

RECIPROCAL BENEFITS IN A PLANT-POLLINATOR MUTUALISM

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SUMMARY ABSTRACT

Mutualisms may facilitate coupled population expansion or decline if changes in either partner species lead to similar changes in the other species. However, few studies have measured how both species in a mutualism respond to changes in each other's abundances. In this study, I tested how reproduction of two mutualists—the bumble bee pollinator, *Bombus appositus*, and the perennial wildflower, *Delphinium barbeyi*—responded to natural and experimental changes in partner density and the resources they derive from each other (food for bees and pollen transfer for plants). From the bumble bee's perspective, bumble bee foragers flew short distances (< 100 m), suggesting that forager density was not strongly limited by floral resource availability or that long-distance flights were costly. Only early forager densities (founding queens) were higher in areas with naturally more flowers, but mid- and late-season forager densities (workers and males) did not increase in areas with more flowers. Bee reproduction was constant across meadows that varied naturally in flower availability, and nectar in *D. barbeyi* flowers was only depleted by 28% throughout the day during the period of colony growth. However, experimental food addition generally increased gyne production. From the plant's perspective, in natural *D. barbeyi* patches, pollen receipt increased linearly with density, but this effect was only significant in meadows and not in forests. Seed production increased across lower flower densities and decreased across higher flower densities, but this effect was only statistically significant in meadows. Experimental flower density did not affect pollinator visitation rates, pollen receipt, or seed production. Neither natural increases in pollinator visitation rate nor supplemental hand-pollinations

affected pollen receipt or seed production. Bumble bee pollinator exclusion generally reduced seed set, but sometimes fly pollinators (which have seed predator offspring) compensated for lost bumble bee pollination services. Together, these results suggest that in this mutualism, neither species should respond rapidly to small changes in partner abundance.

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In rocky canyon, pure content, had bear,
But eaglet's song above him stopped his trek.
Oh bear, she cried, how is the world from there?
He laughed, *Your wings must show you, says my deck.*

But nestled safe, I find no need to fly.
The choice is yours, but if you reach inside,
Your mother's love may lift you to the sky.
And eaglet smiled, she knew she longed to glide.

So up she went, and bear, below her, ran.
Together, they found every mountain pass.
When Sun came up each day they made a plan,
To live and love and learn, they raised a glass.

Remember mother eagle when you fly.
Remember those who love you by and by.

I dedicate this body of work in loving memory of my mother, whose curiosity and love of nature was contagious, a gift I will always treasure.

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SUMMARY INTRODUCTION

Mutualisms may facilitate coupled population growth or coevolutionary relationships if reproduction or survival of both species is limited by the interaction. For example, an increase in the abundance of a host species could lead to an increase in the abundance of its mutualist, and subsequently, that increase could feed back to further increase the abundance of the host species. Therefore, increases in the abundance of key mutualists could facilitate the recovery of threatened partner species (Barrow et al. 1997, Grondona et al. 1997, Thornton et al. 2001, van der Heijden 2004) or fuel the spread of invasive partner species (Schiffman 1994, Wolfe and Klironomos 2005).

Despite the inherent reciprocity of mutualisms, they are often studied from a unilateral perspective, emphasizing the more sessile partner, whose fitness is often easier to measure, such as the plant in a plant-pollinator mutualism (Cushman and Beattie 1991). Knowing how a visitor affects a host mutualist can help explain the ecology and evolution of the host, but if the visitor responds to changes in its partner species (e.g., by an increase or decrease in abundance), this could alter the nature (and our interpretation) of their relationship (Bronstein 1994b). For example, despite the important ecosystem services provided by mutualistic mycorrhizal fungi, nitrogen-fixing microbes, and photosynthetic zooxanthellae (Bever 1999, Simms and Taylor 2002, Hay et al. 2004), little is known about how variation in the abundance of their mutualistic partners affects their fitness or population growth. In plant-pollinator mutualisms, the number and diversity of pollinators is known to affect flowering plant reproduction in many species (Pellmyr and Thompson 1996, Gomez et al. 2007, Sahli and Conner 2007), but the degree

to which floral resource availability or other environmental variables limit pollinator reproduction and population growth is not well understood (National Resource Council 2006). Understanding how both species in a mutualism respond to changes in each other's abundance may help explain how mutualisms can facilitate coupled population growth and coevolutionary processes (Thompson 2005).

The outcomes of mutualisms are inherently context-dependent (Bronstein 1994a). Mutualistic benefits can vary with partner quality, resource availability, the presence or absence of predators, competition for access to a mutualistic partner, and the costs of partner exploitation (Johnson et al. 1997, Herrera 2000, Morales 2000, Egger and Hibbett 2004, Ness et al. 2004, Rudgers and Strauss 2004). Generally, the benefits of a mutualism for a species may be limited by (1) the demand for the mutualistic resources relative to other limiting resources or (2) the costs associated with maintaining the interaction. For example, the benefits of pollination for plant seed production decrease as ovules become saturated with pollen or when water and nutrients limit seed production (Ashman et al. 2004). Plants must attract enough pollinators to maximize seed set, but not so many that visitors remove too much costly nectar or pollen without providing efficient pollination services, that they fertilize so many ovules so that the plant becomes more attractive to seed or fruit predators (Thomson et al. 2000, Herrera et al. 2002). The numerous studies that have quantified how mutualistic benefits are mediated for one side of the interaction or the other have set the stage for examining how the benefits for *pairs* of species are mediated (Holland et al. 2002, Hofstetter et al. 2006, Savage and Peterson 2007).

Understanding reciprocal mutualism benefits is especially challenging in generalized mutualisms where hosts interact with many visitor species and vice versa.

Although mutualisms have traditionally been conceptualized as interdependent species pairs (Bronstein 1994b), many, if not most, mutualisms are actually composed of generalized guilds of interacting species (Waser et al. 1996, Stanton 2003). Pairwise specialization may be rare in mutualisms because specialists often specialize on generalists instead of other specialists (Bascompte et al. 2003, Vazquez and Aizen 2004). However, because generalists are often the most abundant species in the community, they may have to rely primarily on other abundant generalists to provide mutualistic services (Vasquez and Aizen 2006). Therefore, even though many somewhat specialized species associate with generalists, these ‘generalists’ may still receive the bulk of their mutualistic goods and services from a small number of other abundant species. The other species in the community may specialize on and benefit from these abundant species, while only making small contributions to their abundant hosts. For example, in a simulated plant-pollinator community, plants did not respond strongly to the removal of pollinator specialists, but rapid extinctions occurred when generalist bee pollinators were lost (Memmott et al. 2004). Despite the pivotal role of abundant generalists in mutualism communities, no studies have empirically tested the nature of their interdependence.

The responsiveness of two mutualists to each other at any given time could be grouped into one of three scenarios. First, if both partners respond to changes in each others densities, then coupled population processes are more likely. In this scenario, population growth or decline in one partner would lead to accelerating growth or declines in both species—the classic view of a mutualism, where both species benefit from increases in each other’s abundance (Holland et al. 2002). Second, if both partners are limited by other factors outside the mutualism and do not respond to changes in partner

density, then the mutualism is likely to be more resistant to short-term fluctuations in partner density. Third, if only one partner responds to changes in partner density, then coupled population processes are unlikely. However, if one partner changes in abundance for reasons other than the mutualism, then the other partner's response may create the illusion of interdependent population coupling. Thus, if there is a surplus of one partner, then there might be a shortage of the other partner. For example, if there are surplus flowers for pollinator reproduction, there may also be too few pollinators to maximize plant reproduction (Lundberg and Ingvarsson 1998).

Dissertation overview and research questions

I assessed how two mutualists responded to variation in each other's abundance and the resource or service provided by the interaction, focusing on a plant-pollinator mutualism. I used observations and experiments to study how the reproduction of the bumble bee pollinator, *Bombus appositus* (Apidae), and the perennial wildflower, *Delphinium barbeyi* (Ranunculaceae), responded to natural changes in partner density and to experimental variation in the resources they derive from each other. Both species in this mutualism interact with many other species in the community; however, they derive most of their food and pollination services from each other. This study moves beyond defining pairwise benefits (Bronstein 1994b, Holland et al. 2005, Stachowicz and Whitlatch 2005) to understanding the degree to which mutualists embedded in a generalized community respond to changes in each other's densities, resources, or services (Cushman and Beattie 1991, Cushman et al. 1994, Bascompte et al. 2003, Stanton 2003, Hofstetter et al. 2006, Savage and Peterson 2007). To address the responses of pollinator and plant mutualists to

changes in each other's densities, resources, or services, I examined questions from both pollinator and plant perspectives:

Pollinator perspective:

- Chapter 1: Bee foraging. How far do bees fly to forage—and do they fly farther when flower-bee ratios decline at the end of the season? Are bee forager densities related to flower availability within their foraging areas—and do these relationships change over the season?
- Chapter 2: Bee reproduction. Does bee colony reproduction increase in areas with more flowers or with supplemental food?

Plant perspective:

- Chapter 3: Flower density. How does flowering plant density affect pollinator visitation rate, pollen receipt, and seed production?
- Chapter 4: Alternative pollinators. How do primary and secondary pollinators contribute to seed production and selection on floral traits via female reproduction?

Pollinator perspective – Bumble bee reproduction may depend on flower availability since floral nectar and pollen are their sole food resources. However, many other factors could mediate food limitation of bee reproduction and population growth. Bumble bees are annual colonial organisms, and before queens can produce gynes and males, they must survive diapause, establish new nests in the spring, and produce a sterile brood of workers to help collect resources (Alford 1975, Heinrich 1979). Successful

colonies must also survive a suite of potential antagonisms, such as nest predation by mammals, individual predation by birds and spiders, insect parasitism, and fungal infection (Goulson 2003). Several studies have found that bees are more abundant and produce more offspring in areas with more flowers or more natural habitat (Steffan-Dewenter et al. 2001, Goulson et al. 2002, Kremen et al. 2002, Westphal et al. 2003, Greenleaf 2005, Heard et al. 2007, Williams and Kremen 2007). Currently, the only published study that has tested whether field bumble bee colonies are food-limited found that supplemental feeding increased *Bombus impatiens* and *B. ternarius* gyne, worker, and male production in Quebec, Canada (Pelletier and McNeil 2003). Because bumble bees are colonial and active for most of the flowering season, the degree to which flowers are limiting may largely depend on the overall bee population size, which will determine how quickly nectar and pollen resources are depleted in an area (Pyke 1982). However, it is not known if bumble bees are generally limited by food in a variety of habitats.

Plant perspective – Plant seed production may not only vary with visitation rate by common pollinators, but also with the availability and efficiency of alternative pollinators, pollinator visit quality, and abiotic resources (Burd 1994, Stanton 2003, Aizen and Harder 2007). For example, flowers may benefit from being in dense patches if abundant floral resources attract more pollinators and/or provide an ample supply of compatible pollen donors (Kunin 1993, Waites and Ågren 2004, Hegland and Boeke 2006). However, flowers in high density patches may also compete with other flowers for pollinator visits or for abiotic resources necessary for seed production (Steven et al. 2003). In the absence of a plant's primary pollinators, secondary pollinators (i.e., less frequent or less efficient visitors) may provide adequate pollination services, which can

sustain plant reproduction (Kandori 2002, Castellanos et al. 2003, Memmott et al. 2004). However, secondary pollinators might transfer pollen less efficiently, deplete nectar that might attract more efficient pollinators, or produce offspring that could consume fertilized ovules (Schwartz and Hoeksema 1998).

Study System

I studied *Bombus appositus* and *Delphinium barbeyi* reproduction in subalpine meadows and forests near the Rocky Mountain Biological Laboratory in Gothic, Colorado, USA. *Bombus appositus* is one of the three most abundant bumble bee species in this study area and visits at least 20 flower species throughout the season (Pyke 1982, see Chapter 1). Queens emerge from hibernation in early spring (late May to early June) and establish new nests, where they incubate their first brood. About six weeks later, the first worker cohort hatches, and forager density increases sharply. During this increase in bee abundance, while *B. appositus* colonies are provisioning and hatching workers, gynes, and males, over 90% the pollen they collect and the floral visits they make are to *D. barbeyi* (see Chapter 2).

In the study area, *D. barbeyi* occurs in meadows and forest clearings in distinct patches, and *D. barbeyi* flower density varies over three orders of magnitude among 0.5 ha meadow plots (0.8 to 26.2 flowers produced per m², see Chapter 1). With 13.6 ± 0.5 *D. barbeyi* inflorescences per plant (mean \pm 1 SE, n = 420 plants), each bearing 25.4 ± 0.8 flowers per inflorescence (n = 372 inflorescences, S. E. Elliott, *unpublished data*), *D. barbeyi* accounts for $14.0 \pm 4\%$ of all flowers produced in this study area (mean \pm 1 SE, n = 6 meadows, see Chapter 1). On average, flowers contain 1.8 ± 0.05 μ L of nectar per

flower in the morning (before pollinator visits begin, $n = 512$ flowers) with $36.1 \pm 0.7\%$ sugar concentration ($n = 34$ flowers, S. E. Elliott, *unpublished data*).

Delphinium barbeyi flowers are protandrous and self-compatible, but they produce few seeds autogamously (Williams et al. 2001). Flowers from which pollinators are excluded produce at least an order of magnitude fewer seeds than open-pollinated flowers, and therefore, plants require pollinator visits to maximize seed set (Williams et al. 2001). *Delphinium barbeyi* flowers may vary in the degree to which their seed production is pollen-limited (see Chapter 3).

At this study site, the long-tongued bumble bee, *Bombus appositus*, is the most common pollinator of *D. barbeyi* (Inouye 1976). Flowers are also visited less frequently by other bumble bees (*B. flavifrons*, *B. bifarius*, *B. frigidus*, *B. nevadensis*, *B. occidentalis*), hummingbirds (*Selasphorus platycercus*, *Selasphorus rufus*, and *Stellula calliope*), and sphinx moths (*Hyles lineata*) (Inouye 1976, Waser 1982, Williams et al. 2001). Collectively, bumble bees account for over 95% of flower visits to *D. barbeyi*, and *B. appositus* alone accounts for 60-80% of flower visits to *D. barbei* (see Chapter 2 and 3). Seed set does not differ between flowers visited by *B. appositus* and the second most-frequent visitor, *B. flavifrons* (R.E. Irwin, *unpublished data*). However, the relative pollination efficiencies of the other visitors are unknown.

Adult flies also visit *D. barbeyi* flowers. Although the flies only contribute to 0.3-0.6% of flower visits to *D. barbeyi* (R. E. Irwin, *unpublished data*), they may act as secondary pollinators if bumble bees are absent. Female flies also deposit eggs singly or in groups on the carpels prior to fruit expansion, and fly larvae consume ~10% of *D. barbeyi* seeds (see Chapter 3). The flies are probably in the *Botanophila* genus or in a

related genus in the Anthomyiini tribe, but positive identification awaits collection of male individuals. The flies may be consuming pollen, but it is unlikely that they consume nectar, which is contained deep in *D. barbeyi* nectar spurs. Whether these flies also act as secondary pollinators is unknown.

Dissertation outline

A mutualist should be more responsive to changes in partner density if per-capita reproduction or survival is positively associated with variation in partner density or in the resources provided by its partner. Therefore, I combined a variety of observations and experiments to understand how responsive a pollinator and a plant might be to changes in each other's densities.

The bumble bee pollinator and perennial plant operate on very different spatial and temporal scales so therefore, the methods I used to understand each partner varied considerably. Individual bumble bees require resources from thousands of individual flowering host plants to reproduce, and these resources are therefore distributed over large areas—e.g., 100 m – 3,000 m radii areas in other systems (Walther-Hellwig and Frankl 2000, Steffan-Dewenter et al. 2002, Westphal et al. 2006) and throughout the entire season (e.g., about three months in this system). In contrast, a plant may only need as little as one pollinator visit per flower to deliver the pollen necessary to produce seeds. On the other hand, manipulating a sessile partner (e.g., a plant) is easier than manipulating a mobile partner (e.g., a bee). Thus, I used a variety of different observational and experimental approaches for both partners to gain the most complete understanding of how per-capita reproduction of each species might vary with each

other's density, resources, or services. For both species, I first explore natural patterns that provide insight into whether or not they generally have more or less of their partner than they require for reproduction. Next, for both species, I combine experiments and observations to more directly test how variation in partner density or resources affects their per-capita reproduction.

Pollinator perspective:

Chapter 1: Bee foraging – To gain insights into whether bumble bees had ample or scarce floral resources, I measured bee foraging distances using mark-recapture, and I measured natural forager and flower densities in meadows. If floral resources are scarce near the visitor's nest, then I predicted that the visitor should have to forage at longer distances (Cresswell et al. 2000). If host resources limit visitor reproduction or survival, then areas with more hosts may harbor more visitors (Westphal et al. 2003).

I found that bumble bee foragers flew relatively short distances (< 100 m), which could suggest that bumble bees had ample floral resources or that long-distance flights were costly (Cresswell et al. 2000). Early-season foragers (mostly founding queens) were more abundant in meadows with more flowers, but forager-flower density correlations disappeared midway and late in the season. This suggests that initial benefits of high flower densities for colony success could be dampened or reversed by higher predation or parasitism in those areas, and/or that flower density only limits colony establishment, but not colony growth.

Chapter 2: Bee reproduction – I tested whether *B. appositus* reproduction increased in areas with naturally more flowers or with supplemental feeding. I predicted

that if visitor reproduction is food-limited, then visitors in areas with more hosts should produce more offspring (Williams and Kremen 2007). If visitor reproduction is food-limited, then supplemental feeding should increase reproduction (Pelletier and McNeil 2003). If visitor reproduction is not limited by food availability, then visitor reproduction should not increase with feeding; and positive correlations between host density and visitor reproduction may be a result of underlying factors that covary with host density.

I found that bumble bee colony reproduction did not vary across meadows that varied naturally in flower density, possibly because meadows with more flowers housed more nests or because nectar resources may not have been exhausted. Colonies that I fed with supplemental nectar (2006) or nectar and pollen (2007) produced more gynes. These results suggest that *B. appositus* reproduction was food-limited, but that more flowers would not necessarily improve reproduction. I hypothesize that the subalpine colonies may not be able to take full advantage of extra flowers because they may have too few workers to harvest extra resources.

Plant perspective:

Chapter 3: Plant-pollinator densities – I tested how natural flower density affected pollen receipt and seed production and how experimental flower density affected pollinator visitation rates, pollen receipt, and seed production. If patches with higher host density attract disproportionately more visitors, and if host reproduction is limited by visitors, then host reproduction should be positively correlated with host patch density (Kunin 1993). However, if non-mutualistic resources independently promote high host

density and high host reproduction, then host reproduction should not increase with experimental host density (Bosch and Waser 2001).

I found that natural variation in flower density only had minor effects on pollen receipt and seed production. Experimental variation in flower density did not affect pollinator visitation rate, pollen receipt, or seed production. Neither natural increases in pollinator visitation rate nor supplemental hand-pollinations were related to pollen receipt or seed production.

Chapter 4: Alternative pollinators – I tested how bumble bee pollinator exclusion affected *D. barbeyi* seed production and natural selection on floral traits (flower production, blooming date, and spur length) in two sites (one each in forest and meadow habitats) over two study years. If hosts have alternative mutualist visitors, then exclusion of their primary mutualist visitors may not greatly reduce host reproduction (Kandori 2002). If primary visitor mutualists are essential for host fitness, then their exclusion might also alter natural selection on host phenotypic traits associated with visitor attraction or efficiency (Schemske and Bradshaw 1999).

I found that bumble bee pollinator exclusion generally reduced seed set, but in one site in one year, fly pollinators compensated for lost bumble bee pollination services. In the forest site, bumble bees mediated selection for increased flower production and for shorter spurs, and in the meadow, they mediated selection for later blooming.

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CHAPTER I

Subalpine bumble bee foraging distances and densities in relation to flower availability

ABSTRACT

Bees feed almost exclusively on nectar and pollen from flowers. However, little is known about how food availability limits bee populations. To gain insights into the potential for food limitation within subalpine bumble bee populations, I examined bee foraging distances and the relationship between forager and flower densities. If floral resources are limited, then bees should fly farther to forage, and they should be more abundant in areas with more flowers. I estimated foraging distances by calculating forager recapture probabilities at increasing distances (up to 3,766 m) from eight marking locations, and I measured natural variation in forager and flower densities for 11 weeks in six half-hectare plots. Because flower availability should be lowest late in the season when bee density is high relative to flower density, I predicted that late in the season I would: (1) recapture foragers at farther distances and (2) find stronger relationships between forager and flower densities. These predictions were not upheld. Throughout the flowering season, I primarily found marked bees only within 100 m (and never over 1,000 m) from their original marking location, suggesting that they typically did not fly far to forage. Although the density of early season foragers (mostly founding queens) increased with early-season flower density, the density of mid- and late-season foragers (mostly workers and males) did not vary with flower density. In addition, forager densities later in the flowering season were not related to earlier bee or flower densities, suggesting that there were no delayed effects of floral resources or colony productivity on forager density. These results suggest that these high elevation bumble bees may have ample floral resources for reproduction, a hypothesis that warrants further experimental investigation.

INTRODUCTION

Food availability often affects consumer behavior and population growth (Rogers 1987, Guyer 1988, Kreiter and Wise 2001), which may subsequently feed back to affect species interactions (Wise 2006, Pitelka and Batzli 2007). Although it is difficult to experimentally test whether populations of highly mobile consumers are food-limited, examining consumer behaviors and patterns of abundance can provide initial insights into whether consumers have food shortages or surpluses (Petersen et al. 2006, Wirsing and Murray 2007). If food is scarce, mobile consumers should have to spend more time foraging and may travel farther to collect sufficient resources once the resources are exhausted near the nest (Suzuki et al. 2007). If food is scarce and if foraging is energetically costly, then consumers might also produce fewer offspring or have lower survival rates, leading to lower population densities (Schulz et al. 1998, Mduma et al. 1999, Vet 2001). Alternatively, if food is abundant, then consumers could forage near their nests, and their densities might be determined by factors other than food availability (Hairston et al. 1960, Nie and Liu 2005). In this study, to understand whether highly mobile consumers might be food-limited, I measured bumble bee consumer foraging distances, and I assessed whether foragers were more abundant in areas with naturally more flowers (Table 1).

Bee foraging distances are governed by physiological and ecological constraints. Body size determines the possible foraging ranges of bee pollinators (Gathman and Tscharrntke 2002, Greenleaf et al. 2007), and larger colonial bees may also build larger colonies which require more food (Wright et al. 2000, Westphal et al. 2006). Thus, it is often difficult to differentiate between the importance of resource demand and body size

in driving differences in foraging distances among bee species. However, within a species, if nearby resources are scarce, then bees should travel farther to collect sufficient resources to feed themselves and provision their offspring (Cresswell et al. 2000). Instead, if bees have ample resources and if resource quality is constant at near and far sites (but see Batra 1993, Osborne et al. 1999, Heithaus et al. 2005), they could minimize foraging time and energy by feeding closer to their nest (Heinrich 1979b). Estimates of average bumble bee foraging distances vary from 100 m to 2,750 m, with differences found within and among species (Walther-Hellwig and Frankl 2000, Darvill et al. 2004, Knight et al. 2005). Calculations based on bumble bee energy use while foraging and in flight predict that to maximize energy gain, bees should forage within 1,000 m from their nest unless (1) nectar availability per flower drops below one μL per flower near the nest and (2) nectar per flower is greater farther away (Cresswell et al. 2000). Despite the potential for food availability to influence foraging distances, few studies have assessed how temporal or spatial variability in resources affects bee foraging distances (but see Osborne et al. 2008).

Bumble bees may only exhaust local resources when forager density is high and flower density is low. Since bumble bee density increases over the season as colonies grow (Heinrich 1979a, Pleasants 1981), flower availability per bee may be lowest, and therefore most limiting to bee density and colony growth, late in the season (Pyke 1982). For example, in montane areas, bumble bees are more abundant in meadows that are wetter and produce more late-season flowers than in drier meadows that have more early-season flowers (Hatfield and LeBuhn 2007). Similarly, in subalpine meadows, flower composition only influences bumble bee composition near the end of the flowering

season (Bowers 1985).

