and number of seeds per fruit in open-pollinated plants were intermediate to hand-selfed and hand-

outcrossed means. When all plants were considered, seed set was not significantly different between open-pollinated plants and either of the hand-pollination treatments. When only observations with seed data from all three sampled fruits were considered, a significant difference between open-pollinated and hand-selfed plants was detected. Open-pollinated plants had higher seed set than hand-selfed plants, suggesting the occurrence of outcrossing. However, when all plants were considered, hand-outcrossed plants had more seeds per fruit than open-pollinated plants, indicating the occurrence of some selfing in the latter. *Gentianella germanica* also possesses a mixed mating system (Fischer and Matthies 1997). Genetic analysis of open-pollinated seeds would be required to estimate the levels of selfing and outcrossing present in the populations studied.

Animal-pollinated perennials often possess mixed mating systems even when selfed offspring show signs of inbreeding depression (Barrett and Eckert 1990). The ability to self can be crucial when pollinators are unreliable. Pollinator foraging behavior may also lead to the delivery of outcrossed pollen to some stigmas and selfed pollen to others within the same inflorescence or plant (Harder and Barrett 1995). A mixed mating system allows for greater seed production in this scenario.

POLLEN LIMITATION VERSUS RESOURCE LIMITATION

Campbell and Halama (1993) postulated that pollen and resources could affect different measures of reproductive success, and could thus both limit lifetime seed production. They tested this hypothesis by hand-pollinating and fertilizing *Ipomopsis aggregata* (Pursh) V. Grant ssp. *aggregata* (Polemoniaceae) plants. Their results indicated that hand-pollination had the strongest effect on seed production while an increase in flower production resulted from fertilizing.

The results of the present study suggest that resources and pollen also limit different aspects of female reproductive success in *P. bigelovii*. Specifically, fruit set appears to be resource-limited while seed production shows signs of pollen limitation, especially in the absence of pollinators.

All of the pollination treatments had a mean fruit set in excess of 77%. Nineteen percent of the sampled fruits contained no seeds. The combination of high fruit sets and a high percentage of fruits that lacked seeds could suggest that I was not able to discriminate between mature and aborted fruits. However, several other studies report high fruit set in gentians. Petanidou et al. (1995) found 100% fruit set across five pollination treatments and three plots of *G. pneumonanthe* plants. Spira and Pollak (1986) observed mean fruit sets in *Gentiana tenella* Rottb. of 100% for exclusions and 99.5% for open-pollinated plants. Luijten et al. (1998) found that *G. germanica* fruit set across four pollination treatments was nearly 100%. Although

fruits with low seed production are often preferentially aborted, they may be maintained when pollination levels are low and/or resources are plentiful (Stephenson 1981). Dudash (1993) observed that *Sabatia angularis* L. (Gentianaceae) was able to set fruits with as few as nine seeds. So although it is possible that I defined "fruit" too broadly, other studies have also shown that pollen addition or reduction (i.e., exclusion from pollinators) has no effect on fruit set.

Two other findings in my study suggest that fruit set may be limited by resources. First, fruit set was negatively correlated with number of flowers per plant. Second, site had a significant effect on fruit set. Towhee Mesa plants could have had more available resources early in the season which allowed for greater flower production, but lacked the resources required to bring all of these flowers to fruit later in the season. Stephenson (1981) cited many studies where hand-pollination did not increase fruit set, noting that "...the evidence that flower and fruit abortion are a response to limited resources is prodigious."

In a single season, seed set and number of seeds in *P. bigelovii* were clearly limited by the quantity and quality of pollen delivered. Seed production increased when pollinators were not excluded and when outcrossed rather than selfed pollen was delivered. However, hand-outcrossed seed set was not significantly higher than open-pollinated plants, and the increase in number of seeds per fruit in hand-outcrossed plants was only marginally significant. Why is this?

This study may have underestimated the degree of pollen limitation present at Lindsay and Towhee Mesas for several reasons. First, because I was interested in comparing mating systems as well as pollen limitation versus resource limitation, I performed tests for all pairwise comparisons of pollination treatment. The Tukey-Kramer alpha adjustment that I employed controls for Type I error (rejecting the null hypothesis when it should be accepted), but reduces statistical power and increases the probability of Type II error (accepting the null hypothesis when it should be rejected) (Zar 1999). Thus it is probable that a study with more focused comparisons (e.g., one involving only hand-outcrossed and open-pollinated treatments) would have detected more highly significant differences.