To provide insight into whether subalpine bumble bees might have ample or scarce floral resources, I estimated bumble bee foraging distances by calculating recapture probabilities at increasing distances from marking locations, and I measured natural relationships between forager densities and flower densities within their foraging areas. Because subalpine bumble bees have little time to build their colonies (e.g., 9-11 weeks, Pyke 1982), I predicted that subalpine bumble bees would generally forage over short distances (e.g., < 1,000 m), and that forager density would not be correlated with flower density within their foraging areas (Table 1). However, late in the season, when floral resources may be more limiting, I predicted that foraging distances would increase, and there would be stronger relationships between forager and flower densities.

METHODS

Study system

I monitored bumble bee foraging distances and forager and flower densities for 11 weeks (6 June – 28 August, 2004), spanning most of the flowering period, in Gunnison National Forest near the Rocky Mountain Biological Laboratory (RMBL), Gothic, Colorado, USA (2879-2957 m elevation; Figure 1, Appendix I). Twelve bumble bee species and two social nest parasite species are found around the RMBL (Beattie et al. 1973, Pyke 1982). I identified bumble bees (*Bombus* spp., Apidae) to species based on pile color patterns (Stephen 1957). The nest parasites (*Psithyrus* spp., Apidae) could have been either of two species (*P. insularis* or *P. suckleyi*), which are difficult to distinguish in the field, so I identified them to genus only. Bumble bees around the RMBL visit a diversity of

perennial wildflower species, partitioning their resources according to proboscis length and corolla depth or nectar spur lengths (Inouye 1978, Pyke 1982). The three most common bumble bee species in this study, *B. appositus*, *B. flavifrons*, and *B. bifarius*, have long, intermediate, and short proboscises, respectively (Pyke 1982).

Around the RMBL, queen bumble bees emerge from their winter diapauses in May or June (S. E. Elliott, *personal observation*). Each founding queen searches for an underground nest site in which to establish her colony. After producing sterile female workers, the founding queen produces new queens (i.e., gynes) and males. At the end of the season, in late August to early September, gynes and males mate, and only the gynes over-winter. As in most temperate areas, bumble bees produce only one generation per season.

Field Methods

Foraging distance – To estimate foraging distances, I marked and recaptured foragers to determine the spatial scale at which individuals returned to forage. To mark a bee, I painted its thorax with a paint pen (Marvy®Uchida, Torrance, CA), and then I immediately released the bee. Out of 4,280 marked bees, most flew normally when released; only 0.7% crawled on the grass instead of flying. I marked bees in eight 0.5 ha plots, using a unique color for each plot, and I recaptured bees in these plots plus ten additional 0.25 ha plots (Figure 1, Appendix I). I recaptured bees in the 0.25 ha plots in July when bee density was low and required additional search effort. I also marked bees while catching in these additional plots to standardize search effort time. I caught and marked bees in the study plots for approximately four hours per week per plot, while

systematically walking through the meadows. Because new bees hatch and old bees die every two to four weeks, I continuously marked and recaptured bees throughout the entire season. To avoid recapturing recently marked bees (i.e., those that may not have returned to the nest after marking), I did not catch bees with fresh paint. Paint remains on bees for at least two weeks in wild colonies (S. E. Elliott, *personal observation*).

I calculated foraging distances by estimating recapture rates at increasing distances from the marking locations. With eight marking plots and 18 recapture plots, there were 144 plot pairs, representing unique distances over which marked bees could fly and be recaptured, including the possibility for no movement (i.e., recapture in their original marking plot). For each pair of plots, I calculated a recapture rate by dividing the number of bees recaptured in the recipient plot by the number of bees marked in the marking plot, and then weighting this proportion by the search effort (in hours) in the recipient plot.

To determine how recapture rates varied over increasing distances from the marking locations, I divided the 144 plot pairs into categories according to the distance between plot centers. The first category, '0 m', represents recaptures in the same plot where the bees were marked. The categories group plot pairs into 100 m intervals up to the maximum distance of 3,766 m. I calculated the relative recapture rate for each distance category by dividing the average recapture rate (or the single rate for the 901-1,000 m category because there was only one plot pair in this distance category) by the sum of all average recapture rates from each category. Therefore, the recapture probability in each distance category represents the probability that a bee (if recaptured) would be found in that distance interval.

Unlike studies where foragers can move out from marking locations in 360° (Turchin 1991), in this study, I assumed that bees remained in the linear strip of meadows. For example, no marked bees were observed on the southwest side of the river or in the dense spruce forest, which bordered the marking plot (Figure 1), both of which have fewer flowers than in the open south-facing study meadows (S. E. Elliott, *personal observation*). Therefore, this study is more similar to fish mark-recapture studies where fish can only radiate out in two directions, up- or down-stream (Albanese et al. 2003). Unlike mark-recapture studies that have fewer recapture locations relative to the total area available at increasing distances from marking locations (e.g., Turchin and Thoeny 1993, Skalski and Gilliam 2000), because I marked in *multiple* locations distributed evenly throughout the narrow strip of meadows, the proportion of area sampled in each distance interval was constant across distances. Sampling plots that were long distances from any given marking meadow also functioned as plots that were near other marking meadows. In addition, a considerable proportion of the total possible foraging area was sampled at all distances; each 0.5-ha plot incorporated $28.7 \pm 4.1\%$ of the surrounding meadow area (n = 8 plot). Finally, recaptured bees were not prevented from flying longer distances in subsequent foraging bouts because if they were caught in nearby meadows, they were released immediately and could subsequently fly to forage in farther meadows (i.e., they were immediately released after capture).

If bees were frequently caught in a small area, they could have been flying back to a more distant nest between foraging bouts. However, because I marked and recaptures bumble bees in multiple locations, I assumed that bees were flying no farther than the distance between marking meadows (200-300 m) to nest or forage between captures in

the same meadow.

To compare relative recapture rates among bee species, I calculated separate relative recapture probabilities for the three most common species, *B. appositus*, *B. flavifrons*, and *B. bifarius*. To compare relative recapture rates across the season, I grouped all species and calculated separate relative recapture probabilities for early- (weeks 1-4), mid- (weeks 5-8), and late- (weeks 9-11) season recaptures. Recapture rates were too low to test for species-specific recapture rates within each time period.

Forager-flower correlations – To assess whether meadows with more flowers had more bumble bee foragers, I measured natural forager and flower densities over the 11 study weeks in six 0.5 ha plots, each separated by ≥ 288 m (Figure 1). I quantified forager density by recording the rate at which I caught bumble bees in each plot. Each week, I caught bees in each plot for approximately four hours of measured observation time (approximately one hour, twice between 0900 and 1200 and twice between 1230 and 1530). I restricted catching periods to times when there were no storms to avoid storm-related shifts in foraging rates (Peat and Goulson 2005).

During each catching period, I systematically walked the entire plot and captured, marked, and released each bee I encountered. I did not exclude recaptured bees (17% of captures) because I was not trying to determine the total number of unique bees in the meadows, but rather the number of active foraging bouts observed per hour of observation time in each plot. Because I did not recapture recently marked bees, any recaptured bees probably represent two separate foraging bouts from the colony (on the same day or a different day). For each bee, I recorded the species, caste (founding queen, worker, male, or gyne), and the flower species they were visiting. I excluded bees that I

caught while they were grooming on leaves, flying, or crawling on the ground. I included the social nest parasites, *Psithyrus* spp., because they also deplete nectar resources. However, due to their low abundance (Table 2), including or excluding *Psithyrus* did not affect the results. I calculated forager density as the average number of bees caught per hour per 0.5 ha plot (averaged within weeks across the entire season).

I sampled flower density in 16 quadrats (0.25 m x 25 m) per plot (four randomly placed quadrats in each of four 25 m x 50 m subplots per plot, Figure 1). In each quadrat, I recorded the number of open flowers of all flower species, and later I excluded flower species that were not visited by bumble bees during this study. For species that consistently had more than ten open flowers per inflorescence, I counted the number of inflorescences in each quadrat, and I sub-sampled 20 inflorescences to calculate the average number of flowers per inflorescence (or florets per capitulum in the Asteraceae). I multiplied the average number of flowers per inflorescence by the number of inflorescences to calculate total flower number. Although, ideally, I would have standardized flower number to total pollen and nectar availability, per-flower pollen and nectar production estimates were not available for most species (Tepedino and Stanton 1982). However, flower density was dominated by a few species, so flower composition was similar among meadows. To calculate total flower density, I summed average flower density over the 11 sampling weeks in each plot. Because I sampled flowers once per week, I could have missed some flowers that were open for fewer than seven days, and I could have double-counted flowers that were open for more than seven days, but this possible bias was constant across plots.

I used Pearson's correlation coefficients to assess the relationships between

forager density (capture rate per 0.5 ha plot, all bee species combined) and flower density (flowers per m², all bumble bee-visited flower species combined). I combined all bumble bee species because they overlap in flower species use (Appendix III). To determine whether the density of different bumble bee castes increased with flower density, I assessed the relationship between forager density and total flower density, separating analyses by caste. To determine whether forager-flower density relationships varied over the course of the season, I assessed the relationship between forager density (all castes combined) and flower density, separating analyses by early in the season (weeks 1-4), midway through the season (weeks 5-8), and late in the season (weeks 9-11). Finally, to determine whether meadows with more early-, mid-, or late-season flowers also had more total flowers (all weeks combined), I measured correlations between total flower density and early-, mid-, and late-season flower densities.

Because it takes approximately four weeks to provision, develop, and hatch a new cohort of bumble bee offspring (Heinrich 1979a), I also tested for a time lag in the relationships between forager and flower densities. Forager density could be a function of (1) previous flower availability (i.e., food to provision new offspring) and/or (2) previous colony size (i.e., workers available to provision and care for new offspring). Therefore, I tested whether meadows with more early-season flowers or foragers had more mid-season foragers, and whether meadows with more mid-season flowers or foragers had more late-season foragers. All analyses were performed with JMP 4.04 (SAS Institute 2001).

RESULTS

I observed eight *Bombus* species and the bumble bee nest usurper, *Psithyrus* spp., in the study meadows (Figure 1; Table 2). The three most common species, *B. appositus*, *B. flavifrons*, and *B. bifarius*, collectively accounted for $80 \pm 4\%$ of all bees captured (mean ± 1 SE, $n = 6$ plots), and each species individually accounted for $\sim 30\%$ of all bees captured (Table 2). In all plots, I observed bumble bees on 47 flower species from 18 plant families (Appendix II, Figure 1). Foragers primarily visited flower species that were among the top 25% of species based on floral abundance, but uncommon bee species and *B. appositus* workers also frequently visited two patchy uncommon Gentianaceae species: *Frasera speciosa* and *Pneumomanthe parryii* (Appendix III). Flower density was dominated by a few species; only four species contributed to $> 5\%$ of all flowers: *Potentilla pulcherrima* ($41.8 \pm 8.8\%$, Rosaceae), *Delphinium barbeyi* ($14.0 \pm 4.4\%$, Ranunculaceae), *Heliomeris multiflora* ($9.8 \pm 3.5\%$, Asteraceae), and *Erigeron speciosus* ($6.7 \pm 2.9\%$, Asteraceae). Total flower density varied four-fold among meadows (Appendix II). Since flower density peaked midway through the season when bee density was at its lowest (Figure 2A,B), flower availability per bee was lowest early and late in the season (Figure 2C).

Foraging distance – Bees were primarily recaptured in the same plot in which they were marked. Of the 3,258 individual bees marked in the eight 0.5-ha plots 637 were recaptured (19.6%). Of the 637 recaptured individuals, 590 (92.6%) were recaptured in their marking plot. After correcting for sampling effort, there was an 82.3% chance that if a marked bee was recaptured, it would be recaptured in its marking plot (Figure 3). The remaining bees were recaptured within 1,000 m of their marking plot; no bees were recaptured at distances between 1,000 m and 3,766 m from the marking plot. The high

likelihood of recaptures in the marking plot was constant across *Bombus* species (Figure 3A) and across the season (Figure 3B).

Forager-flower correlations – Meadows with more flowers harbored more founding queen foragers ($r = 0.83$, $P = 0.04$; Figure 4A), but this trend disappeared for workers, males, and gynes ($r < 0.36$, $P > 0.4$; Figure 4B-D). Similarly, meadows with more early-blooming flowers had more early-season foragers ($r = 0.85$, $P = 0.03$; Figure 4E), but meadows with more mid- or late-season flowers did not have more mid- or late-season foragers ($r < 0.11$, $P > 0.8$; Figure 4F,G). Since only flower densities from the middle of the season were significantly correlated with total flower densities (late: $r = 0.49$, $P = 0.3$; mid: $r = 0.99$, $P = 0.0001$; early: $r = 0.57$, $P = 0.2$), early season flower densities were not an indicator of future flower availability. Meadows with more flowers or foragers early in the season did not have more foragers midway through the season, nor did meadows with more flowers or foragers midway through the season have more foragers late in the season ($r < 0.51$, $P > 0.2$; Figure 5).

DISCUSSION

Food availability is an important component of consumer behavior, population growth, and species interactions (Rogers 1987, Guyer 1988, Kreiter and Wise 2001, Pitelka and Batzli 2007). As in many consumer-resource mutualisms (Holland et al. 2005), little is known about whether bee pollinator consumers are limited by floral nectar and pollen resources. Because bumble bees often fly long distances to forage, and because their densities are often closely related to flower availability, it is often assumed that bumble bees exhaust local floral resources (Saville et al. 1997, Kremen et al. 2002, Westphal et

al. 2003, Knight et al. 2005, Carvell et al. 2007, Heard et al. 2007, Osborne et al. 2008). However, I found that subalpine bumble bees forage over relatively short distances (< 100 m), and meadows with naturally more flowers only harbor more foragers early in the season. These results suggest that: (1) bumble bees did not exhaust local resources; (2) flower density did not adequately represent resource availability; or (3) bee density was limited by factors other than floral resources (e.g., nest sites or parasites, Table 1).

Although previous studies have demonstrated that bee densities are positively correlated with flower densities at large spatial scales (Kremen et al. 2002, Westphal et al. 2003, Heard et al. 2007), such observational studies (including the current study) are difficult to interpret. For example, if forager density is not correlated with flower density at a specific spatial scale, this could suggest that the bees are not using the resources at that spatial scale (Steffan-Dewenter et al. 2002). Alternatively, bees could be using the resources at that spatial scale but their densities could be controlled by nest site availability or parasitism (Potts et al. 2005, Carvell et al. 2008). Also, since nectar and pollen production per flower may vary among species or among habitats within species, flower number may not adequately represent resource availability (Tepedino and Stanton 1982, Cartar 2004, Goulson et al. 2007). Therefore, to understand whether correlations between forager and flower densities (or lack thereof) indicate that bees are (or are not) limited by floral resources, studies should also measure bee reproduction and population density in response to natural or experimental variation in floral resource availability (Goulson et al. 2002, Pelletier and McNeil 2003, Thomson 2004, Greenleaf 2005, Carvell et al. 2008).

Bumble bee foragers in this study were recaptured over shorter distances than

bumble bees in lower elevation areas. At lower elevations, direct forager observations and inferences based on correlations at different spatial scales suggest that bumble bees typically use floral resources that are located over 1,000 m from their nests (Saville et al. 1997, Steffan-Dewenter et al. 2002, Darvill et al. 2004, Knight et al. 2005, Westphal et al. 2006). Assuming constant flower densities in near and far sites, energetic calculations predict that bumble bees should only fly farther than 1,000 m to forage (1) if nectar availability per flower drops below one μL per flower near the nest and (2) if nectar per flower is greater farther away (Cresswell et al. 2000). In agricultural areas in England, marked *B. terrestris* individuals flew farther than 1,000 m to forage although there were flowers nearby (Osborne et al. 1997, Osborne et al. 2008), but no studies have measured per-flower resource availability in relation to foraging distance. In addition to these low elevation studies, in a higher alpine site in northern Sweden, low *B. alpinus* abundance, coupled with a high number of unique individuals captured in a one ha site, suggested that queens flew farther than 100 m to forage (Stenstrom and Bergman 1998). However, in the current study and in another study in a subalpine habitat in Utah, USA, marked bumble bees were primarily recaptured within a 100 m radius (Bowers 1985). Therefore, it is possible that factors specific to subalpine habitats (e.g., elevation, solar radiation, season length, flower species composition, or pollinator composition) may influence floral rewards and bee foraging distances.

In this study, meadows with more flowers did not harbor more workers, gynes, or males. In contrast, bumble bee forager densities at low elevations (< 200 m) increase in areas with more flowers or more available natural habitat (Kremen et al. 2002, Steffan-Dewenter et al. 2002, Westphal et al. 2003, Darvill et al. 2004, Knight et al. 2005, Carvell

et al. 2007, Heard et al. 2007), and I observed more founding queens in meadows with more flowers. Since I recaptured most of these queens within a 100 m radius, if they were parsing themselves out in proportion to resource availability (Dreisig 1995), then more queens may have over-wintered in meadows with more flowers. Alternatively, the queens may have made a small number of long distance flights before dispersing into the meadow in which they would nest and forage. Because founding queens were the only caste that increased with flower densities, floral resource availability might only determine colony establishment and not colony growth. However, in another subalpine study, in the beginning of the season, meadow size and elevation were better predictors of bumble bee presence or absence than flower species composition and abundance (Bowers 1985).

The subalpine environment (e.g., 2879-2957 m elevation in this study), in contrast to lower elevations, may allow less time for colonies to grow large and exhaust local resources. For bumble bee queens to reproduce, they need at least 1-2 weeks to establish a nest, 3-4 weeks to produce the minimum of one worker brood, and another 3-4 weeks to hatch a reproductive cohort (Alford 1975, Heinrich 1979a). In 1974, bumble bee surveys located near this study site found no gynes or males, suggesting that reproduction was low or did not occur in this area (Pyke 1982). In general, Pyke (1982) found that gynes and males were only produced in areas where the flowering season was greater than 11 weeks. In 2006 and 2007 in this study area, over 50% of *B. appositus* colonies did not produce any gynes or males (see Chapter 2). Since colony growth increases over time, flower requirements per colony may also increase across elevational, latitudinal, or other climatic gradients that influence the length of the flowering period (e.g., snow melt and

precipitation patterns, Inouye et al. 2002).

The relationship between flower and forager densities could have been masked if nest parasitism increased in areas with more flowers or if density was limited by factors besides flower availability. Nest parasites are more common in captive *B. terrestris* colonies in areas with rich agricultural floral resources than in colonies in agricultural areas with few floral resources (Carvell et al. 2008). If more nest parasites were present in the meadows with the most flowers and queens at the beginning of the season, then more colonies in those meadows may have been attacked, canceling the positive effect of flower density on bee density. Bee density could have also been limited by nest sites (Alford 1975). If there were more nest sites in the meadows with more flowers, that could also explain the initial correlations between queen and flower densities.

In conclusion, bumble bees in this study had short foraging distances compared with other large-bodied bumble bees (Greenleaf et al. 2007), and forager densities were only correlated with flower densities early in the season. Short foraging distances could suggest that bees had ample floral resources near their nests, or that the costs of long-distance flights outweighed the benefits of increased resource acquisition (Cresswell et al. 2000). Forager densities may not have increased in meadows with more flowers midway and late in the season because: (1) resources did not limit colony growth; or (2) parallel increases in flower density and parasitism rates masked any relationship between flower densities and the densities of workers, males, and gynes. To understand the role of food availability in driving subalpine bumble bee consumer behavior, population growth, and interactions with their mutualistic plant partners, future work should experimentally test whether bumble bees are food-limited and whether and why food limitation varies among

habitats.

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Table 1. Bee foraging measurements and possible explanations for different outcomes.

Possible outcomes and explanations:		Result
<i>Foraging Distance</i>		
<u>Long</u>	<u>Short</u>	<u>Short-distances</u>
<ul style="list-style-type: none"> • Limited flowers: bees must fly far to find enough resources • OR Benefits of foraging far from the nest outweigh energetic costs of long-distance flights. 	<ul style="list-style-type: none"> • Ample flowers: bees find sufficient resources in a small area • OR Costs of long-distance flights outweigh benefits of increased resource acquisition. 	<p>80 ± 4% of recaptured bees were found in the same 0.5-ha plot in which they were marked. No bees were recaptured between 1,000 m and 3,766 m from where they were marked.</p>
<i>Correlation between forager and flower densities</i>		
<u>Positive correlation</u>	<u>Negative or no correlation</u>	<u>Positive correlation with early queens</u>
<ul style="list-style-type: none"> • Limited flowers: colony number and size controlled by flower density. • OR Forager density controlled by another factor that varies with flower density. 	<ul style="list-style-type: none"> • Ample flowers: colony number and size controlled by factors other than flower density. • OR Increases in colony number and size in meadows with many flowers dampened or reversed by parallel increases in predation, parasitism, or some other unmeasured factor. 	<p><u>only</u> Early forager density increased with early flower density, and founding queen density increased with total flower density. No correlations between mid- or late-season forager densities and flower densities.</p>

Table 2. Bumble bee (*Bombus* spp.) and bumble bee social nest parasite (*Psithyrus* spp.) capture rate (number of bees caught per hour, averaged over 11 weeks) range and averages (± 1 SE) for six subalpine meadows in Colorado, USA.

Species	Range	Mean \pm 1 SE
<i>Bombus appositus</i>	11.9 - 47.8	28.2 \pm 5.2
<i>Bombus bifarius</i>	14.7 - 48.0	33.0 \pm 5.6
<i>Bombus californicus</i>	0.2 - 2.6	1.0 \pm 0.4
<i>Bombus flavifrons</i>	10.1 - 49.9	29.4 \pm 7.2
<i>Bombus frigidus</i>	0.9 - 20.7	6.3 \pm 3.0
<i>Bombus nevadensis</i>	0 - 0.3	0.1 \pm 0.1
<i>Bombus occidentalis</i>	2.7 - 18.5	8.6 \pm 2.6
<i>Bombus sylvicola</i>	0 - 0.6	0.2 \pm 0.1
<i>Psithyrus</i> spp.	2.4 - 8.8	6.1 \pm 1.1

Figure Legends

Figure 1. Study area map showing plots used for mark-recapture study and for measuring forager and flower densities. One plot is enlarged to illustrate subplots and quadrats used for flower density sampling. Plots in meadows used for forager and flower density measurements are labeled 1-6.

Figure 2. Mean (± 1 SE) flower density (A. all flower species combined per m^2), forager density (B. bees caught per hour per 0.5-ha plot, separated by caste: queens, workers, and males), and flower-to-bee ratio (C) among six subalpine meadows.

Figure 3. The probability that if a marked bumble bee was recaptured, that it would be recaptured at increasing distances from where it was marked (Figure 1) for the three most common *Bombus* species (A) and all *Bombus* species divided into early-, mid-, and late-season (B). Recapture probabilities at each distance are corrected for sampling effort (see *Methods*).

Figure 4. Scatterplots and correlation coefficients with significance levels showing the relationships between *Bombus* forager density (average number of bees caught per hour per 0.5-ha plot: labeled 1-6, as in Figure 1) and total flower density (flowers per m^2 over the 11-week study) for founding queens (A), workers (B), males (C), and gynes (D) and for the relationships between forager and flower densities (all casts combined) for early- (E), mid- (F), and late- (G) season.

Figure 5. Scatterplots and correlation coefficients with significance levels showing the relationships between mid-season forager densities and early flower and forager densities (A,B) and between late-season forager densities and mid-season forager and flower densities (C,D) in 0.5 ha plots (labeled 1-6 as in Figure 1).

Figure 1.



Figure 2.

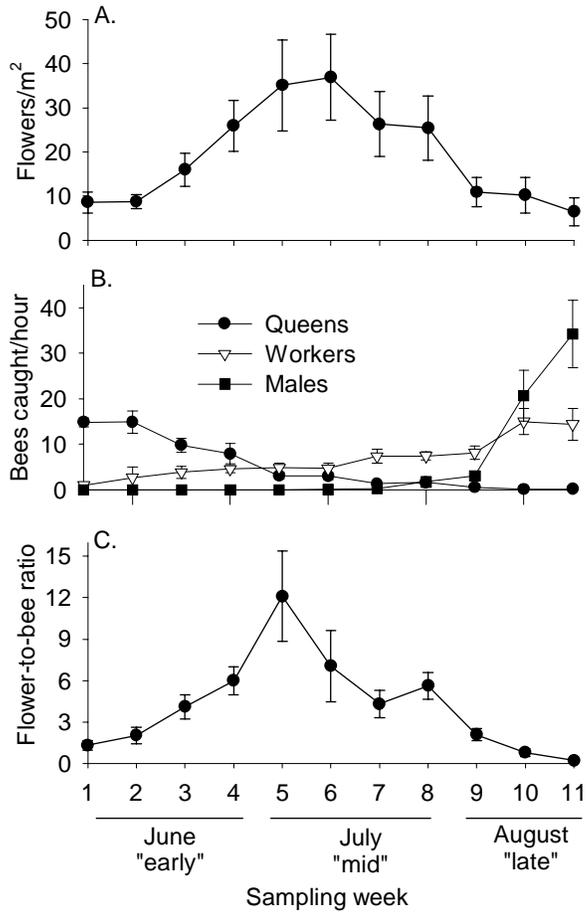


Figure 3.

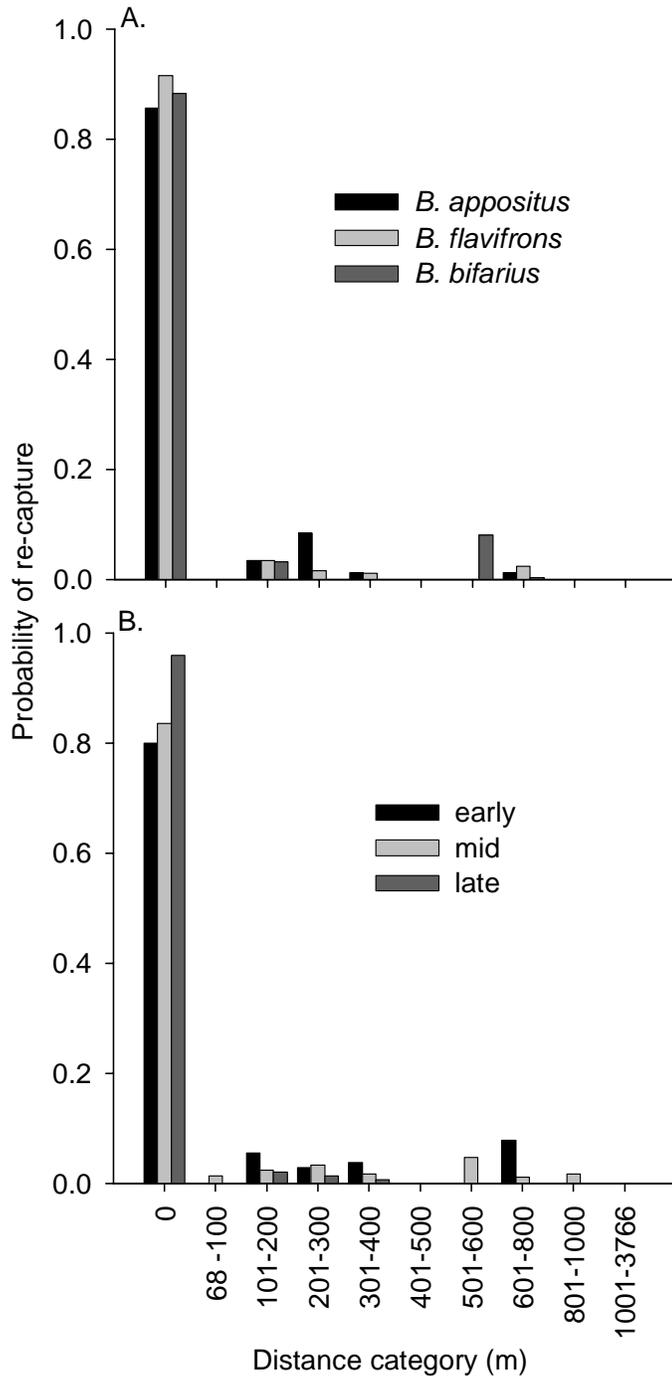


Figure 4.

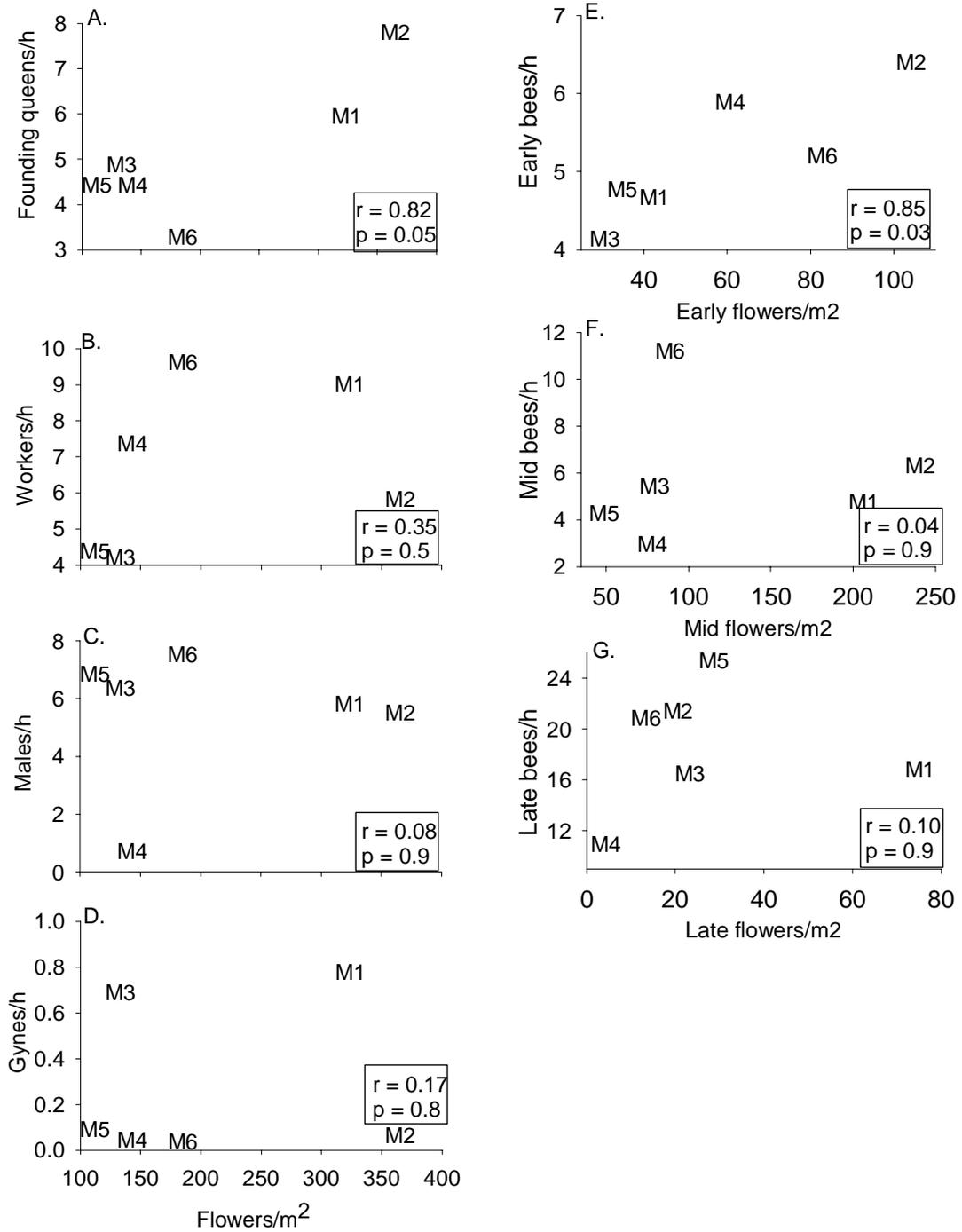
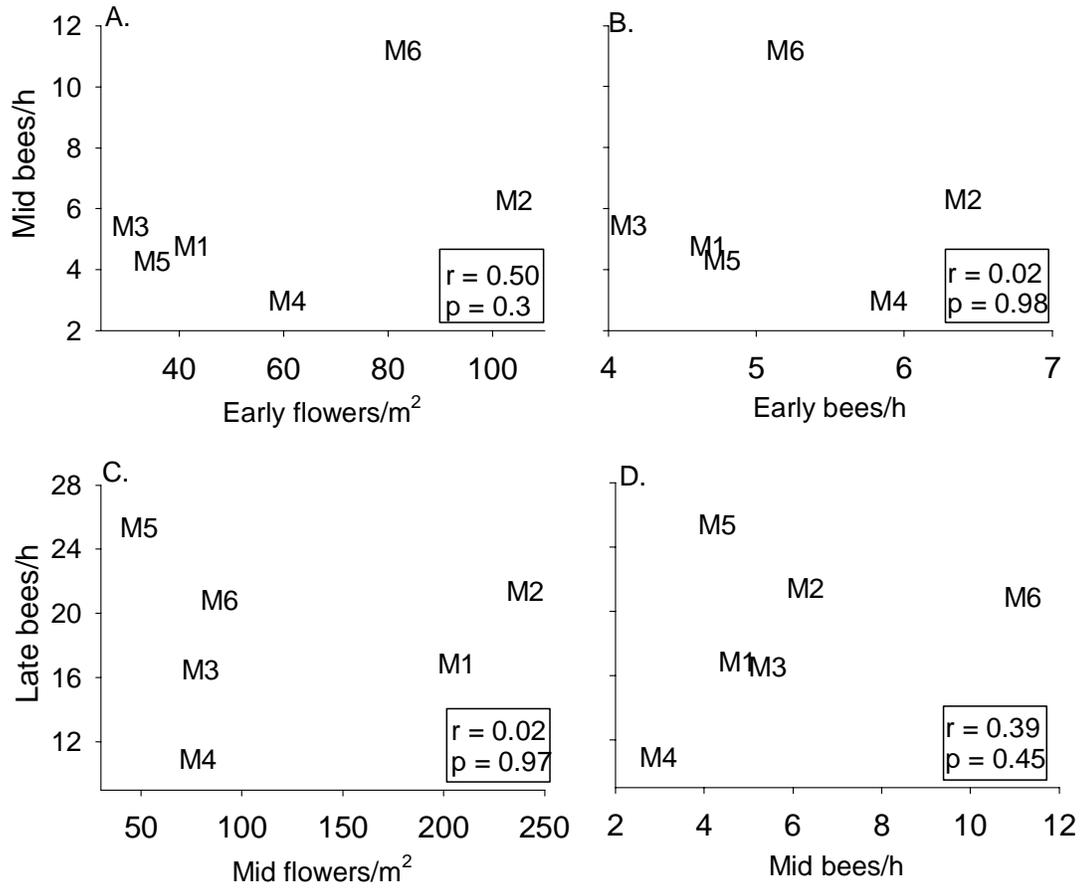


Figure 5.

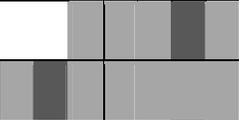
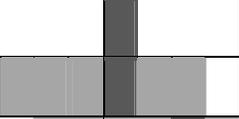
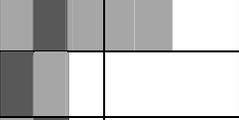
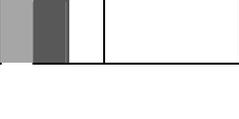


Appendix I. GPS coordinates for the center of study plots used for bumble bee forager-flower observations (ff; M1-6), bee marking (mk), and bee recapturing (rc) (Figure 1).

Meadow Use	GPS coordinates
M1: ff, mk, rc	2°56'8.045"E 38°49'32.593"N
M2: ff, mk, rc	2°56'11.743"E 38°49'21.422"N
M3: ff, mk, rc	2°56'16.742"E 38°49'13.495"N
M4: ff, mk, rc	2°56'33.575"E 38°48'25.544"N
M5: ff, mk, rc	2°56'37.039"E 38°48'10.45"N
M6: ff, mk, rc	2°56'53.293"E 38°47'51.391"N
mk, rc	2°56'4.685"E 38°49'36.911"N
mk, rc	2°56'8.891"E 38°49'27.276"N
rc	2°55'59.784"E 38°49'40.829"N
rc	2°56'1.451"E 38°49'35.723"N
rc	2°56'3.699"E 38°49'31.894"N
rc	2°56'10.137"E 38°49'23.812"N
rc	2°56'13.785"E 38°49'17.877"N
rc	2°56'14.791"E 38°49'14.933"N
rc	2°56'33.048"E 38°48'20.015"N
rc	2°56'35.708"E 38°48'20.239"N
rc	2°56'36.824"E 38°48'13.854"N
rc	2°56'35.477"E 38°48'9.412"N

Appendix II. Average and range of flower densities (flowers per m² summing all weekly averages across the 11-week study) of all bumble bee-visited species blooming in six subalpine meadows (plant names according to Weber 2001). Blooming phenologies indicated with the start dates for each sampling week, denoting whether the species was in bloom (gray) and when its average density peaked (dark gray).

Family	Species	Range	Mean ± 1 SE	June			July				August							
				14	21	28	5	12	19	26	2	9	16	24				
Apiaceae	<i>Heracleum sphondylium</i>	0 - 0.02	0.004 ± 0.004															
Asteraceae	<i>Achillea lanulosa</i>	0.01 - 1.7	0.8 ± 0.3															
Asteraceae	<i>Agoseris glauca</i>	0.01 - 0.3	0.1 ± 0.04															
Asteraceae	<i>Circium eatonii</i>	0 - 0.1	0.03 ± 0.02															
Asteraceae	<i>Erigeron coulteri</i>	0 - 0.3	0.2 ± 0.1															
Asteraceae	<i>Erigeron elatior</i>	0.01 - 5.4	1.3 ± 0.8															
Asteraceae	<i>Erigeron speciosus</i>	0.9 - 19.1	6.6 ± 2.9															
Asteraceae	<i>Helenium hoopsii</i>	0.003 - 0.5	0.2 ± 0.1															
Asteraceae	<i>Helianthella quivinerensis</i>	0 - 0.04	0.02 ± 0.01															
Asteraceae	<i>Heliomeris multiflora</i>	0.1 - 19.7	9.7 ± 3.4															
Asteraceae	<i>Heterotheca villosa</i>	0 - 12.2	2.2 ± 2.0															
Asteraceae	<i>Pyrocoma croacea</i>	0 - 0.04	0.01 ± 0.01															
Asteraceae	<i>Senecio crassulus</i>	0.003 - 2.0	0.5 ± 0.3															
Asteraceae	<i>Senecio integerrimus</i>	0 - 0.2	0.1 ± 0.04															
Asteraceae	<i>Senecio serra</i>	0 - 0.1	0.04 ± 0.02															
Asteraceae	<i>Solidago multiradiata</i>	0 - 2.2	0.4 ± 0.4															
Asteraceae	<i>Taraxacum officinalis</i>	0.01 - 0.6	0.3 ± 0.1															
Boraginaceae	<i>Mertensia ciliata</i>	0 - 1.9	0.5 ± 0.3															
Boraginaceae	<i>Mertensia fusiformis</i>	0 - 0.05	0.01 ± 0.01															
Brassicaceae	<i>Barbarea vulgaris</i>	0 - 2.6	0.5 ± 0.4															

Brassicaceae	<i>Cardamine cordifolia</i>	0 - 1.6	0.3 ± 0.3	
Brassicaceae	<i>Erysimum inconspicuum</i>	0 - 1.7	0.4 ± 0.3	
Campanulaceae	<i>Campanula rotundiflora</i>	0 - 3.6	0.8 ± 0.6	
Caprifoliaceae	<i>Lonicera involucrate</i>	0 - 0.3	0.1 ± 0.05	
Caryophyllaceae	<i>Arenaria congesta</i>	0 - 11.5	2.8 ± 2.0	
Fabaceae	<i>Lathyrus lecanthemum</i>	0.0 - 7.6	1.9 ± 1.2	
Fabaceae	<i>Trifolium fragiferum</i>	0 - 0.2	0.03 ± 0.03	
Fabaceae	<i>Vicia Americana</i>	0.2 - 2.9	1.2 ± 0.5	
Gentianaceae	<i>Frasera speciosus</i>	0 - 0.2	0.1 ± 0.04	
Gentianaceae	<i>Pneumomanthe parryii</i>	0 - 0.3	0.1 ± 0.1	
Hydrophyllaceae	<i>Hydrophyllum capitatum</i>	0 - 2.2	0.4 ± 0.4	
Linaceae	<i>Linum lewisii</i>	0.1 - 6.0	2.6 ± 0.8	
Onograceae	<i>Epilobium angustifolium</i>	0 - 0.5	0.1 ± 0.1	
Polemoniaceae	<i>Ipomopsis aggregata</i>	0 - 2.5	1.1 ± 0.5	
Polemoniaceae	<i>Polemonium foliosissimum</i>	0 - 0.03	0.01 ± 0.01	
Polygonaceae	<i>Eriogonum umbellatum</i>	0 - 3.2	0.6 ± 0.5	
Ranunculaceae	<i>Acconitium colombianum</i>	0 - 5.5	1.8 ± 0.9	
Ranunculaceae	<i>Aquilegia caerulea</i>	0 - 0.1	0.01 ± 0.01	
Ranunculaceae	<i>Delphinium barbeyi</i>	0.8 - 26.2	13.8 ± 4.4	
Ranunculaceae	<i>Delphinium nuttalianum</i>	0.3 - 7.4	2.9 ± 1.1	
Rosaceae	<i>Geum triflora</i>	0 - 0.5	0.1 ± 0.1	
Rosaceae	<i>Pentaphylloides floribunda</i>	0 - 14.7	3.0 ± 2.3	
Rosaceae	<i>Potentilla pulcherrima</i>	11.5 - 65.4	41.2 ± 8.7	
Scrophulariaceae	<i>Castilleja sulfura</i>	0 - 5.7	1.0 ± 0.9	
Scrophulariaceae	<i>Pedicularis bracteosa</i>	0 - 0.3	0.1 ± 0.05	
Violaceae	<i>Viola adunca</i>	0 - 0.2	0.04 ± 0.04	
Violaceae	<i>Viola nuttallii</i>	0 - 0.01	0.002 ± 0.002	

Appendix III. Percent of bumble bee visits to all flower species in six 0.5-ha plots, sorted by the percent contribution that each flower species made to total flower density (of the 40 plant species that received visits in the six density meadows; Figure 1) for the entire 11-week study. Visits to each flower species are separated by caste (founding queen, worker, or male) and into five species categories: All (all *Bombus* and *Psithyrus* spp.), Ap (*B. appositus*), Fl (*B. flavifrons*), Bi (*B. bifarius*), and Ot (all remaining bumble bee species, see Table 2), with the top two most-frequently visited flower species for each caste-species combination bolded.

Flower species with % abundance (mean \pm 1 SE)	Cast:	Founding queens					Workers					Males				
	Species: N bees:	All	Ap	Fl	Bi	Ot	All	Ap	Fl	Bi	Ot	All	Ap	Fl	Bi	Ot
<i>Potentilla pulcherrima</i>	41.8 \pm 8.8	3.9	4.9	0.9	8.7	27.9	12.1	3.6	2.6	15.5	22.5	0	0	0	0	0
<i>Delphinium barbeyi</i>	14.0 \pm 4.4	18.4	37.3	14.9	3.7	17.2	11.7	30.8	17.5	0.7	6.3	0.2	0.4	0	0	0.9
<i>Heliomerus multiradiata</i>	9.8 \pm 3.5	0	0	0	0	0	35.7	17.2	47.0	50.9	19.6	94.5	85.9	95.7	98.0	97.8
<i>Erigeron speciosus</i>	6.7 \pm 2.9	0.2	0	0	1.9	0	0.8	0	0.6	1.8	0	0.1	0.3	0	0	0
<i>Pentaphylloides floribunda</i>	3.1 \pm 2.4	0.2	0	0.2	0	3.6	3.9	0	1.8	5.3	7.2	0.1	0.5	0	0	0
<i>Arenaria congesta</i>	2.9 \pm 2.0	0.2	0	0	2.3	0	0.5	0	0	1.1	0.3	0	0	0	0	0
<i>Delphinium nuttalianum</i>	2.9 \pm 1.1	45.4	50.4	54.1	8.0	12.5	0.1	0.6	0	0	0	0	0	0	0	0
<i>Linum lewisii</i>	2.6 \pm 0.8	0.6	0	0.8	0	3.9	0.2	0	0.4	0.3	0	0	0	0	0	0
<i>Heterotheca villosa</i>	2.3 \pm 2.1	0	0	0	0	0	0.6	0	0	1.2	0.3	2.2	7.6	0.4	0.7	0
<i>Lathyrus lecanthemum</i>	1.9 \pm 1.2	3.1	1.9	3.8	3.1	3.6	0.3	0	0.4	0.2	1.1	0	0	0	0	0
<i>Acconitium colombianum</i>	1.9 \pm 1.0	3.3	1.9	4.8	0	0	5.4	4.2	19.9	2.8	0.9	0.2	0	0.8	0	0
<i>Erigeron elatior</i>	1.4 \pm 0.9	0	0	0	0	0	0.1	0	0	0.2	0	0.1	0	0	0	0.7
<i>Vicia Americana</i>	1.2 \pm 0.5	1.5	1.7	1.6	0	5.8	0.3	0.3	1.1	0	0.4	0	0	0	0	0
<i>Castilleja sulfura</i>	1.1 \pm 1.0	0.7	0	1.0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Achillea lanulosa</i>	0.9 \pm 0.3	0.2	0	0	0	5.8	0.1	0	0	0	0.5	0	0	0	0	0
<i>Campanula rotundiflora</i>	0.8 \pm 0.6	0	0	0	0	0	0.4	0	0	0.9	0	0	0	0	0	0

<i>Eriogonum umbellatum</i>	0.6 ± 0.5	0.2	0	0	1.8	0	0.5	0	0	0.8	0.9	0	0	0	0	
<i>Senecio crassulus</i>	0.6 ± 0.3	0	0	0	0	0	2.0	0.3	1.1	3.7	1.4	0.4	0.7	0.8	0.2	0
<i>Mertensia ciliata</i>	0.5 ± 0.3	2.8	0	4.7	0	0	0.1	0	0.9	0	0	0	0	0	0	0
<i>Erysimum inconspicuum</i>	0.4 ± 0.3	0.1	0.4	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Solidago multiradiata</i>	0.4 ± 0.4	0.2	0	0.3	0	0	1.5	0	0	3.0	1.6	0.4	1.5	0	0	0
<i>Hydrophyllum capitatum</i>	0.4 ± 0.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Cardamine cordifolia</i>	0.3 ± 0.3	0	0	0	0	0	0.1	0	0	0	0.3	0	0	0	0	0
<i>Taraxacum officinalis</i>	0.3 ± 0.1	2.6	0.5	0.4	18.6	3.6	0.5	0	0	0.6	1.2	0	0	0	0	0
<i>Helenium hoopsii</i>	0.2 ± 0.1	0	0	0	0	0	0.3	0	0	0.2	0.8	0	0	0	0	0
<i>Erigeron coulteri</i>	0.2 ± 0.1	0	0	0	0	0	0.1	0	0.5	0	0	0	0	0	0	0
<i>Geum triflora</i>	0.1 ± 0.1	0.2	0	0.2	1.2	0	0.1	0	0	0.1	0.4	0	0	0	0	0
<i>Senecio integerrimus</i>	0.1 ± 0.4	0.5	0	0	2.5	8.8	0.8	0	0.4	2.0	0	0	0	0	0	0
<i>Agoseris glauca</i>	0.1 ± 0.4	0	0	0	0	0	0.1	0	0	0	0.3	0	0	0	0	0
<i>Epilobium angustifolium</i>	0.1 ± 0.1	0	0	0	0	0	0.1	0	0	0.2	0	0.5	0.8	0.8	0.2	0
<i>Pneumomanthe parryii</i>	0.1 ± 0.1	0	0	0	0	0	11.0	41.8	2.4	1.2	0.3	0.6	0	0.8	0.9	0
<i>Lonicera involucrate</i>	0.1 ± 0.5	6.7	0	9.5	9.3	0	0.2	0	0.5	0.2	0.3	0	0	0	0	0
<i>Pedicularis bracteosa</i>	0.1 ± 0.5	0.3	0	0.5	0	0	0.1	0	0	0	0.3	0	0	0	0	0
<i>Frasera speciosus</i>	0.1 ± 0.4	8.2	1.0	2.1	37.9	3.9	8.0	0	1.1	4.3	29.8	0.1	0	0	0	0.7
<i>Senecio serra</i>	< 0.1	0.1	0	0	1.0	0	0.1	0	0	0	0.3	0	0	0	0	0
<i>Trifolium fragiferum</i>	< 0.1	0	0	0	0	0	0.4	0	0.4	0.9	0	0	0	0	0	0
<i>Circium eatonii</i>	< 0.1	0.1	0	0	0	3.6	1.3	0.9	0.8	1.1	2.6	0.2	0	0.7	0	0
<i>Helianthella quivinerensis</i>	< 0.1	0	0	0	0	0	0.1	0	0	0.2	0	0.1	0.4	0	0	0
<i>Aquilegia caerulea</i>	< 0.1	0.1	0	0.2	0	0	0.1	0	0	0	0.5	0	0	0	0	0
<i>Pyrocoma croacea</i>	< 0.1	0	0	0	0	0	0.4	0.3	0.5	0.6	0	0.5	1.9	0	0	0

CHAPTER II

Food limitation in the subalpine bumble bee, *Bombus appositus*

ABSTRACT

Mutualisms may cause coupled population expansion or decline if both partners respond to variation in the other's abundance. Many studies have shown how the abundance of animal mutualists affects plant reproduction, but less is known about how the abundance of plant mutualists affects animal reproduction. Over two years, I compared reproduction of the bumble bee, *Bombus appositus*, across meadows that varied naturally in flower density, and I compared reproduction between fed colonies and unfed control colonies. Colony reproduction (gyne, worker, and male production) was constant across meadows that varied naturally in flower density. Forager densities per flower did not vary among meadows, and daily nectar depletion was consistently low across meadows, suggesting that bees had ample nectar in all meadows. Yet, colonies directly fed with supplemental nectar and pollen generally produced over twice as many gynes as control colonies. Feeding did not affect male or worker production. Although colonies may benefit from food supplementation at the nest, I hypothesize that they may not benefit from additional flowers because they have too few workers to collect extra resources.