Second, many hand-pollinations undoubtedly missed peak stigmatic receptivity and/or pollen viability, as the duration of both was not tested. The large sample size (over 1800 flowers were hand-pollinated) and the occurrence of snowstorms during the study period made it difficult to visit all of the hand-pollinated plants as frequently as would have been required to achieve maximum pollination success.

Thirdly, if pollen and resources equally limit seed production, one would not expect pollen addition to increase seed production unless resources were added as well (Haig and Westoby 1988). In this scenario, pollen would still be a limiting factor because a decrease in its availability would result in decreased seed production.

The exclusions do not provide a test for this hypothesis, however, because of the confounding effects of self-incompatibility. Hand-outcrossed plants never achieved 100% seed set, suggesting that pollen was not the only limiting factor (but see above discussion regarding timing of hand-pollinations).

Other gentian studies also followed this pattern where means of measurements of female reproductive success were highest in hand-outcrossed plants, but did not differ significantly from open-pollinated means (Barnes and Rust 1994; Fischer and Matthies 1997). These studies may have been plagued with some of the problems discussed above.

One might hypothesize that the high mean seed mass in *P. bigelovii* exclusions was related to low number of seeds per fruit. However, the correlation results do not support this hypothesis. If mean seed mass were resource limited, it should have been correlated with one of more of the variables tested. It is noteworthy that the two lowest pollination treatment means for mean seed mass occurred in hand-pollinated plants. Fischer and Matthies (1997) found no significant difference in mean seed mass between exclusions and hand-pollinated *G. germanica* plants, and Dudash (1993) was unable to detect a significant difference in this measure between open-pollinated and hand-outcrossed *S. angularis* plants. Oostermeijer et al. (1995) found that seed weight and seed set were negatively correlated in *G. pneumonanthe*. Campbell and Halama (1993) found no significant effects of pollen or resource

supplementation on mean seed mass in *I. aggregata* ssp. *aggregata*. Thus, it is difficult to interpret why exclusions and hand-outcrossed plants would differ significantly in terms of mean seed mass if not because of allocation of fewer resources with the production of more seeds.

Besides the negative relationship between number of flowers per plant and fruit set which has already been discussed, the other correlations tested did not reveal any other signs of resource limitation of female reproductive success. Herbivory also was not implicated in influencing reproductive output.

Overall, it appears that in these two populations of *P. bigelovii* in the year studied, resources may have limited early stages of reproduction (i.e., fruit set), pollen availability and quality were partial limits to seed production, and the factors that influenced seed mass are not yet understood.

CHAPTER 5

CONCLUSIONS

Pneumonanthe bigelovii appears to be pollinated only by bumblebee queens. Higher levels of seed production are realized when plants are not excluded from pollinators. This gentian species is capable of spontaneous selfing, and a mixture of selfing and outcrossing appear to occur in the populations studied. Hand-selfed plants exhibit signs of inbreeding depression. Overall, it appears that in these two populations in the year studied, resources may have limited early stages of reproduction (*i.e.*, fruit set) while pollen availability and quality may have partly limited seed production. The factors that influenced seed mass are not yet clear.

The results of this study suggest that the following areas of inquiry may yield a better understanding of the pollination biology of this species. More careful observation of flower visitors might identify additional pollinators, and could indicate whether the number of observed pollinators was typical. Genetic analyses could provide more information on selfing and outcrossing rates. Selfed and outcrossed seeds could be germinated and measures of fitness in other stages of development

beyond seed production could be compared to test for inbreeding depression . More work is necessary to explain why exclusions produced more massive seeds than hand-outcrossed plants.

Pollination biology not only recognizes the interrelatedness of different kinds of organisms, it embraces the idea that these connections must be studied if the individual components of a system are to be understood. The results often lead to the conclusion that the system is more complex than was imagined. Recent studies seem to confirm that limits to female reproductive success in plants are influenced by many environmental factors, and that these limits may be different from one individual to the next or even from one week to the next. No one model can be expected to incorporate all of these factors.

Human population growth will introduce more stresses and obstacles to plant and pollinator systems. Attempting to understand these systems while they still function is critical not only because pollination is vital to human economy and survival, but also because the planet is richer for having big, furry bumblebees and deep blue gentians.

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