INTRODUCTION

Mutualisms are often considered from a unilateral perspective, emphasizing the more sessile or larger partner, whose fitness is often easier to measure (e.g., the plant in a plant-pollinator mutualism: Cushman and Beattie 1991). Knowing how a visitor affects a host mutualist can help explain the ecology and evolution of the host, but if the visitor responds to changes in its partner species (e.g., by an increase or decrease in abundance), this could alter the nature (and our interpretation) of their relationship (Bronstein 1994b,

Thompson 2005). For example, little is known about how the fitness or population growth of mutualistic mycorrhizal fungi, nitrogen-fixing microbes, and photosynthetic zooxanthellae vary with the frequency of their mutualistic partners (Bever 1999, Simms and Taylor 2002, Hay et al. 2004). In plant-pollinator mutualisms, flowering plant reproduction often varies with the number and diversity of pollinators (Pellmyr and Thompson 1996, Gomez et al. 2007, Sahli and Conner 2007). However, the sensitivity of pollinator reproduction to changes in floral resources or other environmental variables is poorly understood, especially in North America (National Resource Council 2006).

The outcomes of mutualisms are often highly context-dependent (Bronstein 1994a). The benefits of mutualistic associations can vary with partner quality, resource availability, the presence or absence of predators, competition for access to a mutualistic partner, and the costs of partner exploitation (Johnson et al. 1997, Herrera 2000, Morales 2000, Egger and Hibbett 2004, Ness et al. 2004, Rudgers and Strauss 2004). In plant-pollinator mutualisms, unless the pollinator also acts as a seed predator, there is likely little to no cost of extra pollinator visits for seed production (but see Young and Young 1992). Similarly, there is probably no cost to bees for living in areas where there are surplus flowers unless bee parasites are positively correlated with flower density (Carvell et al. 2008). The benefits of pollinator abundance to female plant reproduction and changes in population growth (through effects on seed production) may saturate when abiotic resources become limiting for seed production (Burd 1994, Ashman et al. 2004). Plants may compensate for pollinator shortages if they can self-pollinate without inbreeding costs, reproduce asexually, or endure unfavorable periods via longevity or dormancy by living longer or having seed banks (Pake and Venable 1996, Morgan et al.

2005). In contrast, most insect pollinators are short-lived organisms, so they must reproduce even when resources are scarce. With very few exceptions, bee pollinators cannot reproduce without harvesting pollen and nectar to feed themselves and their offspring (Michener 2007). But is pollinator per-capita reproduction food-limited?

The length of the bee life-cycle, relative to flower phenologies, influences the contribution of different flower species to the bee diet (Minkley and Roulston 2006). For example, bumble bees (*Bombus* spp.) must visit a diversity of flower species because their colonies are typically active longer than the time any one flower species is in bloom (Heinrich 1979, Carvell et al. 2007). However, although bumble bees visit a variety of flowers, they may be functionally specialized if they derive most of their resources from the most rewarding flower species (Dramstad and Fry 1995, Stenstrom and Bergman 1998, Goulson and Darvill 2004).

Positive correlations between bee and flower densities could indicate that bees have higher reproduction, recruitment, or survival in areas with more flowers (Steffan-Dewenter et al. 2002, Westphal et al. 2006), but these relationships could also be influenced by other limiting factors such as parasitism and nest site availability. Recent studies from low elevation areas (< 200 m) confirm that bee reproduction of solitary and colonial bee species is greater in areas with higher natural or experimental levels of floral resources (Goulson et al. 2002, Pelletier and McNeil 2003, Greenleaf 2005, Williams and Kremen 2007, Carvell et al. 2008). However, since bees at high elevations have shorter growing seasons and consequently less time to establish nests, collect resources, and produce offspring, their reproduction may instead be time-limited (Pyke 1982). To determine whether subalpine bumble bees have surplus flowers for colony reproduction, I

measured bumble bee reproduction across meadows that varied naturally in flower density and in response to supplemental feeding at the nest. Time limitation of bumble bee reproduction was not addressed experimentally but is discussed within the context of the experimental findings of this study.

The focal bumble bee in this study may visit at least 20 flower species throughout the season in the study area (Pyke 1982, see Chapter 1), but it collects much of its pollen and nectar from the abundant perennial wildflower, *Delphinium barbeyi* (Ranunculaceae). *Delphinium barbeyi*, the second most abundant flower species in these meadows, is in bloom in the middle of season while *B. appositus* colonies are provisioning and hatching workers, gynes, and males (see Chapter 1). During this time, *D. barbeyi* accounts for the majority of pollen collection and flower visitation by *B. appositus*. To test how floral resources affect *Bombus appositus* reproduction (i.e., worker, gyne, and male offspring production), I asked the following questions:

1. *Does colony reproduction vary across meadows that vary naturally in flower density?* If bee colony density is independent of flower density and if colony reproduction is food-limited, then colonies in meadows with more flowers should produce more offspring, assuming that flower number adequately represents pollen and nectar availability (Tepedino and Stanton 1982). Alternatively, if meadows with more flowers have disproportionately more colonies, flower availability per colony (and reproduction per colony if reproduction is flower-limited) might be lower in meadows with more total flowers. If flower availability per colony is greater in some meadows, then flowers in those meadows should have lower per-flower pollinator visitation rates,

and nectar in those meadows should be depleted less quickly than in meadows with more flowers per colony.

2. *Is bee colony reproduction food-limited?* If bee reproduction is food-limited, then colonies that are fed at the nest with supplemental nectar and pollen should have higher reproduction than unfed control colonies. Also, fed colonies may reduce foraging to avoid costs associated with foraging outside the colony (Dukas and Morse 2003) or to benefit the colony by devoting more time to brood care or colony defense (Cartar 1992).

METHODS

Study System

In 2006 and 2007, I studied reproduction in the long-tongued bumble bee, *Bombus appositus* (Apidae), in subalpine meadows in the East River Valley near the Rocky Mountain Biological Laboratory in Gothic, Colorado, USA (2952-2969 m elevation, Figure 1). *Bombus appositus* is one of the three most abundant bumble bee species in this study area (Pyke 1982, see Chapter 1). Inseminated bumble bee queens emerge from hibernation in early spring (late May to early June) and establish new nests, where they incubate their first brood. About six weeks later, the first worker cohort hatches, and forager density increases sharply. During this increase in bee abundance, the common perennial wildflower, *Delphinium barbeyi* (Ranunculaceae), accounts for 91.2% of the pollen collected by *B. appositus* and 94.6% of the floral visits made by *B. appositus* (median contributions, Appendix I).

In the study area, *D. barbeyi* occurs in meadows and forest clearings in distinct patches, and *D. barbeyi* flower density varies over three orders of magnitude among 0.5

ha meadow plots (0.8 to 26.2 flowers produced per m², see Chapter 1). With 13.6 ± 0.5 *D. barbeyi* inflorescences per plant (mean \pm 1 SE, n = 420 plants), each bearing 25.4 ± 0.8 flowers per inflorescence (n = 372 inflorescences, S. E. Elliott, *unpublished data*), *D. barbeyi* accounts for $14.0 \pm 4\%$ of all flowers produced (mean \pm 1 SE, n = 6 meadows, see Chapter 1). On average, flowers contain 1.8 ± 0.05 μ L of nectar per flower in the morning (before pollinator visits begin, n = 512 flowers) with $36.1 \pm 0.7\%$ sugar concentration (n = 34 flowers, S. E. Elliott, *unpublished data*). Flowers are protandrous, with anthers dehiscing over a period of about three days (S. E. Elliott, *personal observation*).

Field Methods

1. Does colony reproduction vary across meadows that vary naturally in flower density?

To determine whether bees in meadows with more flowers produce more offspring, I compared *B. appositus* colony reproduction in three meadows that varied naturally in *D. barbeyi* density. The meadows were separated by ~ 400 m (Figure 1). Although large-bodied bees like *B. appositus* are physiologically able to fly up to 9.8 km (Goulson and Stout 2001), in this study system, marked bees, if recaptured, have an 82% chance of being found within a 100 m radius of their original marking location (see Chapter 1). Therefore, I assumed that bees did not fly among focal meadows.

Flower density – In 2007, to measure the degree to which *D. barbeyi* flower density varied among study meadows, I counted the number of *D. barbeyi* flowering inflorescences in a 200 m radius around each shelter that housed captive bee colonies (one shelter per focal meadow, described below). I estimated *D. barbeyi* density by

counting the number of flowering inflorescences within a 200 m radius around each shelter (Figure 1). Flower number per inflorescence did not vary with inflorescence density ($r = 0.09$, $P = 0.8$, $n = 10$ meadows, 60 inflorescences averaged per meadow). This analysis and all subsequent analyses were performed with JMP v. 4.04 (SAS Institute, 2001).

Although *D. barbeyi* density in these subalpine meadows is correlated with total flower density, the most abundant flower species, *Potentilla pulcherrima* (Rosaceae), which is frequented by shorter-tongued bumble bees, only contributed to 2.6% of all *B. appositus* visits and therefore probably does not contribute strongly to colony reproduction (see Chapter 1). In 2007, to determine whether colonies in meadows with fewer *D. barbeyi* flowers switched to foraging on *P. pulcherrima*, I compared corbicular pollen loads carried by *B. appositus* in each meadow. *Potentilla pulcherrima* pollen is bright orange and easily distinguished from *D. barbeyi*'s cream-colored pollen. I used a one-way ANOVA to compare the proportions of cream and orange pollen loads carried by foragers returning to the colonies (described below) among the three study meadows, using colony as the unit of replication.

Bumble bee colonies – In 2006 and 2007, I monitored captive *B. appositus* colony reproduction in the three focal meadows. To obtain colonies, I set out ~100 wooden nest boxes in the study meadows, providing each box with cotton for nest insulation. Each year, I had ~10% success rate in attracting emerging *B. appositus* queens from the study meadows to the nest boxes. I also started colonies in the laboratory using nest-searching queens collected from the study meadows (2006: three colonies, one per meadow, 2007: one colony, used in one meadow only). As soon as field or laboratory colonies laid their

first brood, I distributed them evenly among the shelters (one fenced and covered shelter per meadow to reduce damage from porcupine, bear, and rain), where they stayed for the duration of the experiment. Therefore, this study tests the effects of flower density (and supplemental feeding, see below) on colony growth and reproduction (as in Goulson et al. 2002, Pelletier and McNeil 2003, Thomson 2004, Greenleaf 2005) and not on colony establishment. The queens in the captive colonies fed their offspring (including the developing larvae from the first cohort) with nectar and pollen obtained in the study meadows. The study colonies contributed to a small proportion of ambient bee density (Appendix II). The shelters were fenced enclosures that reduced, but did not completely eliminate, porcupine and bear disturbances. After excluding porcupine- and bear-disturbed colonies, I was left with seven (2006) and eight (2007) control colonies (i.e., two to three colonies per meadow each year). Once per week at night, I counted the number of individuals present in each colony (usually a founding queen, mostly workers, and at the end of the season, gynes and occasionally males).

Colony reproduction – After colonies were abandoned at the end of the season, I quantified three aspects of offspring production per colony: number of gyne cocoons, number of worker cocoons, and number of males. I counted all cocoons produced (which remain intact after individuals hatch), and I measured the diameter of each cocoon to the nearest 0.1 mm. Cocoons that had housed gynes were roughly three times larger than worker or male cocoons, as with most *Bombus* species that feed offspring individually (Goulson 2003). Gyne cocoons ranged from 9.6 - 18.8 mm in diameter and non-queen cocoons ranged from 5.6 - 9.9 mm in diameter. For intermediate sizes, I assigned the caste based on the cocoon size, relative to the other cocoons in the colony. I measured

gyne cocoon diameter as a proxy for gyne body size, which can affect diapause survival (Beekman et al. 1998). I quantified male production as the sum of all males seen in the colonies during weekly night censuses. This is a conservative estimate of male production since males leave the colonies two to three days after hatching, and I only visited colonies once per week. I estimated worker production as the number of small (non-gyne) cocoons minus the number of males observed during night censuses. Therefore, worker production is slightly inflated because I probably missed seeing some males if they hatched and dispersed between censuses. I used MANOVAs (one for each year) to test whether the number of gynes, workers, and males per colony varied among meadows. I did not use flower density as a covariate because by using meadow as a categorical predictor variable, I could detect differences among meadows that may not vary linearly with flower density. I used a MANOVA because reproduction among castes may be correlated (Pelletier and McNeil 2003). Given a marginally significant effect in one year, I then used separate one-way ANOVAs to test how the number of gynes, workers, and males per colony varied among meadows. I could not test for meadow effects on average gyne cocoon diameter because some meadows had only one colony that produced gynes. I first separated analyses by year and then combined years to assess overall effects.

Foraging behavior and nectar availability – In 2007, to determine if foragers were distributed in proportion to flower availability, I calculated flower visitation rates in ~ 20 m² patches (12 15-minute observation bouts between 0900 and 1500, spread over three weeks in the middle of *D. barbeyi*'s blooming period). I recorded visitation rates in the three focal meadows plus nine adjacent meadows. However, because I never saw

marked (captive colony) bees in the surrounding meadows, I only report visitation rates in the three focal meadows. A sample size of 12 observation bouts per meadow provides sufficient power to detect among-meadow variation in visitation rates (Appendix III). Before each 15-minute observation period, I recorded the number of open inflorescences I was observing. I also counted the number of open flowers per inflorescence from 20 inflorescences per meadow to calculate per-flower pollinator visitation rates. I recorded visits by all bee and bird species, although *B. appositus* made up the majority of all flower visits (Appendix IV). For each visitor, I recorded the species and the number of flowers they visited. I used a one-way ANOVA to test whether *B. appositus* per-flower visitation rates (i.e., percent of open flowers visited by *B. appositus* per minute) varied among the three study meadows, using observation period as the unit of replication.

In 2007, to determine whether per-flower nectar availability varied among meadows, I compared pollinator visitation rates per flower (all pollinator species) and daily nectar depletion per flower across the three meadows. I collected flowers to measure nectar availability and depletion on three days (5, 18, and 25 July) in the middle of *D. barbeyi*'s blooming period. Each day, I collected flowers from different plants spread throughout each meadow in the mornings (before 0800 when *D. barbeyi* pollinators become active) and in the late afternoons (after 1600 when most foraging on *D. barbeyi* has slowed or stopped). I collected 20 flowers per meadow at each time period on each day, but I excluded flowers that were damaged so total sample size per meadow varied from 32-48 morning flowers and 24-38 afternoon flowers per meadow (total n = 217 flowers). I picked flowers from the middle of inflorescences to standardize flower age. I kept the flowers in a cooler and extracted the nectar (from the two nectar spurs per

flower) within 24 hours, using 2- μ L capillary tubes. Nectar re-absorption in unvisited flowers in *D. barbeyi* has not been reported in published studies. To determine if meadows varied in nectar availability and if nectar availability dropped over the course of a day, I used a 2-way ANOVA to test the effects of meadow and time period (morning versus afternoon) on nectar volume per flower. I also tested whether nectar depletion varied among meadows (meadow x time period interaction), using flower as the unit of replication.

2. *Is bee colony reproduction food-limited?* In 2006 and 2007, to test if colonies were food-limited, I fed two colonies (one from each of the first two meadows) and compared their reproduction and activity levels with the control (unfed) colonies (described above). I could not feed multiple colonies per meadow because (1) sample size was limited; (2) I wanted to maximize control colony sample size to establish baseline variation in reproduction across meadows; and (3) I did not want to inflate forager number (and resource depletion) by feeding multiple colonies. I also fed one colony in the third meadow each year, but they were destroyed by porcupine and bear (2006 and 2007, respectively) early in their development, and therefore excluded from analyses. Each week, I fed the colonies with 50 mL of sugar-water (50:50 sucrose and water, with one mL of honey mixed in) with two plastic pipette feeders. The colonies typically drained the feeders in three days. I added Colorado wildflower honey (Ambrosia Honey Co., Parachute, CO) so that natural non-sugar constituents would also be present (Baker and Baker 1982). The 50 mL nectar volume is comparable to the total amount of nectar held within one third of all of the *D. barbeyi* flowers in an average meadow (given an average of 3,856 inflorescences per meadow x 25 flowers per inflorescence x 1.8 μ L of

nectar per flower). In 2007, to determine if colonies were limited by both nectar and pollen, I also gave the two fed colonies supplemental pollen. Each week, I gave the colonies ~ 500 mg of honey bee-collected pollen moistened with artificial nectar (Kearns and Thomson 2001), which is slightly more pollen than all of the workers in a colony would bring in on an average day (19 ± 5 corbicular pollen loads per day, S. E. Elliott, *unpublished data*; 21 mg pollen per load, Heinrich 1979). I used pollen collected from honey bees that were foraging in similar subalpine habitats in Colorado.

I compared four metrics of offspring production between fed and control colonies: number of gyne cocoons, worker cocoons, and males censused, and gyne cocoon diameter (described above). First, I used MANOVAs (one for each year) to test whether the number of gynes, workers, and males per colony varied among meadows. Given significant differences in both years, I then tested the effect of feeding on these four variables with separate two-tailed t-tests for each year and for both years combined. For males and gynes, I used pooled estimates of variance for the two treatments (Levene's test of the null hypothesis that equal variance are equal were all non-significant at $\alpha = 0.05$), but for worker production, I could not assume equal variances, so I did not pool variances.

To determine whether individuals in fed colonies foraged more frequently (e.g., because of increased energy to fuel foraging efforts) or less frequently (e.g., to reduce foraging costs) than unfed control colonies, I compared foraging activity of fed and control colonies. In 2006, I recorded foraging activity for 30 minutes per colony on one day toward the end *D. barbeyi*'s blooming period. In 2007, I increased observation time to 12 30-minute observation periods per colony, spread over two weeks at peak *D.*

barbeyi bloom. For each colony, I divided the total number of entries into and exits out of the colony by the number of individuals and by the 30 minutes of observation time to calculate an activity rate per individual per minute. I used two-tailed t-tests to compare activity rates between fed and control colonies, with colony as the unit of replication, analyzing each year separately.

RESULTS

1. Does colony reproduction vary across meadows that vary naturally in flower density?

Despite five-fold variation in flower abundance (Figure 1), bee reproduction was relatively constant among meadows (MANOVAs: 2006, $F_{6,4} = 0.4$, $P = 0.9$; 2007, $F_{6,4} = 5.7$, $P = 0.056$; Figure 3, Table 1). During both years, control colonies produced 0-12 gynes (median = 0 gynes per colony), and average gyne production did not vary among meadows (2006: $F_{2,4} = 0.4$, $P = 0.7$; 2007: $F_{2,5} = 0.5$, $P = 0.6$; both years: $F_{2,12} = 0.5$, $P = 0.6$; Figure 3A, Table 1). Colonies produced 0-27 workers (median = 11 workers per colony), and average worker production did not vary across meadows for both years combined ($F_{2,12} = 0.5$, $P = 0.6$; Figure 3B, Table 1) and in 2006 alone ($F_{2,4} = 0.1$, $P = 0.9$; Figure 3B, Table 1). However, in 2007, colonies in the meadow with intermediate-high flower density (Figure 1) produced marginally more workers than colonies in the other two meadows ($F_{2,5} = 6.1$, $P = 0.06$; Figure 3B, Table 1). Males were seen in five out of 16 control colonies during night censuses from the two years, with a maximum of five males observed per colony (median = 0 males per colony). In 2006, very few males were seen in any of the meadows, but in 2007, males were seen more frequently in the meadow with

intermediate-high flower density (2006: $F_{2,4} = 0.1$, $P = 0.9$; 2007: $F_{2,5} = 7.0$, $P = 0.04$; both years: $F_{2,12} = 2.7$, $P = 0.1$; Figure 3C, Table 1).

In 2007, *B. appositus* flower visitation rate (i.e., percent of *D. barbeyi* flowers visited by *B. appositus* per minute) did not vary among meadows ($F_{2,33} = 1.1$, $P = 0.4$; Figure 4). Similarly, flower visitation rate of all visitor species combined (Appendix IV) did not vary among meadows ($F_{2,33} = 0.9$, $P = 0.4$; Figure 4). Although nectar volume per flower was highest in the meadow with the fewest *D. barbeyi* flowers ($F_{2,211} = 3.9$, $P = 0.2$; Figure 4), nectar depletion did not vary among meadows (meadow x time period: $F_{2,211} = 1.7$, $P = 0.2$; Figure 4). Nectar decreased by 28.4% from morning to late afternoon (time effect: $F_{1,211} = 14.5$, $P = 0.0002$; Figure 4).

The proportion of cream-colored pollen loads brought back by foragers, indicating *D. barbeyi* pollen use instead of orange *P. pulcherrima* pollen, varied among meadows ($F_{2,6} = 13.3$, $P = 0.006$). The lowest *D. barbeyi* use was in the meadow with the most *D. barbeyi* flowers, which was also the meadow with intermediate *P. pulcherrima* density in 2004 (Mean \pm 1 SE percent cream pollen loads per colony: Meadow 1 = $42.7 \pm 7.5\%$, Meadow 2 = $85.6 \pm 6.5\%$, Meadow 3 = $96.4 \pm 9.2\%$).

2. *Is bee colony reproduction food-limited?* In individual years, there were some significant differences in reproduction between control and fed colonies (MANOVA 2006 $F_{3,5} = 8.0$, $P = 0.02$; MANOVA 2007 $F_{3,5} = 8.5$, $P = 0.02$; Figure 3, Table 1). While all four fed colonies produced gynes, only eight out of the 15 control colonies produced gynes (Figure 3d). In 2006, fed colonies produced five times more gynes than control colonies ($t_7 = 3.0$, $P = 0.02$; Table 1). However, in 2007, feeding did not significantly affect gyne production ($t_9 = 0.3$, $P = 0.8$; Table 1). The non-significant effect of feeding

on gyne production for both years combined was caused by a control colony in 2007 with very high reproduction (with this outlier excluded, $t_{16} = 3.9$, $P = 0.001$; Figure 3D). Feeding did not affect gyne cocoon diameter in either year (2006: control = 10.9 ± 0.5 mm, fed = 11.9 ± 0.6 mm, $t_5 = -1.2$, $P = 0.3$; 2007: control = 11.7 ± 0.2 , fed = 11.5 ± 0.3 mm, $t_4 = 0.4$ $P = 0.7$; both years: control = 11.3 ± 0.3 mm, fed = 11.7 ± 0.4 mm, $t_9 = 0.9$, $P = 0.4$). Worker production also did not vary between control and fed colonies in either year (2006: $t_{6,9} = 0.4$, $P = 0.7$; 2007: $t_{7,9} = 1.8$, $P = 0.1$; Figure 3E, Table 1). Males were found in the colonies at night in all four fed colonies, yet they were only observed in one third of the control colonies (Figure 3F). However, there were no statistically significant differences in the number of males censused between control and fed colonies in either year (2006: $t_7 = 2.0$, $P = 0.08$; 2007: $t_7 = 0.8$ $P = 0.9$; Table 1). With both years combined, there were no statistically significant effects of feeding on gyne, worker, or male production (gynes: $t_{17} = 1.4$, $P = 0.2$; workers: $t_{17,0} = 1.1$, $P = 0.3$; males: $t_{17} = 0.6$, $P = 0.6$; Figure 3D-F; Table 1).

In 2006, individuals in fed colonies made more entries and exits in and out of their nest boxes than individuals in control colonies (mean \pm 1 SE activity per individual: fed = 0.38 ± 0.06 , control = 0.06 ± 0.03 , $t_7 = 4.8$, $P = 0.0021$). However, in 2007, individual activity rates in fed colonies did not differ from control colonies (fed = 0.60 ± 0.36 , control = 0.57 ± 0.18 , $t_8 = 0.08$, $P = 0.9$).

DISCUSSION

In contrast to the wealth of studies that test for pollen limitation of plant reproduction, very few studies have tested for flower limitation of pollinator reproduction (Cushman

and Beattie 1991). Studies from low elevations suggest that bumble bee pollinator reproduction is limited by floral resources (Goulson et al. 2002, Pelletier and McNeil 2003, Greenleaf 2005). However, in subalpine meadows, I found that bumble bee reproduction was marginally *food*-limited (i.e., fed colonies generally produced more gynes), but there was no evidence that reproduction was *flower*-limited (i.e., nectar was not depleted to a large degree and reproduction did not vary across a natural gradient in flower density). One explanation for this pattern may be that unless bumble bee colonies have ample workers, they may not be able to take full advantage of abundant flowers.

Natural flower density may not have affected colony reproduction if colony density increased proportionally with flower density, resulting in a constant ratio of bees to flowers. At the population scale (i.e., changes in population density instead of behavioral responses to flower density), areas with more flowers may harbor more foragers (Steffan-Dewenter et al. 2002, Westphal et al. 2006). However, relationships between forager and flower densities may not hold if pollen and nectar production per flower varies across habitats (Cartar 2004, Goulson et al. 2007). In 2007, I found that forager abundance per *D. barbeyi* flower was constant across meadows, confirming that forager density was proportional to flower density. Also, although recapture rates were low overall, marked bees from the captive colonies contributed to a greater proportion of flower visitors in the meadow with the fewest *D. barbeyi* flowers (Appendix IV). Therefore, there were probably fewer natural bee colonies where there were fewer flowers.

Colony reproduction also may have been constant across meadows if all meadows had similar nectar availability. Because daily *D. barbeyi* nectar depletion was minimal, it

is unlikely that bees exhausted the available nectar in 2007. In 2005, bees depleted *D. barbeyi* nectar from a starting level of $0.68 \pm 0.03 \mu\text{L}$ per flower down to $0.23 \pm 0.04 \mu\text{L}$ per flower over the course of the day (S. E. Elliott, *unpublished data*). Therefore, the average $1.3 \pm 0.1 \mu\text{L}$ of nectar remaining in *D. barbeyi* flowers at the end of the day in 2007 was probably not too deep within the nectar spur for *B. appositus* to reach. The three nectar sampling dates included the period of colony growth up to peak colony size (Figure 1, first three census dates). Later in the season, if less nectar was available, then it would have primarily affected hatching, but not provisioning, of gynes and males. However, fed colonies did not hatch a greater proportion of their gynes than control colonies (95% confidence interval for the mean percent of gynes that did not hatch per colony: control = -13.1%-41.6%, fed = -23.7%-48.7%). Therefore, there was probably ample nectar available for colony growth (i.e., production of new individuals). After the period of colony growth, bee colonies may have needed less nectar.

Food limitation for bee colonies depends on both pollen and nectar availability. In 2006, foragers in fed colonies were six times more active than foragers in control colonies (i.e., more movements in and out of the hive, scaled by colony size). Fed colonies may have used the extra nectar to increase foraging efforts, enabling them to harvest even more resources, to produce more gynes. In contrast, in 2007, colonies that were fed with extra nectar and pollen, instead of nectar only, were not more active than control colonies. If foraging activity increases when colonies need more pollen (Plowright et al. 1993, Rasheed and Harder 1997), then this could also explain why bees in fed colonies in 2007 were not more active than bees in control colonies—i.e., they already had plenty of pollen so they did not need to leave the colony and forage more (as

in Cartar 1992, Weinberg and Plowright 2006). Future studies could compare ambient pollen and nectar availability and the relative pollen and nectar requirements for colony reproduction.

In the feeding experiment, I found conflicting evidence for nectar (2006) or nectar and pollen (2007) limitation of bee reproduction. In 2006, gyne (but not worker or male) production increased with nectar additions. However, in 2007, supplemental nectar and pollen additions did not affect reproduction. The feeding effect size was so low that 80% power to detect a significant effect of feeding on gyne production would require a sample size of 73 colonies, which would require many years of study, given the low success rates of rearing captive colonies (Kearns and Thomson 2001). Small sample sizes *per meadow* are ideal since increasing captive colony number per meadow could inflate natural levels of resource depletion. Therefore, future work should increase sample sizes by studying colonies in more meadows (instead of more colonies per meadow). It is important to note that supplemental food could have had a stronger effect on natural colonies if my captive colonies had been smaller than natural colonies. Small colony sizes (i.e., few workers per colony) may prevent colonies from taking advantage of extra flowers and reduce the overall demand for food. Also, supplemental flowers might have had a larger effect on colony establishment than on colony growth, which occurs when flower availability per bee is lower than during the colony growth phase (see Chapter 1).

The few studies that have tested whether bumble bee colony productivity in the wild is sensitive to food supplementation or natural variation in floral resources suggest that at lower elevations (< 200 m), bumble bees are food-limited (Goulson et al. 2002, Pelletier and McNeil 2003, Thomson 2004, Greenleaf 2005, Carvell et al. 2008). For

example, in the only other feeding experiment with field colonies, fed colonies of *Bombus impatiens* and *B. ternarius* colonies in Quebec, Canada produced more gynes, workers, and males, than unfed control colonies (Pelletier and McNeil 2003). Reduced resource availability in conventional agricultural habitats versus natural habitats or flower rich suburban habitats also reduced colony reproduction of *B. vosnesenskii* and *B. terrestris* in California (Greenleaf 2005) and the UK (Goulson et al. 2002). In the UK, worker production was higher when *B. terrestris* colonies were placed near flower-rich oil-seed rape fields versus flower-poor wheat fields, but rich resources were also associated with higher social parasitism (colonies terminated before measuring gyne production, Carvell et al. 2008). Finally, in California, USA, proximity to honey bee competitors reduced *B. occidentalis* gyne and male production (Thomson 2004). In all of these systems, colonies had longer foraging seasons and grew to be larger than the colonies in this study.

Subalpine bees may be at one end of a food limitation gradient, being less food-limited than other bees. As the length of the growing season increases, colonial bees have more time to build large colonies, and multivoltine solitary bees have more time to produce additional generations (Minckley et al. 1994, Goodwin 1995, Thiele 2005, de la Hoz 2006, Packer et al. 2007). Because the growing season at high elevation is shorter than at low elevation, high elevation bumble bee reproduction may be time-limited (Pyke 1982) instead of food-limited. For example, in this study, colonies produced a maximum of only 21 workers (Figure 2), while the average number of workers alive at any given time in the Quebec study ranged from 30-49 workers per control colony (Pelletier and McNeil 2003). Small colonies may never have enough foragers to collect extra resources

even if they are readily available in nearby flowers. Food supplementation not only provides more total resources, but they are also resources that workers can use while staying in the colony and maintaining colony defense and brood care (Cartar 1992). Therefore, the benefits of food additions for reproduction may not necessarily mirror the benefits of flower addition for bee reproduction.

Recent pollinator shortages have heightened awareness that pollinator declines may be linked to declines in plant populations (Buchmann and Nabham 1996, Kearns et al. 1998). For example, plants that require pollinators for out-crossing and bees have declined in Britain and the Netherlands (Biesmeijer et al. 2006). Because bumble bees visit a diversity of flower species (even if they receive most of their resources from a few abundant species), their removal from plant-pollinator communities could have especially detrimental effects on plant-pollinator assemblages (Memmott et al. 2004). However, this study illustrates that floral resources are not the sole driver of bee reproduction, and that future studies should test food and flower limitations for bee reproduction across a variety of habitats. In areas with surplus flowers for pollinator reproduction, there could also be a higher probability that seed production is limited by pollinator visits (Alonso 2005). This study highlights the need for more studies that examine flower and food limitation of pollinator reproduction in multiple locations and habitats.

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Table 1. Mean (± 1 SE) *Bombus appositus* offspring production (queen cocoons, worker cocoons, and male sightings), compared across three sub-alpine meadows (1-3) and between fed and control (unfed) colonies.

Factor	Year	Meadows or Feeding	N colonies	Queen cocoons	Worker cocoons	Male sightings
Meadows	2006	1	2	1.5 \pm 1.5	18.0 \pm 9.0	0.0 \pm 0.0
		2	2	0.0 \pm 0.0	13.0 \pm 8.0	0.0 \pm 0.0
		3	3	1.3 \pm 1.3	15.0 \pm 3.6	0.7 \pm 0.7
	2007	1	2	0.0 \pm 0.0	5.0 \pm 1.0	0.0 \pm 0.0
		2	3	1.7 \pm 0.7	16.3 \pm 4.4	3.7 \pm 0.9
		3	3	4.0 \pm 4.0	7.3 \pm 7.3	0.7 \pm 0.7
	both years	1	4	0.8 \pm 0.8	11.5 \pm 5.3	0.0 \pm 0.0
		2	5	1.0 \pm 0.5	15.0 \pm 3.6	2.2 \pm 1.0
		3	6	2.7 \pm 2.0	11.2 \pm 4.0	0.7 \pm 0.4
Fed vs. Control	2006	F	2	5.0 \pm 1.0	14.0 \pm 1.0	1.5 \pm 0.5
		C	7	1.0 \pm 0.7	15.3 \pm 3.1	0.3 \pm 0.3
Control	2007	F	2	3.0 \pm 0.0	16.5 \pm 1.5	1.6 \pm 0.7
		C	8	2.1 \pm 1.5	10.1 \pm 3.4	1.5 \pm 0.5
	both years	F	4	4.0 \pm 0.7	15.3 \pm 1.0	1.5 \pm 0.3
		C	15	1.6 \pm 0.8	12.5 \pm 2.3	1.0 \pm 0.4

Figure Legends

Figure 1. Study meadows (A) in the East River Valley, Gunnison National Forest, CO, USA and total number of *Delphinium barbeyi* inflorescences (B) in 200 m radii around each captive colony location (1-3; GPS coordinates = 1: 2°56'8"E 38°49'33"N, 2: 2°56'12"E 38°49'21"N, 3: 2°56'17"E 38°49'13"N).

Figure 2. *Bombus appositus* colony size (number of live hatched individuals) over the season for captive colonies placed in three study meadows in order of decreasing flower abundance (see Figure 1). Open circles represent control colonies and closed circles represent colonies fed with supplemental nectar (2006) and nectar and pollen (2007). Arrows point to disturbances (P = porcupine, U = nest usurpation by U(A) (*B. appositus*) or U(P) (*Psithyrus* sp.), and B = bear damage). The '?' points to a colony that was found after the start of the study so its starting size is unknown. In 2007, the fed colony with bear damage had the same colony size and is thus plotted over a control colony that had no bear damage.

Figure 3. *Bombus appositus* colony reproduction across meadows (unfed control colonies: A-C) and between control and fed treatments (D-F) in terms of queens (A,D), workers (B,E), and males (C,F) produced per colony.

Figure 4. Comparison of pollinator abundance and nectar availability per flower for *Delphinium barbeyi* growing in three subalpine meadows in Gunnison National Forest, CO, USA (in decreasing order of flower abundance, see Figure 1) showing: (A) flower

visitation rate (percent of flowers visited per minute), and (B) ambient nectar volume per flower measured before (AM) and after (PM) pollinator foraging. Error bars represent \pm 1 SE around meadow averages.

Figure 1.

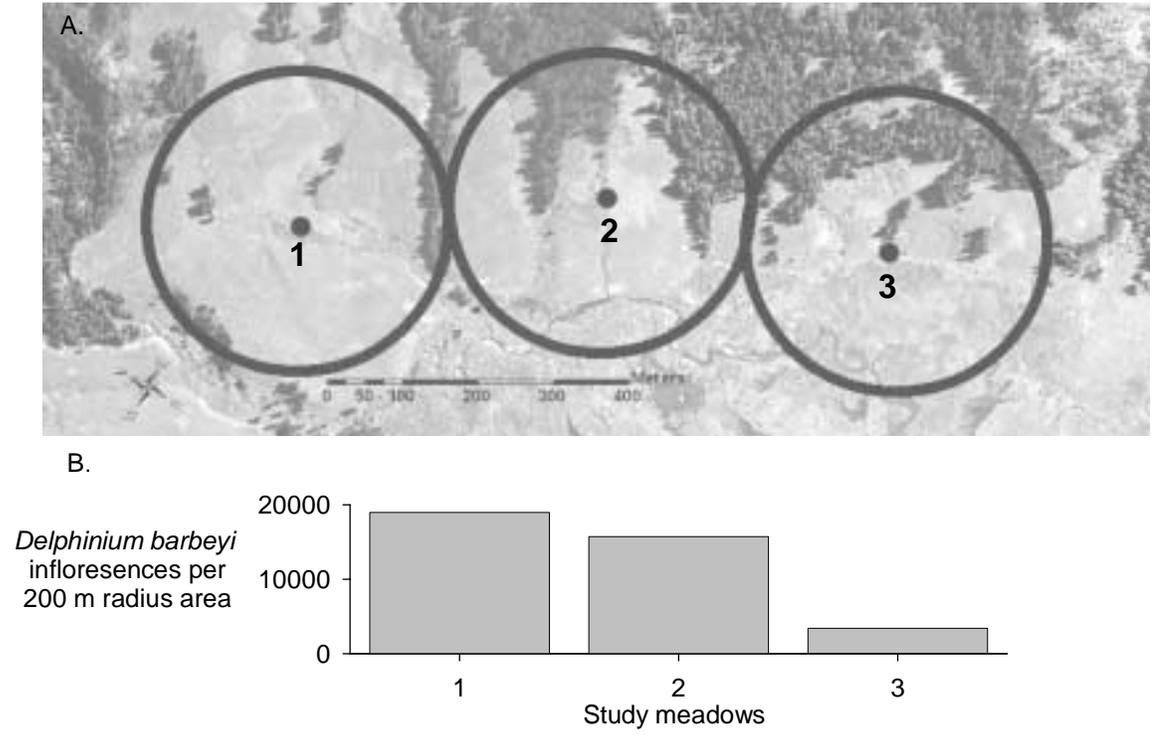


Figure 2.

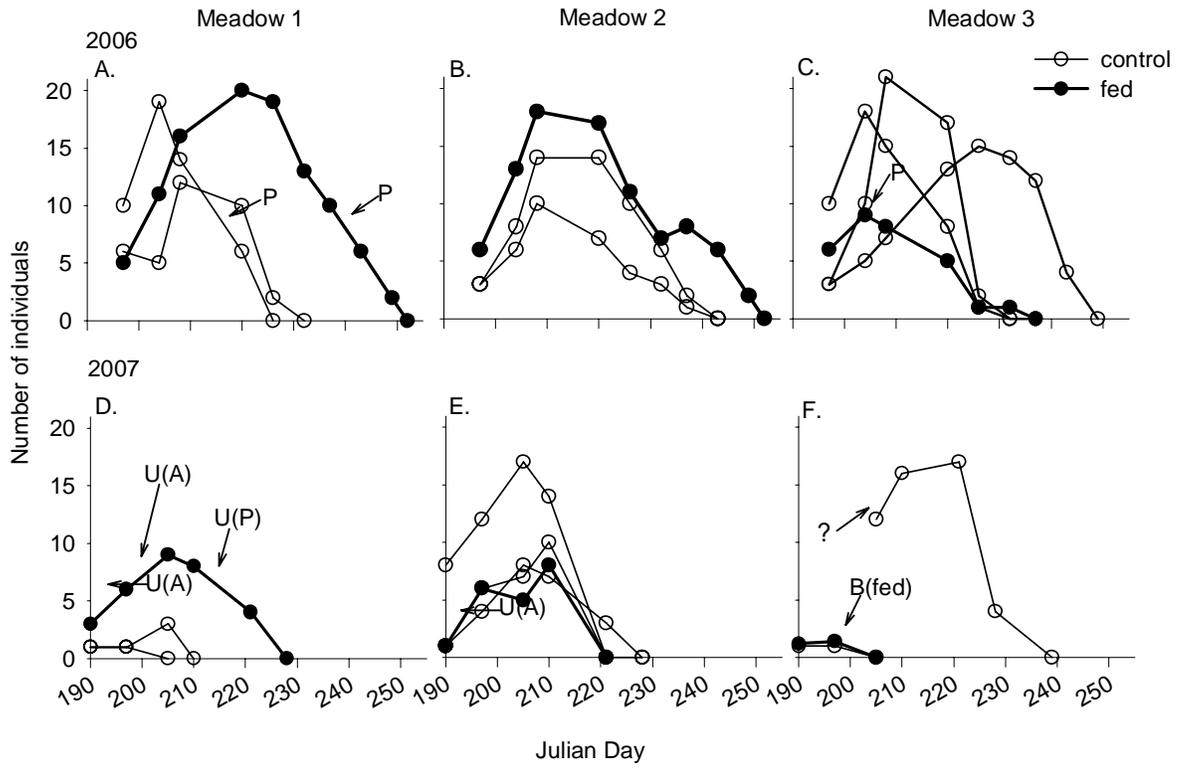


Figure 3.

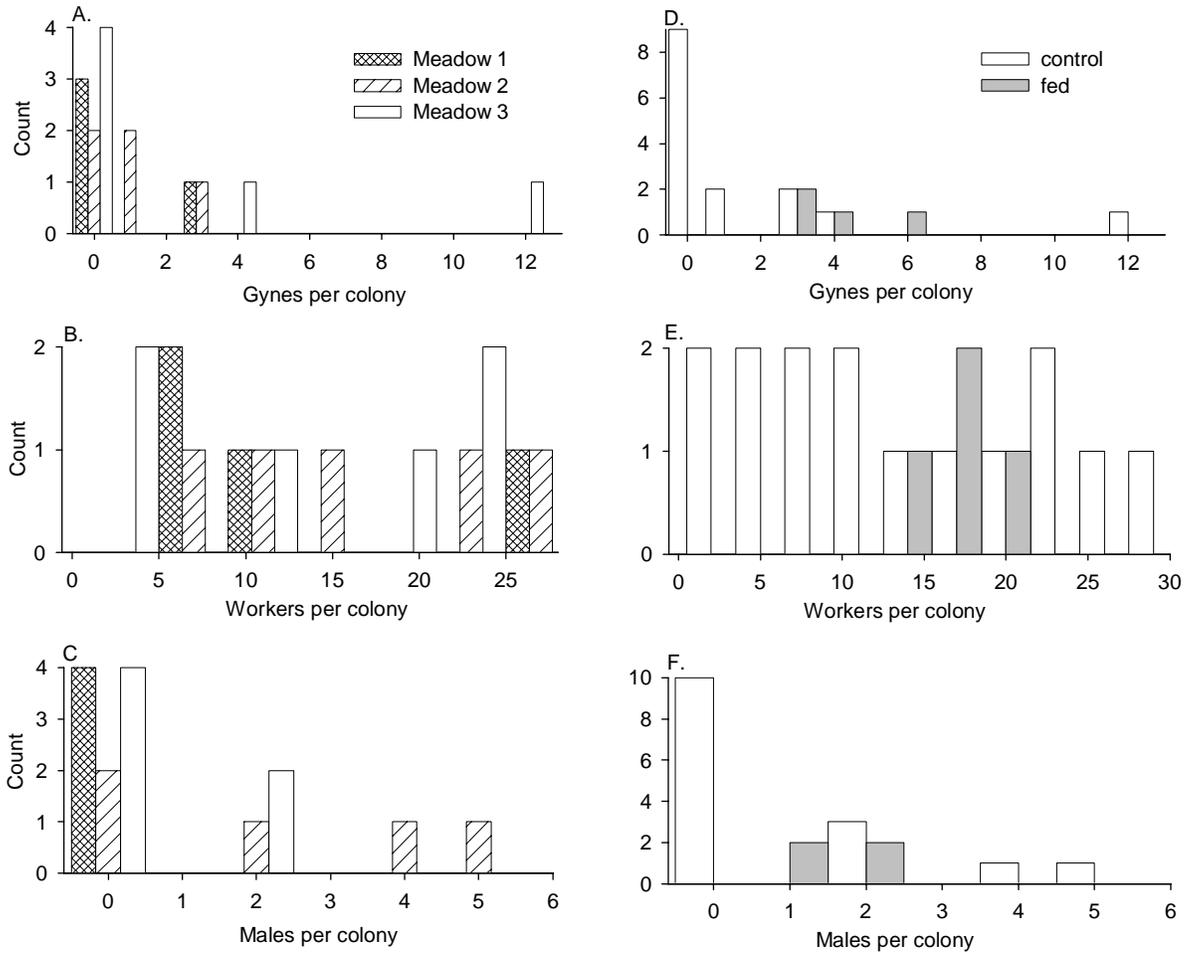
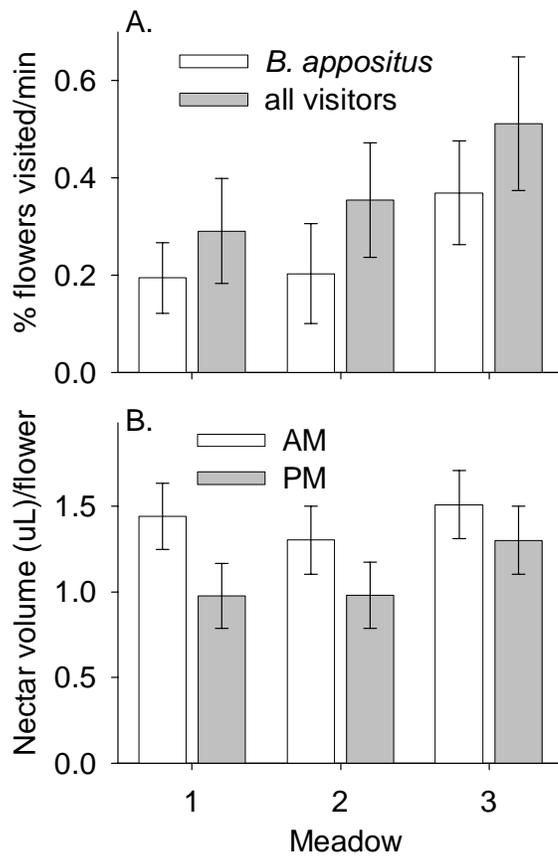


Figure 4.



Appendix I. Contributions of *Delphinium barbeyi* to *Bombus appositus* pollen collection (A) and flower visits (B).

A. In 2004, I sampled 112 corbicular pollen loads caught from *B. appositus* workers and queens (collected over the entire season) from study meadows around the Rocky Mountain Biological Laboratory. After mixing the pollen with ethanol and sonicating the pollen loads, I determined flower species composition from two samples of the pollen load under a compound microscope. After surveying the slide to identify all flower species present, I visually split the sample into four quadrats. With the microscope micrometer over each quadrat at 200x magnification, I counted the number of pollen grains of each species within the micrometer. For each replicate pollen load, I calculated the proportion of *D. barbeyi* pollen grains in the four quadrats and averaged the percent of *D. barbeyi* pollen in the two microscope slide samples. Median percent *D. barbeyi* in pollen provisions was 91.2% (interquartile range = 38.4%).

B. Percent of *B. appositus* flower visits to *D. barbeyi* over the 11-week flowering season (14 June – 28 August) in the three subalpine study meadows also used in this study (Figure 1, for 2005 sampling methods, see Chapter 1). No *B. appositus* were found in Meadow 3 in week 7.

Week:	1	2	3	4	5	6	7	8	9	10	11
Meadow 1	0	0	0	83.3	100.0	100.0	100.0	66.7	95.0	8.3	0
Meadow 2	0	0	0	100.0	84.6	60.0	100.0	94.1	100.0	0	0
Meadow 3	0	0	0	0	66.7	100.0	n/a	88.9	83.3	0	0

Average	0	0	0	61.1	83.8	86.7	100	83.2	92.8	2.8	0
SE	0	0	0	30.9	9.6	13.3	0	8.4	4.9	2.8	0

Appendix II. I used mark-recapture (unique paint color used to mark bees for each meadow) to estimate ambient colony density in each study meadow. *Bombus appositus* mark-recapture data is from bees in captive colonies in three focal meadows (1-3 in decreasing order of flower density; Figure 1). The low recapture rates from individuals in captive colonies suggest that captive colonies did not contribute strongly to total bee density in the focal meadows. Higher recapture rates in the meadow with the fewest flowers suggest that there were fewer natural colonies and/or foragers in the meadow with the fewest flowers.

I estimated natural *Bombus appositus* colony abundance in each meadow by recording the ratio of captive to natural foragers in the field. During nighttime colony censuses, I marked each individual with a dot of paint on its thorax so I could distinguish it from non-captive foragers. The following day, I recorded the species and paint color (if present) of visitors to *D. barbeyi* flowers. In 2006, I recorded pollinator visits for a total of 140, 40, and 90 minutes in the three meadows, respectively (observation bouts that lasted 15-20 minute, distributed over one week). In 2007, I recorded pollinator visits to each meadow for a total of 180 minutes per meadow (12 15-minute bouts spread over three weeks at peak *D. barbeyi* bloom) between 0900 and 1500. All captive bees were seen foraging within the meadow nearest their respective colonies, suggesting that they did not fly among study meadows (as in Chapter 1).

I used the ratio of marked to unmarked *B. appositus* individuals, the total number of individuals marked, and the total number of colonies marked to estimate natural colony density in the three meadows (i.e., number of captive colonies x unmarked bees caught x (marked bees caught)⁻¹).

Year, meadows	# Colonies marked	Search effort (min)	# Marked/Unmarked bees caught	Natural colony estimate
2006				
1	3	134	2/26	39
2	3	40	0/4	Insufficient data
3	4	90	1/3	12
2007				
1	2	200	0/35	Insufficient data
2	4	200	0/29	Insufficient data
3	3	200	4/49	37

Appendix III. ANOVA demonstrating significant among-meadow variation in *B.*

appositus flower visitation rates from n = 12 15-minute observation periods per meadow

(n = 12 study meadows).

Source	DF	SS	MS	F Ratio	P
Model	11	2298.0	208.9	2.9	0.002
Error	132	9450.7	71.6		
Total	143	11748.7			

Appendix IV. *Delphinium barbeyi* flower visitors (i.e., bumble bees (*Bombus* spp.), hummingbirds (*Selophorus platycerus* and *S. rufus*), sphinx moths (*Hyles lineata*), and one solitary bee in the genus *Osmia*) and the contribution of each visitor type to all patch approaches (i.e., visitor entered an observation patch and visited at least one flower) and the total number of flowers visited.

Species	Bee	Approaches		Flowers visited	
	cast	Total	(% of total)	Total	(% of total)
<i>Bombus appositus</i>	W	151	(38.5%)	6979	(49.9%)
	Q	107	(27.3%)	3255	(23.3%)
<i>Bombus flavifrons</i>	W	63	(16.1%)	2088	(14.9%)
	Q	35	(8.9%)	1058	(7.6%)
<i>Selophorus</i> spp.		19	(4.8%)	279	(2.0%)
<i>Bombus nevadensis</i>	Q	4	(1.0%)	131	(0.9%)
<i>Bombus occidentalis</i>	W	5	(1.3%)	77	(0.6%)
<i>Bombus californicus</i>	Q	2	(0.5%)	49	(0.4%)
	W	2	(0.5%)	31	(0.2%)
<i>Bombus frigidus</i>	W	2	(0.5%)	19	(0.1%)
<i>Hyles lineata</i>		1	(0.3%)	16	(0.1%)
<i>Osmia</i> sp.		1	(0.3%)	4	(<0.1%)

CHAPTER III

Effects of flowering plant density on pollinator visitation, pollen receipt, and seed production in *Delphinium barbeyi*

ABSTRACT

Flowering plant density can have conflicting effects on pollination and seed production. For example, dense flower patches may attract more pollinators, but flowers in those patches may also compete for pollinator visits, potentially nullifying the initial benefits of pollinator attraction. We examined how natural conspecific flower density affected pollen receipt and seed production in the protandrous bumble bee-pollinated wildflower, *Delphinium barbeyi* (Ranunculaceae) in natural habitats. To control for underlying variation in abiotic resources, we also tested whether experimentally manipulated flowering plant density affected pollinator visits, pollen receipt, and seed production per flower. In experimental plots, we measured variation in floral sex ratios, pollinator visitation rates, and pollen limitation of seed set from early to late in the season to assess whether these factors were related to seasonal variation in pollen receipt and seed production. In natural plots, pollen receipt increased linearly with density. Seed production increased across lower densities and decreased across higher densities. Experimental density did not affect pollinator visitation rate, pollen receipt, or seed production. Despite an order of magnitude increase in pollinator visitation rate from early to late in the season, pollen receipt decreased over this time period, possibly because male-to-female flower ratios also decreased. Seed set also decreased from early and late in the season, but seed set was not pollen-limited in either part of the season. This study demonstrates that flowering plant density has only minor effects on *D. barbeyi* pollination and seed production.

INTRODUCTION

Group size can influence mating success in both plants and animals (Tejedo 1993, Aizen 1997, Carlsson-Graner et al. 1998, Roed et al. 2002). While large groups may contain more possible mates, individuals in large groups may compete more strongly for those mates (Alberts and Altmann 1995), depending on the group sex ratio and the gender of the individuals. For example, flowers may benefit from being in dense patches if abundant floral resources attract more pollinators and/or provide an ample supply of compatible pollen donors (Kunin 1993, Waites and Ågren 2004, Hegland and Boeke 2006). However, flowers in high density patches may also compete with other flowers for pollinator visits or for abiotic resources necessary for seed production (Steven et al. 2003). Natural relationships between flower density and seed production can be deceptive because they may be driven by other underlying factors (Bosch and Waser 2001). Therefore, it is not surprising that past studies have shown that plant density can have positive, negative, or no effects on pollinator visitation, pollen receipt, and seed production, depending on the spatial scale of the study and habitat characteristics (Ghazoul 2005). To understand the effects of flowering plant density on seed production, and some of the mechanisms involved, we focused on how flowering plant density affects multiple steps in the pollination and seed production process, from pollinator visitation to pollen receipt to seed production, and subsequent seed predation and how density relationships with seed production vary between habitats and between natural and experimental results.

To maximize nectar or pollen acquisition, pollinators can change their foraging behavior in response to flower density (Dreisig 1995, Cresswell and Osborne 2004).

Dense patches may be more attractive to pollinators because they allow pollinators to gather their resources in one place instead of flying from patch to patch (Kacelnik et al. 1986). However, while pollinators such as bumble bees often prefer large patches, they tend to visit a smaller proportion of the flowers (reviewed in Goulson 2003). If increases in per-flower visitation rates are less than proportional to increases in flower density, then plants in dense plots may receive fewer pollinator visits per flower (Goulson 2000). If seed set is pollinator-limited, and if pollinators visit a smaller proportion of flowers in dense patches, then seed set per flower may decline in dense patches (Garcia-Robledo et al. 2005). Alternatively, if seed set is sensitive to inbreeding, then shorter pollinator visits per patch could reduce within-patch pollen movement among more closely related individuals (Field et al. 2005).

Flower densities may not only affect the frequency of pollinator visits, but also the amount of pollen transferred per visit (Aizen 1997). For example, bee-pollinated plants often lose a large proportion of their pollen when bees groom, fly, or brush against non-reproductive floral parts (Rademaker et al. 1997, Castellanos et al. 2003). Therefore, in bee-pollinated flowers, an abundant supply of pollen donors could increase the amount of pollen that reaches stigmas. If pollen donor availability influences pollen receipt, then variation in patch sex ratios might be as important as flower density for pollen transfer and seed production (Lalonde and Roitberg 1994, Aizen 1997, Ishihama et al. 2006).

Abiotic resource availability and biotic interactions other than pollination may also influence the relationship between flower density and seed production. If abiotic resources are scarce, then any effect of flower density on pollinator visitation and pollen receipt may not translate into an effect on seed production (Burd 1994, Ashman et al.

2004). Also, if abiotic resources promote higher plant density and seed production independently, then natural gradients in water and nutrients could create spurious positive correlations between plant density and seed production (Bosch and Waser 2001). Seed predators (or other herbivores) could mask the benefits of higher pollination rates for seed production (Herrera et al. 2002), especially if dense flower patches attract more pollinators as well as more seed predators or other herbivores or florivores (Steffan-Dewenter et al. 2001).

We used observations and experiments to test how conspecific flowering plant density affected pollen receipt and seed production of the protandrous bumble bee-pollinated wildflower, *Delphinium barbeyi* (Ranunculaceae). *Delphinium barbeyi* is naturally patchy, with plant density varying within patches (see *Results*), and bumble bee pollinators are known to vary their foraging behavior according to flower density within plant patches (Pleasants 1981). We addressed three questions: (1) *Is natural flowering plant density related to pollen receipt and seed production?* We examined relationships among flowering plant density, pollen receipt, and seed production in *D. barbeyi* patches located in natural habitats. (2) *Does experimental flowering plant density affect pollinator visitation rate, pollen receipt, and seed production?* To control for underlying variation in abiotic resources, we tested how experimentally manipulated flowering plant density affected pollinator visitation rate, pollen receipt, and seed production. (3) *Do seasonal changes in floral sex ratios, pollinator visitation rate, or pollen limitation of seed set correspond to seasonal changes in pollen receipt and seed production?* In the experimental plots, we compared floral sex ratios, pollinator visitation rate per flower, and pollen limitation of seed set early and late in the season. Then we compared those

changes with changes in pollen receipt and seed production in flowers that bloomed early and late in the season.

We tested the null hypothesis that flower density does not affect pollinator visitation, pollen receipt, or seed production. Alternatively, flowering plant density may lead to higher or lower pollen receipt and higher or lower seed production, depending on the degree to which flowers in denser plots facilitate or compete for pollination services (Feldman et al. 2004). However, if underlying abiotic resources drive density relationships in natural populations, then we should not see positive density relationships when we experimentally manipulate flowering plant density (Bosch and Waser 2001). Also, floral sex ratios are more male biased and pollinator abundance is lower in the first half of *D. barbeyi*'s blooming period. Therefore, if floral sex ratios and pollinator density are more important than flowering plant density for pollen receipt and seed set, then we should see more variation in pollen receipt and seed set from early to late in the season than among plots that vary in density throughout the season.

METHODS

Study System

We examined the relationship between flowering plant density and reproduction in the herbaceous perennial wildflower, *Delphinium barbeyi* (Ranunculaceae, Huth), in July and August, 2005, at the Rocky Mountain Biological Laboratory (RMBL, elevation 2831-3441 m) in Gunnison County, Colorado, USA. At our field site, *D. barbeyi* grows in moist meadows and forest clearings in distinct patches. Plants bear an average of 13.6 ± 0.5 inflorescences per plant (mean \pm 1 SE), with each inflorescence producing 25.4 ± 0.8

flowers (S. E. Elliott, *unpublished data*). *Delphinium barbeyi* flowers are protandrous and self-compatible, but they produce very few seeds autogamously (Williams et al. 2001). In the absence of pollinator visits, flowers produce at least an order of magnitude fewer seeds than open-pollinated flowers (Williams et al. 2001); therefore, plants require pollinator visits to maximize seed set. Individual plants vary in the degree to which their seed production is pollen-limited (Williams et al. 2001). Specifically, in a single-year study, hand-pollinated flowers on three out of six plants produced twice as many seeds as control flowers, but hand-pollinated flowers on the remaining three study plants produced 21-37% fewer seeds than control flowers (Williams et al. 2001).

At our study site, the long-tongued bumble bee, *Bombus appositus*, is the most common pollinator of *D. barbeyi* (Inouye 1976). Floral visitation rates by *B. appositus* increase over *D. barbeyi*'s blooming period because *D. barbeyi* blooms midway through the season when bumble bee colonies are hatching new workers (see Chapters 1 and 2). *Delphinium barbeyi* flowers are also visited less frequently by other bumble bees (*B. flavifrons*, *B. bifarius*, *B. frigidus*, *B. nevadensis*, *B. occidentalis*), hummingbirds (*Selasphorus platycercus*, *Selasphorus rufus*, and *Stellula calliope*), and sphinx moths (*Hyles lineata*) (Inouye 1976, Waser 1982, Williams et al. 2001, see Chapter 3). Seed set does not differ between flowers visited by *B. appositus* or *B. flavifrons* (R. E. Irwin, *unpublished data*), but the relative pollination efficiencies of the other visitors are unknown.

Adult flies also visit *D. barbeyi* flowers. In a nearby site, flies contributed to 0.3-0.6% of flower visits to *D. barbeyi* in 2005 and 2006 (R. E. Irwin, *unpublished data*). The flies are probably in the *Botanophila* genus or a related genus in the Anthomyiini tribe,

but positive identification awaits collection of male individuals. Female flies deposit eggs singly or in groups on the carpels prior to fruit expansion. At our study site in 2006, approximately 10% of all seeds were lost to seed predators; $22.9 \pm 1.8\%$ of fruits per inflorescence were attacked, and attacked fruits lost $53.3 \pm 0.8\%$ of their seeds (mean \pm 1 SE, $n = 129$ inflorescences). In 2006 and 2007 at this study site, seed production of *D. barbeyi* flowers visited by flies only did not significantly differ from plants with no visitors, suggesting they are not important pollinators of *D. barbeyi* (see Chapter 4).

Field Methods

*1. Is natural flowering plant density related to pollen receipt and seed production? We examined natural relationships among flowering plant density, pollen receipt, and seed production in 2005. Because bumble bees are more likely to respond to flower density in larger plots (i.e., 100-2,000 m² versus 1-81 m²) and restrict individual foraging bouts to areas within 18 m (Osborne and Williams 2001, Johnson et al. 2003), we used circular 100 m² plots, each separated by 20 m. We placed 148 plots throughout the East River Valley near the RMBL and in adjacent drainages. We positioned plots in *D. barbeyi* patches that included a wide range of flower densities (0.01-0.68 flowering plants per m²). We measured flowering plant density as the number of *D. barbeyi* plants with buds or flowers within each plot. There was no relationship between plant size (inflorescences per plant) and flowering plant density ($r = 0.10$, $P = 0.2$, $n = 148$ plots).*

Pollen receipt – We quantified average stigmatic pollen receipt per flower from a sub-sample of flowers in each plot. During peak bloom, we collected stigmas from 12 flowers per plot (three flowers per inflorescence from two inflorescences per plant on two

focal plants per plot). In two plots, each with only one plant, we sampled four inflorescences per plant. We collected stigmas after petals had fallen, indicating that stigmas were no longer receptive. We marked sampled flowers with a dot of paint on their pedicels so we could revisit those flowers when the fruits had matured. In another perennial wildflower, *Ipomopsis aggregata* (Polemoniaceae), collecting stigmas at this stage does not affect fruit or seed production (Waser and Fugate 1986). In the laboratory, we mounted stigmas on microscope slides, used basic fuchsin dye to stain the pollen (Kearns and Inouye 1993), and counted the number of conspecific and heterospecific pollen grains with a compound microscope. We only present analyses of conspecific pollen because heterospecific pollen was rare ($4.6 \pm 0.4\%$ of grains, median = 0.0%, $n = 1128$ flowers). Each flower has three stigmas, and we summed pollen receipt from all stigmas for total pollen receipt per flower.

Seed production – We quantified average seed production per flower from the same sub-sample of flowers from which we collected stigmas. If flowers aborted, we included them in the analyses as producing zero seeds per flower. For flowers that produced fruits, we counted the number of seeds surviving and seeds consumed by fly larvae. We could count the number of consumed seeds because their seed coats remain intact after larvae consume the endosperm. To determine how many ovules developed into mature seeds, we summed surviving and consumed seeds. Unless there were qualitative differences in effects on developed seeds, we reported seed production as *surviving* seeds per flower.

Statistical analyses – We used linear regression to test the effects of natural flowering plant density on pollen receipt. To analyze the effect of natural flowering plant

density on seed production, we included a quadratic term in the regressions because bivariate scatter plots suggested unimodal relationships (Figure 1). We evaluated spatial patterns in regression residuals by calculating Moran's I values (Legendre 1993). We also used simultaneous autoregressive models to analyze density relationships and compared models with and without accounting for fine-scale autocorrelations using model fit (R^2) and Akaike information criterion (AIC) (Lichstein et al. 2002, Kissling and Carl 2008). Finally, we tested for correlations between pollen receipt and seed set because failure of density to affect seed set could occur if pollen receipt does not affect seed set. We performed spatial analyses with SAM (Spatial Analysis in Macroecology, Rangel et al. 2006). We performed all other analyses with JMP 4.04 (SAS Institute 2001).

2. Does experimental flowering plant density affect pollinator visitation rate, pollen receipt, and seed production? To control for underlying variation in abiotic resources, in one meadow area, we tested whether experimentally manipulated *D. barbeyi* flowering plant density affected pollinator visitation rate per flower, pollen receipt per flower, and seed production per flower. Since floral sex ratios and pollinator visitation rates (either of which could mediate or mask density effects) change over *D. barbeyi*'s blooming period, we measured these variables twice: early and late in the blooming period (hereafter, referred to as early and late in the season, see Question 3 methods below).

We experimentally manipulated flowering plant density in circular 100 m² plots that initially had medium to high flowering plant densities (33-77 flowering plants per plot). We randomly assigned plots to density treatments, which consisted of 4, 8, 12, 14,

16, 18, 22, 28, or 32 unclipped plants remaining per plot, with three to four replicates per density for a total of 31 plots. These experimental densities spanned the lower 67% density range found in the natural patches (median natural density = 24 plants per plot). We used a range of experimental densities so we could detect potential nonlinear relationships, such as saturating, sigmoidal, or unimodal pollinator responses to flower density (Goulson 2000, Feldman 2006). We clipped inflorescences, rather than removing entire plants, to avoid altering competition for water or nutrients that might fuel pollinator rewards. For example, neither morning nectar volume per flower nor floral display size varied with experimental densities (nectar volume: $F_{1,13} = 0.01$, $P = 0.92$; floral display: $F_{1,29} = 0.5$, $P = 0.5$). We also clipped inflorescences from a 10 m buffer around each plot to ensure that bumble bee pollinator responses to experimental densities were not confounded by neighboring flower densities (Osborne and Williams 2001).

Pollinator visitation rate and floral sex ratios – We monitored pollinator visitation rate in a subset of experimental plots that spanned the full range of density treatments (16 plots early in the season and 14 plots late in the season). In each part of the season, we monitored pollinator visits to four focal plants per plot for three to four 30-minute intervals (total = 1.5-2 hours of observation per plot) between 0900-1700 (peak hours of bumble bee pollinator activity). In each 30-minute interval, we recorded each pollinator species (and caste for bumble bees: queen, worker, or male) that visited a focal plant. We also recorded the number of flowers visited before the pollinator left the patch. Before each observation period, we counted the total number of open male and female flowers on each focal plant. We used flower counts to calculate pollinator visitation rate per flower (i.e., proportion of flowers visited per minute of observation time) and to

calculate male-to-female flower ratios.

Pollen receipt and seed production – After each pollinator observation period, we collected stigmas from 16 flowers per plot (four flowers per plant from four focal plants per plot) to quantify pollen receipt (described above). We collected fruits from the flowers from which we had collected stigmas in eight experimental plots that included the full density range.

Statistical analyses – We used linear regressions to test the relationships between experimental flowering plant density and (1) pollinator visitation rate per flower, (2) pollen receipt per flower, and (3) seed production per flower, with separate analyses for early and late in the flowering season. Positive relationships between density and all three response variables could suggest that flowers in dense plots benefited from increased pollinator attraction, and that seed set was pollinator-limited. A negative effect of density on visitation could indicate that there was increased competition for pollinators in dense plots. Increases in visitation, but not pollen receipt, could occur if pollen receipt was not limited by pollinator visits. Increases in pollen receipt, but not seed production, could occur if seed set was not limited by pollen receipt. Therefore, we also tested for correlations between (1) pollinator visitation rate and pollen receipt, (2) pollinator visitation rate and seed production, and (3) pollen receipt and seed production, with separate analyses for early and late in the season.

3. Do seasonal changes in floral sex ratios, pollinator visitation rate, or pollen limitation of seed set correspond to seasonal changes in pollen receipt and seed production? In experimental plots, we measured floral sex ratios, pollinator visitation

rates, pollen receipt, seed production, and pollen limitation or seed production at two time periods: early (19 July – 23 July) and late (29 July – 4 August) in the flowering season.

These time intervals represent the midpoints of the first and second halves of *D.*

barbeyi's blooming period. Because flower density within plots did not differ from early to late in the season (95% confidence interval for mean difference between early and late = -61.4 – 55.2 open flowers per plant, $n = 12$ plots), any potential changes in pollinator visitation rate per flower were not driven by decreases in available flowers.

We used one-tailed paired t-tests to test whether male-to-female flower ratios decreased, and whether per flower pollinator visitation rates increased, from early to late in the season. We used two-tailed paired t-tests to test whether pollen receipt and seed production changed over the season. Increases in pollen receipt and seed set throughout the season could suggest that high pollinator visitation rates benefit seed set. Decreases in pollen receipt and seed set throughout the season could suggest that costs of lower male-to-female flower ratios, or some other factor, outweigh benefits of high pollinator visitation rates.

Pollen limitation – To test for pollen-limitation of seed set, we hand-pollinated flowers and compared their seed production to paired open-pollinated control flowers. On each focal plant in ten experimental plots that spanned the full experimental density range, we assigned one inflorescence to a hand-pollination treatment and a second inflorescence to a control treatment. We hand-pollinated any open female-phase flowers once during each pollinator-observation period. We collected dehiscing anthers from at least ten plants growing at least five meters away to avoid potential pollen incompatibility and because plants in nature may receive pollen from multiple donors.

We added pollen to stigmas by brushing dehiscing anthers onto receptive stigmas. To control for flower handling in the hand-pollination treatment, we handled a similar number of flowers in the control treatment. Combining both observation periods, the 29 treated hand-pollination inflorescences each had an average of 14.3 ± 1.4 hand-pollinated flowers (4.1% of all flowers; see *Study System*). Given that we hand-pollinated such a small proportion of the flowers on each focal plant, it is unlikely that there was competition for resources among flowers on the same plant in the hand-pollinated and control treatments (but see Knight et al. 2006). To determine whether hand-pollination increased stigmatic pollen receipt, we collected stigmas from 230 early flowers (111 hand-pollinated and 119 control) and 120 late flowers (75 hand-pollinated and 45 control), and we counted pollen grains (described above).

In the early pollinator-observation period, we treated 29 and 28 inflorescences in the hand-pollination and control treatments, respectively. In the late period, we treated 28 and 26 inflorescences from the hand-pollination and control treatments, respectively. The sample size varied because we could only treat assigned inflorescences that had open female flowers. The number of open female flowers per inflorescence ranged from 2 – 22, with averages of 10 and 9 open female flowers per inflorescence for early hand-pollination and control treatments, respectively, and an average of 5 open female flowers per inflorescence for late hand-pollination and control treatments.

To measure seed production, we collected fruits from the treated flowers and counted seeds. We used two two-tailed t-tests to compare pollen receipt and seed production between control and hand-pollination treatments, with plot as the unit of replication for the effects on seeds, and with flower as the unit of replication for the

effects on pollen receipt. We used a fully-crossed 2-way ANOVA (density treatments x hand-pollinations) to test whether hand-pollinating flowers altered density effects on seed set.

RESULTS

1. Is natural flowering plant density related to pollen receipt and seed production? Pollen receipt per flower increased linearly with flowering plant density ($r^2 = 0.06$, $P = 0.004$, Figure 1A). Seed production per flower also increased with flowering plant density ($r^2 = 0.03$, $P = 0.03$, Figure 1B), but a polynomial model describing an increase in seed set over a low density range and decrease in seed set over a high density range explained more of the variation in seed production than a linear relationship ($r^2 = 0.09$, $P = 0.001$; Figure 1B, Tables 1 and 2). Pollen receipt and seed production regression residuals showed little spatial autocorrelation (max Moran's $I < 0.25$; Table 1). Adding geographic coordinates in the spatial autoregressions slightly improved the fit of the models describing variation in pollen receipt and seed production (Table 2). Pollen receipt was best explained by a combination of variation in plant density and geographic location (Table 2). Similarly, seed production was best explained by a combination of plant density (polynomial relationship) and geographic location (Table 2). Seed production increased in plots with higher pollen receipt (developed seeds: $r = 0.31$, $P = 0.0001$, $n = 116$ plots), and this relationship was dampened but not masked by accounting for seed predation (surviving seeds: $r = 0.22$, $P = 0.01$; Table 3).

2. Does experimental flowering plant density affect pollinator visitation rate, pollen receipt, and seed production? Unlike natural plots, manipulation of flowering

plant density had virtually no effect on pollination or seed production per flower. Early in the season, pollinator visitation rates increased with experimental flowering plant density, but this relationship was not statistically significant ($r^2 = 0.19$, $P = 0.09$; Figure 2A, Table 4). Late in the season, the relationship between pollinator visitation rate and flowering plant density disappeared ($r^2 = 0.002$, $P = 0.9$; Figure 2A, Table 4). Flowering plant density had no effect on early season pollen receipt ($r^2 = 0.001$, $P = 0.9$; Figure 2B, Table 4). Late in the season, pollen receipt increased with experimental flowering plant density, but this relationship was not statistically significant ($r^2 = 0.01$, $P = 0.1$; Figure 2B, Table 4). Experimental flowering plant density did not affect seed production early ($r^2 = 0.01$, $P = 0.8$; Figure 2C, Table 4) or late in the season ($r^2 = 0.10$, $P = 0.4$; Figure 2C, Table 4).

Neither pollen receipt nor seed production per flower varied with pollinator visitation rate per flower (Table 3). As with natural plots, early in the season, seed production increased with pollen receipt (developed seeds: $r = 0.72$, $P = 0.04$), but this effect disappeared after accounting for seed predation (surviving seeds: $r = 0.39$, $P = 0.3$; $n = 8$ plots, Table 3). In contrast, late in the season, seed production did not vary with pollen receipt (Table 3).

3. Do seasonal changes in floral sex ratios, pollinator visitation rate, or pollen limitation of seed set correspond to seasonal changes in pollen receipt and seed production? In experimental plots, the male-to-female flower ratio decreased by 87% from early to late in the season ($t_{11} = 2.03$, $P = 0.03$) and pollinator visitation rates increased by an order of magnitude ($t_{11} = 4.1$, $P = 0.001$, Figure 3A,B). *Bombus appositus* dominated the pollinator assemblage in both parts of the season (Appendix). As with

male flower availability, pollen receipt decreased by 36% from early to late in the season ($t_{24} = 3.6$, $P = 0.002$, Figure 3C).

Pollen receipt did not differ between control and hand-pollinated flowers early ($t_{228} = 0.7$, $P = 0.5$; average pollen grains per flower ± 1 SE: hand-pollinated = 140 ± 10 pollen grains, control = 131 ± 9 pollen grains) or late in the season ($t_{118} = 0.7$, $P = 0.5$; hand-pollinated = 97 ± 9 pollen grains, control = 86 ± 12 pollen grains). Thus, not surprisingly, there was no difference in developed seeds produced by hand-pollinated and control flowers early ($t_9 = 1.5$, $P = 0.15$) or late in the season ($t_9 = -0.1$, $P > 0.9$; Figure 3D). Experimental densities did not alter the effect of hand-pollinating flowers on seed set in either part of the season (early: $F_{1,16} = 0.5$, $P = 0.5$; late $F_{1,16} = 0.2$, $P = 0.7$; Table 5). Seed production of hand-pollinated flowers decreased by 12% over the season, although this decrease was not statistically significant ($t_9 = -0.9$, $P = 0.4$; Figure 3D). Similarly, ambient seed production decreased by 44% over the season ($t_8 = -2.7$, $P = 0.03$, Figure 3E).

DISCUSSION

Flowering plant density can have conflicting effects on pollination and seed production. Flowers in high density patches may benefit from attracting more pollinators, but sometimes at the cost of competing with other flowers for pollinator visits or water and nutrients to fuel seed production (Callaway and Walker 1997, Steven et al. 2003). In this study, we found that *Delphinium barbeyi* pollen receipt increased linearly with natural increases in conspecific flowering plant density. Seed production per flower increased over a low density range and decreased over a high range. In contrast, experimental

densities did not significantly affect pollinator visitation rate, pollen receipt, or seed production per flower. The effects of *D. barbeyi* flowering plant density on pollination and female reproductive success are minor, but the strength of these effects may increase where pollinators or male flowers are limiting to pollen receipt and seed production.

Several factors could explain why we only detected significant density effects in natural plots, and not in experimental plots. If underlying variation in abiotic resources caused the increase in seed production with flowering plant density over the lower natural density range, then this could explain why we did not see similar trends in experimental plots (Bosch and Waser 2001). In addition, since pollen receipt increased with natural flowering plant density, abiotic resources may have also affected per-flower nectar and pollen rewards used to attract pollinators, creating spurious positive correlations between pollen receipt and seed production (Carroll et al. 2001). Also, pollen receipt and seed production were only weakly related to natural flowering plant density; only 3-9% of the variation in pollen receipt and seed production was explained by flowering plant density in natural plots. In addition, some of the variation in pollen receipt and seed production was explained by fine-scale spatial autocorrelation, suggesting that patchiness in abiotic resources may contribute to density-pollen and density-seed relationships. Although we removed some of the underlying variation in environmental variables that could have facilitated density relationships in experimental plots, there was some evidence in that (1) pollinator visitation rates increased with flower density when pollinators were most limiting (i.e., early in the season), and (2) pollen receipt increased with flower density when male flowers were most limiting (i.e., late in the season). Therefore, flower density

may have stronger effects on pollination and seed production in areas where (1) pollinators are scarce or (2) female-phase flowers outnumber male flowers.

For density to affect pollen receipt through increases in per-flower pollinator visitation rate, there needs to be a strong relationship between pollen receipt and visitation rates. However, pollinator visitation rates to *D. barbeyi* flowers were not correlated with pollen receipt in either part of the season. Also, despite an order of magnitude increase in pollinator visitation rates across the season, pollen receipt decreased. In other flower species, the number of pollen grains or unique sires per flower may increase with pollinator visitation rate (Engel and Irwin 2003, Karron et al. 2006), and *D. barbeyi* pollen receipt decreases by 71% when all pollinators are excluded (S. E. Elliott, *unpublished data*). However, our hand-pollinated flowers did not receive more pollen than open-pollinated control flowers, suggesting that stigmatic surface area was limited. Alternatively, by hand-pollinating flowers, we could have knocked off more pollen than we added. Also, if pollinators make multiple visits to stigmas, the stigmatic surface area may become saturated (Brown and Kephart 1999), and additional visits might leave only a small net increase in pollen receipt. To determine whether flowers received multiple pollinator visits, we multiplied per-flower pollinator visitation rates by the length of time that pollinators were active each day (i.e., eight hours). Assuming that all flowers were visited equally, we calculated that open flowers would receive ~1.5 and ~17.8 visits per day, early and late in the season. Thus, all flowers probably received multiple visits, and pollen receipt may saturate at low visitation rates.

With male biased sex ratios, pollinators may transfer more compatible pollen grains to stigmas. For example, in the gynodioecious plant, *Daphne laureola*, plants in

male biased populations receive more pollen on their stigmas and produce more pollen tubes per style (Alonso 2005). Similarly, *Silene dioica* flowers are more strongly pollen-limited in populations that have fewer male flowers per female flower (Carlsson-Graner et al. 1998). In a system similar to ours, pollinator visits to flowers of the protandrous perennial herb, *Alstroemeria aurea*, increase late in the season when there are fewer male flowers available, and consequently, bumble bees deliver an order of magnitude fewer pollen grains per visit (Aizen 2001).

Changes in pollinator behavior throughout the season could have also contributed to the decrease in pollen receipt per flower. In the field, we could not distinguish between visits to male versus female flowers. Therefore, we calculated visitation rates as the percent of all flowers visited per minute. If bees were primarily collecting pollen, then they might have preferentially visited male flowers. If bees were preferentially collecting nectar, then they might have preferentially visited female flowers because female flowers contain 22% more nectar per flower than male flowers (mean nectar volume per female flower \pm 1 SE: 0.61 ± 0.04 μ L per flower, $n = 179$ flowers; male flowers: 0.50 ± 0.03 μ L per flower, $n = 275$ flowers; $t_{452} = 2.3$, $P = 0.02$). If bees had preferentially visited male or female flowers (as in Carlsson-Graner et al. 1998), then we may not have adequately described visitation rates to female flowers, which we ultimately hoped to link to pollen receipt. In addition, pollination efficiency of *Bombus appositus* individuals (i.e., the most frequent visitors) could have varied from early to late in the season since late-season *Bombus appositus* worker and queen foragers spent 29% less time per flower than they did early in the season (mean time per flower \pm 1 SE, 2.99 ± 0.07 seconds per flower, $n =$

233 individuals) compared to earlier in the blooming period (2.12 ± 0.07 seconds per flower, $n = 218$ individuals; $t_{449} = 8.7$, $P < 0.0001$).

The late season decrease in seed production was probably not due to increased pollen limitation. For example, in the herbaceous perennial, *Lithophragma parviflorum*, seed set of hand-pollinated late-blooming flowers was lower than early blooming hand-pollinated flowers (Pellmyr and Thompson 1996). *Delphinium barbeyi* could have invested fewer resources in late-blooming flowers if their early blooming flowers had already produced sufficient seeds. For example, in sequentially blooming plants, seasonal changes in available mates may alter investment in male or female flowers (Brunet and Charlesworth 1995). For *D. barbeyi*, shortages of male flowers late in the season may have caused the decrease in pollen receipt, but a shortage of available abiotic resources or resources allocated to late blooming flowers was more likely to have caused the decrease in seed set.

Seed predators (or other herbivores) could mask the effects of flower density or pollen receipt on seed production (Steffan-Dewenter et al. 2001). To increase the likelihood that their offspring will have seeds to consume, adult insects may oviposit on flowers that have been or will be heavily pollinated (Brody 1992). In our study, pre-dispersal fly seed predators dampened or masked the relationships between pollen receipt and seed production (i.e., surviving seeds) in natural plots and in early blooming flowers in experimental plots. However, seed predators had little to no effect on the relationship between flowering plant density and seed production. Similarly, beetle fruit predators of the terrestrial aroid, *Xanthosoma daguense*, masked the benefits of increased pollinator visitation rates for fruit production, but had little to no effect on pollinator-mediated

benefits of plant density (Garcia-Robledo et al. 2005).

Although visitation rates were unrelated to pollen receipt and seed production, they could still affect pollen export and male reproductive success. In other species, high visitation rates may only affect pollen export and donation (Stanton et al. 1991). In *D. barbeyi*, pollen export may have been most sensitive to flowering plant density early in the season when female flowers were least common, and therefore more pollen may have been lost during visits between male flowers. If male and female fitness components are both positively correlated with density, but at different times of the season, then this would reinforce a net positive effect of flower density on plant fitness.

Our results support the growing evidence that interaction outcomes are highly contingent on the surrounding biotic and abiotic environment (Thompson 1999, Strauss and Irwin 2004, Tschardtke and Brandl 2004). The consequences of variation in interaction rates alone, such as pollinator visitation rates, did not translate into interaction outcomes, such as pollen receipt and seed production. In plant species that produce many flowers over a long blooming period, and that separate male and female flowers spatially or temporally, fluctuations in factors external to the plant-pollinator interaction (e.g., abiotic resources, sex-ratios, and seed predation) may mask the fitness effects of variation in partner abundance (e.g., plant and pollinator densities).

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Table 1. Regression statistics and maximum spatial autocorrelation (Moran's I for regression residuals) for the relationships between *Delphinium barbeyi* natural flowering plant density, pollen receipt, and surviving seeds per flower in 100 m² plots: (1) Pollen grains received per flower = Intercept + b₁*Flowering plants(m⁻²); (2) Surviving seeds per flower = Intercept + b₁*Flowering plants(m⁻²); (3) Surviving seeds per flower = Intercept + b₁*Flowering plants(m⁻²) + b₂*(Flowering plants(m⁻²))².

Model	Intercept	b ₁	b ₂	Max Moran's I
(1) Pollen	81.5(±4.9)	45.6(±15.6)		0.128
	t = 16.8	t = 2.9		
	P < 0.0001	P = 0.004		
(2) Seeds, linear	17.5(±1.3)	9.0(±4.1)		0.248
	t = 13.6	t = 2.2		
	P < 0.0001	P = 0.03		
(3) Seeds, polynomial	18.0(±1.3)	14.7(±4.5)	-72.8(±24.1)	0.216
	t = 14.2	t = 3.3	t = 3.0	
	P < 0.0001	P = 0.001	P = 0.003	

Table 2. Comparison of models incorporating density and spatial location (model parameters in Table 1) to predict *Delphinium barbeyi* pollen receipt and seed production in 100 m² plots.

Response	Predictors	R ²	AIC
Pollen receipt	Density + space	0.07	1445.7
	Density	0.06	1448.8
Seeds per flower	Density + Density ² + space	0.11	1045.5
	Density + space	0.08	1048.1
	Density + Density ²	0.09	1049.2
	Density	0.03	1055.6

Table 3. Correlations between *Delphinium barbeyi* per-flower pollinator visitation rates (experimental plots only), pollen receipt, and seed production (developed and surviving seeds) for experimental plots (sampled early and late in the blooming period) and for natural plots. Correlation coefficients are reported with p-values in parentheses and sample size (number of plots) below.

	Natural pollen	Early visitation	Late visitation	Early pollen	Late pollen
Pollen		-0.27 (0.4) n = 14	-0.04 (0.9) n = 14		
Developed seeds	0.31 (0.0001) n = 148	0.64 (0.2) n = 5	-0.38 (0.4) n = 8	0.72 (0.04) n = 8	0.47 (0.2) n = 10
Surviving seeds	0.22 (0.01) n = 148	-0.21 (0.7) n = 5	-0.38 (0.4) n = 8	0.39 (0.3) n = 8	0.47 (0.2) n = 10

Table 4. Regression statistics for the relationships between experimental *Delphinium barbeyi* flowering plant density, pollinator visitation rate per flower, pollen receipt, and surviving seeds per flower in 100 m² plots: (1) Percent of flowers visited per min = Intercept + b₁*Flowering plants(m⁻²), (2) Pollen grains received per flower = Intercept + b₁*Flowering plants(m⁻²); (3) Surviving seeds per flower = Intercept + b₁*Flowering plants(m⁻²).

<u>% of flowers visited per min:</u>		<u>Pollen grains received per flower:</u>		<u>Surviving seeds per flower:</u>	
Intercept	b ₁	Intercept	b ₁	Intercept	b ₁
Experimental early season plots:					
0.16(±0.1)	0.0082(±0.005)	148.7(±23.2)	0.22(±1.2)	25.6(±6.7)	-0.1(±0.4)
t = 1.8	t = 1.8	t = 6.4	t = 0.2	t = 3.8	t = -0.3
P = 0.1	P = 0.09	P < 0.0001	P = 0.9	p = 0.01	P = 0.8
Experimental late season plots:					
3.5(±1.4)	0.0099(±0.07)	71.3(±15.5)	1.2(±0.8)	10.0(±4.2)	0.2(±0.2)
t = 2.6	t = 0.1	t = 4.6	t = 1.5	t = 2.4	t = 0.9
P = 0.02	P = 0.9	P = 0.0001	P = 0.1	P = 0.05	P = 0.4

Table 5. ANOVA testing the effects of experimental *Delphinium barbeyi* flowering plant density in 100 m² plots, hand-pollinations (HP), and their interactions on developed seeds per flower, with separate analyses for early and late in *D. barbeyi*'s blooming period.

	<u>Early</u>			<u>Late</u>		
	DF	F-ratio	P-value	DF	F-ratio	P-value
Full Model	3,16	1.0	0.4	3,16	0.4	0.8
HP	1,16	2.3	0.2	1,16	0.02	0.9
Density	1,16	0.3	0.6	1,16	0.9	0.4
HP*Density	1,16	0.5	0.5	1,16	0.2	0.7

Figure legends

Figure 1. Relationship between natural *Delphinium barbeyi* flowering plant density (plants per m² in 100 m² plots), pollen receipt per flower, and surviving seeds per flower (n = 148 plots). Regression parameters and statistics reported in Table 1.

Figure 2 Relationship between experimental *Delphinium barbeyi* flowering plant density (plants per m² in 100 m² plots) and percent of open flowers visited by pollinators per minute (A), pollen receipt per flower (B), and surviving seeds per flower (C) for flowers blooming early (closed circles) and late (open circles) in *D. barbeyi*'s blooming period. Regression lines (early = solid, late = dashed) and associated statistics (r² and p-value) for equations reported in Table 1.

Figure 3. Variation in *Delphinium barbeyi* seed production and factors contributing to seed production measured in 100 m² experimental plots: male-to-female flower ratio (A, n = 12 plots), percent of open flowers visited per minute (B, n = 12 plots), pollen receipt per flower (C, n = 25 plots), developed seeds per flower (D, solid = open control flowers, hatched = hand-pollinated flowers, n = 9 plots), and surviving seeds per flower (E, n = 9 plots) early and late in the blooming period. Error bars represent ± 1 SE around plot means.

Figure 1.

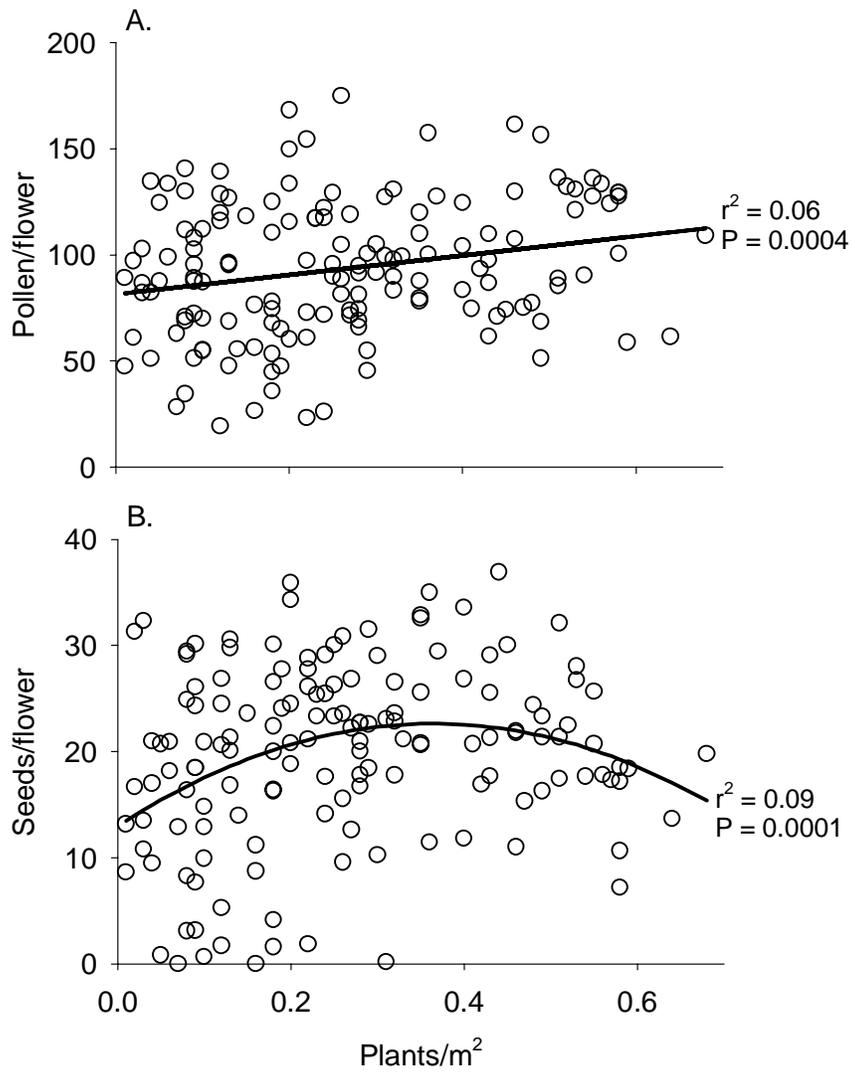


Figure 2

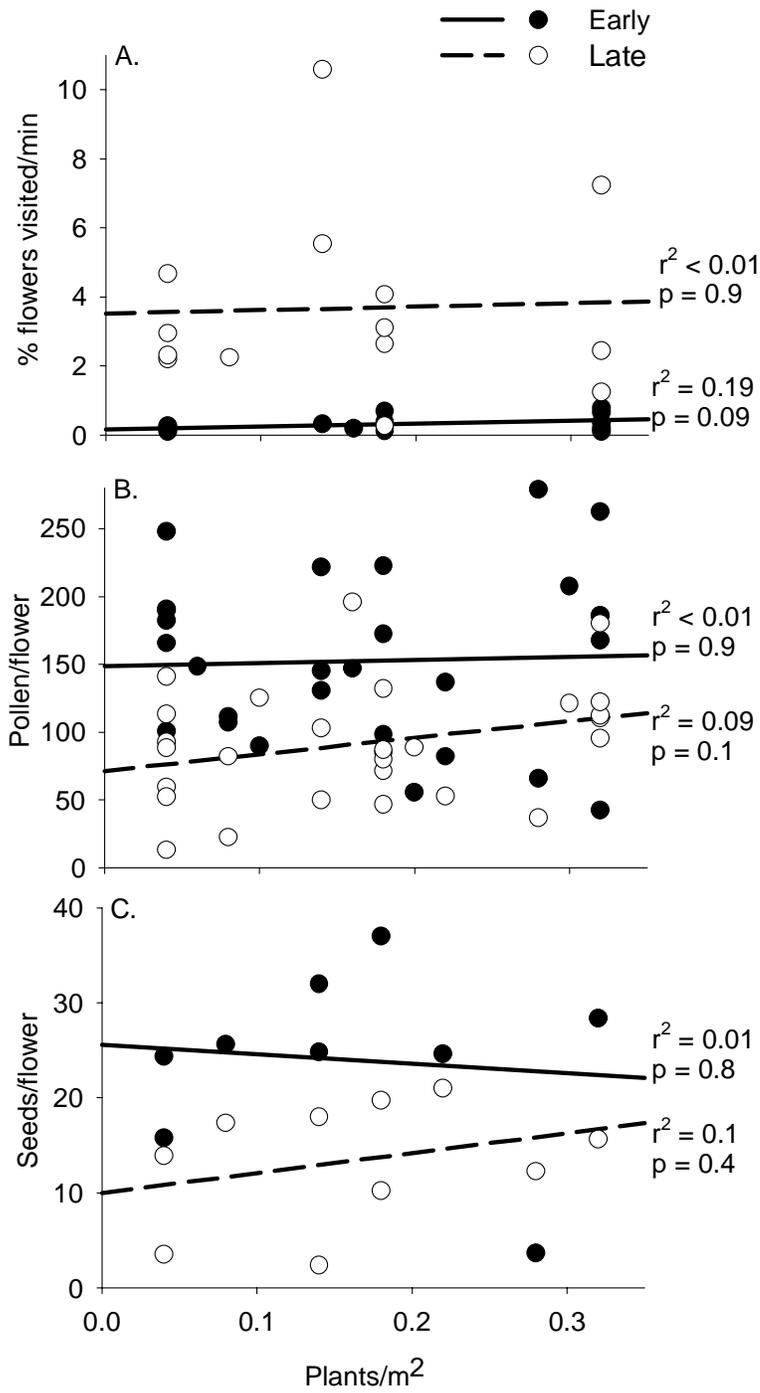
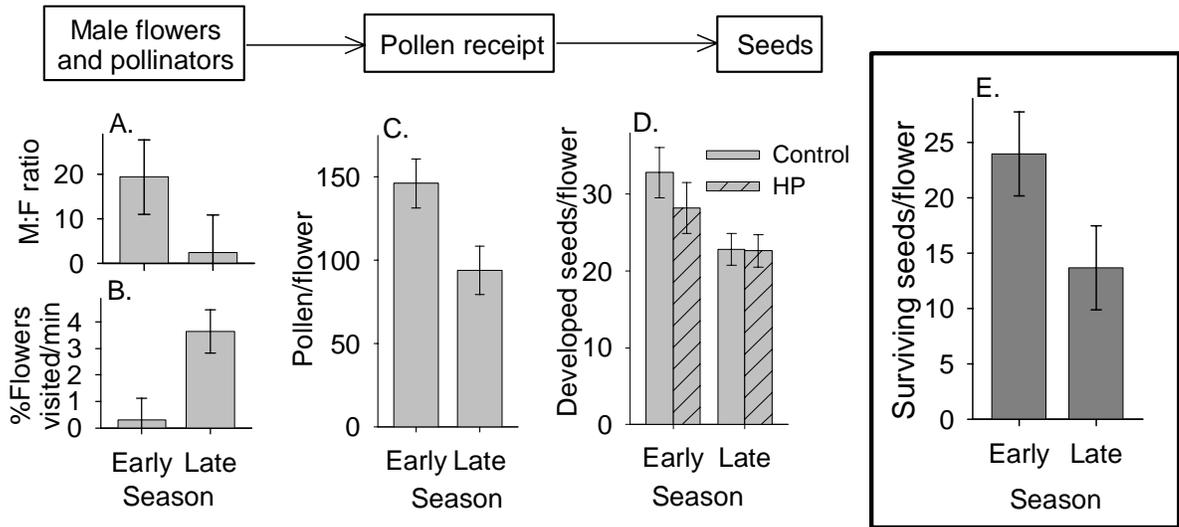


Figure 3



Appendix. Percent of visits to *Delphinium barbeyi* flowers by different species (and castes for bumble bees), early and late in the blooming period.

Species	Caste	Early		Late	
		By caste	Total	By caste	Total
<i>Bombus appositus</i>	worker	75.5		76.9	
	queen	2.0	77.5	0.9	79.9
	male	0.0		2.1	
<i>Bombus flavifrons</i>	worker	7.2		13.3	
	queen	2.5	9.7	2.0	15.3
	male	0		0	
<i>Bombus nevadensis</i>	worker	2.8		< 0.1	
	queen	2.7	5.5	< 0.1	< 0.1
	male	0		0	
<i>Bombus californicus</i>	worker	4.1		4.5	
	queen	0	4.1	0.3	4.8
	male	0		0	
<i>Hyles lineata</i>			2.1		0
<i>Selophorus platycerus</i>			0.9		0
<i>Selophorus rufus</i>			0.1		0

CHAPTER IV

Effects of primary and secondary pollinators on seed production and selection on floral traits in *Delphinium barbeyi*

ABSTRACT

While pollinators can have strong effects on plant reproduction and natural selection on floral traits, the strength and direction of these effects may depend on the assemblage of other floral visitors. Secondary (less common or less efficient) pollinators could either magnify or mask the effects of primary (more common or more efficient) pollinators, depending on their floral visitation preferences and on the relative costs and benefits of their interactions. We excluded pollinators to test how primary pollinators (here, bumble bees) and secondary pollinators (here, flies) affected seed production and natural selection on floral traits through female reproduction in the perennial wildflower, *Delphinium barbeyi* (Ranunculaceae). In two sites (one each in forest and meadow habitats), over two study years, we manipulated floral visitors by allowing visits by: (1) all visitors (control), (2) secondary fly pollinators only (excluding all bumble bees, the most common visitors, and hummingbirds), or (3) no visitors (all visitors excluded). To test whether floral traits were associated with female reproduction and whether pollinators mediated this association, we measured three floral traits (flower production, blooming time, and spur length) on control plants in 2006 and on control and fly only plants in 2007. In 2006, secondary fly pollinators compensated for the pollination services lost by the exclusion of primary bumble bee pollinators in the forest site, but not in the meadow site. In 2007, flies did not compensate for bumble bee exclusion in the forest, and meadow seed production was nearly zero regardless of visitor treatment. Primary pollinators mediated the relationships between seed production and (1) flower production in the forest, (2) spur length in the forest, and (3) blooming date in the meadow. Spatial and temporal variation in secondary pollinator contributions to seed set

may mediate the importance of primary pollinators for determining patterns of female reproduction and phenotypic selection on floral traits.

INTRODUCTION

The ecology and evolution of plant species is often closely tied to the behavior and population dynamics of animal species with which they interact (Leimu and Lehtila 2006, McCall and Irwin 2006, Kolb et al. 2007, Styrsky and Eubanks 2007), but the outcomes of plant-animal interactions may vary with changes in the biotic or abiotic environment (Wootton 2002, Reynolds et al. 2003, Strauss and Irwin 2004, Agrawal et al. 2006). For example, pollinators can have strong effects on plant reproduction and natural selection on floral traits, but the strength and direction of pollinator effects may depend on the assemblage of other floral visitors (Aigner 2001, Greenleaf and Kremen 2006). Variation in the floral visitor assemblage associated with plant species can create spatial and temporal mosaics of plant fitness and phenotypic selection on floral traits (Nattero and Cocucci 2007).

The relative role of a plant's primary pollinators in mediating seed production and natural selection on phenotypic traits may depend in part on the availability of secondary pollinators (i.e., less common or less efficient pollinators). When a plant's primary pollinators are scarce or absent, secondary pollinators may provide adequate pollination services to sustain plant reproduction (Scott 1989, Kandori 2002, Castellanos et al. 2003, Memmott et al. 2004). However, in the presence of primary pollinators, if secondary pollinators transfer pollen less efficiently, deplete nectar that might attract more efficient primary pollinators, or produce offspring that could consume fertilized ovules, then

secondary pollinators may have a net cost for plant reproduction (Schwartz and Hoeksema 1998, Stanton 2003, Thomson 2003). Depending on their floral visitation preferences, secondary pollinators could also magnify or mask selection pressures exerted by primary pollinators (Castellanos et al. 2004, Janecek et al. 2007). Thus, secondary pollinators may have mixed effects (positive, null, or negative) on natural selection and natural selection on floral traits. Yet, few studies have experimentally manipulated the pollinator assemblage to test whether pollinators mediate natural selection on floral traits (Strauss et al. 2005).

If certain floral traits are correlated with pollinator visitation and plant fitness, a variety of factors could still combine to affect the overall relationship between floral traits and seed production. Pollinators can mediate natural selection on a variety of floral traits that influence pollinator attraction or pollinator efficiency (Schemske and Bradshaw 1999, Huang and Fenster 2007). For example, high flower production could indirectly benefit male or female plant fitness by increasing pollinator attraction (Grindeland et al. 2005, Ishii and Harder 2006). However, flower production may also directly affect seed production by providing more ovules for seed production (Cariveau et al. 2004, Zhao et al. 2007). Similarly, flowering phenology may not only affect plant fitness through synchronizing blooms with pollinator abundance, but also by synchronizing blooms with abiotic resource availability for seed production, pollen donor (male flower) availability, or seed predator abundance (Gross and Werner 1983, Pellmyr and Thompson 1996, Brody 1997, Wright and Meagher 2003, Hirao et al. 2006).

The potential roles of primary and secondary pollinators in mediating seed production and selection on floral traits raise several questions. When and where might a

plant's primary versus secondary pollinators be essential for female reproduction (Scott 1989)? If primary pollinators are more important than secondary pollinators for seed production, does it necessarily follow that they are also more important in mediating selection on floral traits (Thomson et al. 2000, Thompson and Cunningham 2002)? These questions remain largely unexamined in most plant species. Thus, in this study, we tested how excluding primary pollinators affected seed production and natural selection on floral traits through female reproduction in the perennial wildflower, *Delphinium barbeyi*, in two sites (forest and meadow populations) over two years.

The spurred flowers of *D. barbeyi* attract bumble bee pollinators, which comprise 96-100% of floral visits, as well as flies, which lay their eggs on immature *D. barbeyi* carpels. While bumble bees have been conventionally considered the primary pollinators of *D. barbeyi* (Inouye 1978, Waser 1982, Williams et al. 2001), the degree to which adult flies also facilitate seed production as secondary pollinators is unknown. In addition, we do not know if and how either visitor type (bumble bees versus flies) mediates natural selection on floral traits. We combined observations and pollinator exclusions to test the effects of primary and secondary pollinators on *D. barbeyi* seed production and natural selection on floral traits. Specifically, we addressed three questions:

1. *How do primary and secondary pollinators affect female reproduction in D. barbeyi?* In two years and two sites, we compared seed production among plants that could be visited by: (a) all visitors, (b) flies only, or (c) no visitors. If flies can compensate for the loss of bumble bee pollination services, then control and fly only plants should perform similarly, and both should outperform plants with all visitors excluded, assuming that floral visitors are important for female plant reproduction.

2. *To what degree are floral traits associated with D. barbeyi pollen receipt and seed production?* In 2006, we quantified the natural relationships between (a) floral traits and pollen receipt per flower and (b) floral traits and seed production per flower and per plant. Pollinator-mediated selection on floral traits via pollinator attraction or visitation efficiency should affect pollen receipt and seed production per flower.

3. *How do primary pollinators affect the relationship between D. barbeyi floral traits and seed production?* In 2006, all three floral traits were correlated with pollen receipt and/or seed production per flower in one or both sites. Therefore, in 2007, we experimentally tested whether primary pollinators mediated selection on floral traits by comparing the floral trait-seed production relationship between control plants and plants from which primary pollinators were excluded. If primary pollinators mediate selection on floral traits, we expected to find no relationship between floral traits and seed production in the absence of primary pollinators.

METHODS

Study system

Delphinium barbeyi (Ranunculaceae, Huth) is an herbaceous perennial that grows in distinct patches in moist shaded and open sites (Kershaw et al. 1998). We studied two *D. barbeyi* populations near the Rocky Mountain Biological Laboratory in Colorado, USA (elev. 2900 m). The first population was in a series of forest clearings along the Copper Creek drainage (4,316,079°24'17.7"N, 330, 626°8'0.3" E: 3119 m). The second population was in a meadow near the junction of Copper Creek and the East River, 3.9 km from the forest site (4,313,678°20'16.5"N, 327,779°2'2.4" E: 2871m). *Delphinium*

barbeyi plants in the forest population were about half as dense (0.25 ± 0.05 plants/m², n = 12 100 m² plots) as they were in the meadow (0.47 ± 0.02 plants/m², n = 30 100 m² plots). Across habitats, flowering *D. barbeyi* plants produce 13.6 ± 0.5 inflorescences (mean \pm 1 SE, n = 420 plants), and each inflorescence bears 25.4 ± 0.8 bluish-purple flowers (n = 372 inflorescences).

Delphinium barbeyi flowers are protandrous and self-compatible, but they produce few seeds autogamously (Williams et al. 2001). Flowers from which pollinators are excluded produce at least an order of magnitude fewer seeds than open-pollinated flowers (plants with all pollinators excluded = 1.1 ± 0.6 seeds per flower (n = 8 plants), open control plants = 13.4 ± 4.5 seeds per flower (n = 7 plants), Williams et al. 2001). Therefore, plants require pollinator visits to maximize seed set. *Delphinium barbeyi* plants may vary in the degree to which their seed production is pollen-limited. In 1990, hand-pollinated flowers on three out of six plants produced twice as many seeds as control flowers, but hand-pollinated flowers on the remaining three study plants produced 21-37% fewer seeds than control flowers (Williams et al. 2001). In 2005, average *D. barbeyi* seed set was not pollen-limited in one meadow site (see Chapter 3). However, pollen receipt and seed production varies among populations and habitats (forest versus meadow), suggesting that pollen limitation of seed set may also vary among habitats (see Chapter 3). *Delphinium barbeyi* flowers produce nectar in two nectar spurs that are enclosed by a modified sepal. In the morning, before pollinator visits begin, individual flowers contain 1.8 ± 0.05 μ L of nectar with $36.1 \pm 0.7\%$ sugar concentration (mean \pm 1 SE, n = 512 flowers for nectar and n = 34 flowers for sugar, S. E. Elliott, *unpublished data*).

At our study site, the long-tongued bumble bee, *Bombus appositus*, is the most common pollinator of *D. barbeyi* (Inouye 1976, see Chapter 3). Flowers are also visited less frequently by other bumble bees (*B. flavifrons*, *B. bifarius*, *B. frigidus*, *B. nevadensis*, *B. occidentalis*), hummingbirds (*Selasphorus platycercus*, *Selasphorus rufus*, and *Stellula calliope*), and sphinx moths (*Hyles lineata*) (Inouye 1976, Waser 1982, Williams et al. 2001). In 2005, in the first and second halves of *D. barbeyi*'s blooming period, all bumble bee species collectively accounted for 96.9% and 100% of all flower visits in the meadow study site (see Chapter 3). In 2007, in a nearby stretch of meadows, bumble bees accounted for 97.9% of all flower visits (see Chapter 2). Thus, bumble bees are the primary pollinators of *D. barbeyi* based on visitation rates. Seed set does not differ between flowers visited by *B. appositus* and the second most-frequent visitor, *B. flavifrons* (R.E. Irwin, *unpublished data*). However, the relative pollination efficiencies of the other visitors are unknown.

Adult flies also visit *D. barbeyi* flowers and may act as secondary pollinators of *D. barbeyi* flowers. In 2005 and 2006, in nearby meadows, we recorded flower interactions with all visitors, including flies and found that flies contributed to 0.3-0.6% of flower visits to *D. barbeyi* (R. E. Irwin, *unpublished data*). Most fly visitors are probably in the *Botanophila* genus or in a related genus in the Anthomyiini tribe, but positive identification awaits collection of male individuals. The flies may be consuming pollen, but it is unlikely that they consume nectar, which is contained deep in *D. barbeyi* nectar spurs. Along with visiting flowers legitimately through the front of the flower, female flies deposit eggs singly or in groups on the carpels prior to fruit expansion. At our study site in 2006, approximately 10% of all seeds were lost to these fly seed

predators; $22.9 \pm 1.8\%$ of fruits per inflorescence were attacked, and attacked fruits lost $53.3 \pm 0.8\%$ of their seeds (mean \pm 1 SE, $n = 129$ inflorescences). We do not know whether these flies are also pollinators, but we test their importance as secondary pollinators in this study.

Field Methods

*1. How do primary and secondary pollinators affect female reproduction in *D. barbeyi*?*

In 2006 and 2007, we compared seed production among three treatments: all visitors (open control), flies only (bumble bees and other large visitors excluded), and no visitors (all visitors excluded). The all visitors (open control) treatment was dominated by bumble bees (see *Study system*). We excluded bumble bees (as well as hummingbirds and sphinx moths) by bagging inflorescences with 0.635-cm mesh. We refer to this treatment as ‘fly only’ because flies were the only organisms we ever saw in the bags, and no other small visitors were observed (one Megachilidae bee was observed on an unbagged flower in 2007, but it contributed to $< 0.1\%$ of observed visits, and we do not know if it would enter the mesh). We excluded all visitors by bagging inflorescences with bridal veil (~ 0.1 -cm mesh). We refer to this treatment as ‘no visitors’.

We bagged one randomly selected inflorescence per plant, and we used inflorescence as the unit of replication (hereafter, we simply refer to these single inflorescences per plant as plants). Plants probably did not reallocate resources from bagged to open inflorescences because flowers on open inflorescences with bagged inflorescences on the same plant produced similar numbers of seeds as flowers on plants with no bags (Appendix I). In 2006, we had 21-24 plants per treatment per site (one site

each in the meadow and forest), and in 2007, we had 38-62 plants per treatment per site. To control for any potential constricting effects of bags on inflorescence growth, we tied a plastic label around the control plants in the same manner that we tied bags to treated plants. Bags did not affect blooming (Appendix I).

We measured seed production as (1) the probability that plants produced any seeds and (2) the average seed production per inflorescence. We collected mature fruits from the focal inflorescences and counted the number of seeds per fruit. We distinguished between seeds consumed by the fly larvae (seed coats remain intact) and seeds surviving past seed predation. To determine total seeds developed, we summed seeds consumed and seeds surviving.

Statistical analyses – We used chi-squared tests to compare the proportion of plants that produced any seeds between control and fly only treatments. We performed four tests—one for each year-site combination. We used a mixed-model ANOVA to compare the number of seeds surviving per inflorescence among treatments (open control, fly only, or no visitors), sites (forest or meadow), and years (2006 or 2007). We treated year as a random factor, site as a random factor nested within year, and visitor treatment as a fixed factor. We tested for interactions between year and treatment and between site and treatment. We \log_{10} -transformed surviving seed production to reduce right skew. We do not report results for developed seeds because the results were qualitatively the same for surviving seeds per inflorescence, which is a more realistic measure of final reproductive output. We used Tukey-Kramer multiple comparisons to test for significant differences ($\alpha = 0.05$) in mean seed production among treatments,

separating analyses by site and year. These and all subsequent analyses were performed with JMP v. 4.04 (SAS Institute 2001).

2. To what degree are floral traits associated with D. barbeyi pollen receipt and seed production? We focused on three floral traits (flower production, blooming date, and spur length) because they have been linked to pollinator visitation and/or female reproduction in other systems (Gross and Werner 1983, Herrera 1992, Norberg et al. 1993, Johnson and Steiner 1997, Ohashi and Yahara 2000, Lacey et al. 2003, Cariveau et al. 2004, Lavergne et al. 2005, Whittall and Hodges 2007). In 2006, we measured floral traits, pollen receipt, and seed production on open-pollinated plants only (not the same individuals used in Question 1).

Flower production – We defined flower production as the number of flowers produced per focal inflorescence. Flower production may affect seed set by attracting more pollinator visits or by affecting the amount of outcrossing via increased within-plant movements (Williams et al. 2001). High flower production is also often associated with increased fruit and seed set because a plant with more flowers has more ovules to potentially produce seeds (Ohashi and Yahara 2000, Cariveau et al. 2004). Flower production per inflorescence varies more among plants than within plants and increases with the number of inflorescences per plant (Appendix II).

Blooming date – We quantified blooming date as the date of the first open flower on each focal plant. Pollinator visitation rate to *D. barbeyi* increases and male-to-female flower ratio decreases over *D. barbeyi*'s flowering season, creating a potential trade-off

for optimal blooming time (see Chapter 3). Blooming date for plants in 2007 was positively correlated with their blooming date in 2006 (Appendix II).

Spur length – We measured spur length as the distance from the opening of the spur to its tip (ignoring minor spur curvature) to the nearest 0.01 mm with digital calipers. We collected up to six flowers from the non-focal inflorescences, once during the first half of the blooming period, and again during the second half of the blooming period (6-12 flowers per plant). Measuring six flowers per inflorescence provides a reliable estimate of spur length for detecting among-plant variation (spur length varies more among than within plants), and flowers from inflorescences on the same plant do differ significantly from each other in average spur length (Appendix II).

Pollen receipt and seed production – To determine whether floral traits were associated with pollen receipt and seed production (either by attracting more pollinators or improving pollinator efficiency), we quantified pollen receipt and seed production per flower. We sampled stigmas from flowers from the focal inflorescence after corollas had fallen off and before carpels started to expand. We staggered stigma collection throughout the blooming period (forest: 10 July-11 August, meadow: 5 July-10 August), collecting a total of six flowers per focal inflorescence. We mounted stigmas in fuchsin dye and counted conspecific pollen grains with a compound microscope (Kearns and Inouye 1993). We counted seeds from all fruits on the focal inflorescences. We divided seed number by flower number to calculate seeds per flower (for seeds that developed and survived seed predation). To estimate seed production per plant, we multiplied the number of flowering inflorescences per plant by the number of seeds per focal inflorescence. Seed production (developed seeds per inflorescence) is more variable

within plants than among plants, and seed production varies between sites (two inflorescences sampled per plant on 98 plants in two sites; Overall nested ANOVA model $F_{1,97} = 10.4$, $P < 0.0001$; Site $F_{1,1} = 49.1$, $P < 0.0001$; Plant(Site) $F_{1,48} = 9.5$, $P < 0.0001$).

Statistical analyses – To determine whether floral traits were associated with pollen receipt and seed production (1) we used two-tailed t-tests to compare floral traits between plants that produced seeds and plants that did not produce seeds, and (2) we used multiple regressions to test the effects of floral traits on pollen receipt per flower and seed production (per flower and per plant, for seeds developed and for seeds surviving seed predation). We averaged per flower pollen receipt and seed production for the focal inflorescences, so the focal plant is the unit of replication in all cases. We separated analyses by site because the range in floral traits varied between sites and because we sampled the sites at different times due to different peak blooming times. If floral traits influence the frequency or efficiency of pollinator visits per flower, then floral traits should be related to seed production per flower, assuming that seed production is pollinator-limited and pollen-limited. We standardized floral traits to a mean of zero and a standard deviation of one and used relative seed production (i.e., we calculated standardized selection gradients, Lande and Arnold 1983). To determine whether traits were correlated with each other, we tested correlations among traits within sites and years. We also calculated variance inflation factors (VIFs) to test for multicollinearity among traits within sites; all VIFs were small (< 1.27), so we included all three traits in the analyses (Neter et al. 1996).

3. *How do primary pollinators affect the relationship between D. barbeyi floral traits and seed production?* In 2007, we measured floral traits on open control and fly only plants (same plants used in 2007 for Question 1). If primary pollinators mediate the benefits of floral traits for seed production, then excluding primary pollinators should result in no relationship between seed production and floral traits.

Statistical analyses – We used logistic regressions to test the effects of primary and secondary pollinators (control = both primary and secondary; fly only = secondary only), floral traits, and interactions between pollinators and floral traits on the probability of producing seeds. We did not test for effects on average seed production per inflorescence because the majority of plants produced no seeds in 2007, irrespective of pollinator treatment. We used 2-way ANOVAs to test: (1) whether floral traits differed between plants that produced seeds and plants that did not produce seeds and (2) whether this effect was stronger for control plants than fly only plants. We separated analyses by trait and site. Because some of the interaction terms (trait x seeds y/n) were marginally significant ($0.05 < P < 0.08$), we used two-tailed t-tests to compare traits between plants that did and did not produce seeds, separating analyses by treatment.

RESULTS

1. *How do primary and secondary pollinators affect female reproduction in D. barbeyi?*

In the forest, fly only plants were less likely to produce seeds than control plants in 2007, but not in 2006 (2007: $\chi^2_{1,1} = 7.91$, $P = 0.005$, 2006: $\chi^2_{1,1} = 1.69$, $P = 0.2$; Figure 1A). In contrast, in the meadow, fly only plants were less likely to produce seeds than control plants in 2006, but this trend was not statistically significant, and in 2007, fly only and

control plant were equally likely to produce seeds (2006: $\chi^2 = 2.82$, $P = 0.09$, 2006: $\chi^2 = 0.57$, $P = 0.5$; Figure 1B).

Visitor treatment (open control, fly only, or no visitors) affected *D. barbeyi* seed production per inflorescence, but the magnitude of this effect varied between sites and years (Site(Year) x Visitor treatment: $F_{4, 424} = 8.69$, $P < 0.0001$; Figure 2, Table 1). In 2006, fly only plants produced fewer seeds than control plants, but the difference was only statistically significant in the meadow (Figure 2). In 2006, seed production of fly only plants was higher than no visitor plants in the forest, but not in the meadow (Figure 2). In 2006, control plants produced 80% more seeds in the meadow than in the forest (Figure 2). In contrast, in 2007, most meadow flowers produced no seeds, regardless of visitor treatment (Figure 2B), and meadow control plants produced ten times *fewer* seeds than in the forest (Figure 2A). In 2007, seed production of fly only plants did not differ from plants in either control or no visitor treatments, but control plants produced more seeds than no visitor plants (Figure 2D). Visitor treatment did not affect seed weight or germination rate (Appendix I).

2. *To what degree are floral traits associated with D. barbeyi pollen receipt and seed production?* Flower production, blooming date, and spur length varied between sites and years (Figure 3). There were also some site- and year-specific correlations among some of the floral traits (Table 2). Flower production decreased with blooming date in the forest in both years and in the meadow in 2007 (Table 2). In 2006 only, spur length increased with blooming date in the meadow (Table 2).

In 2006, flower production, blooming date, and spur length were all related to relative seed production at some plant scale (total seeds per plant or average seeds per flower per inflorescence) in one or both sites (Table 3). Flower production had the strongest and most consistent effects on relative seed production. Plants that produced seeds had 62-75% higher flower production than plants that did not produce seeds (Forest: $t_{136} = 3.9$, $P = 0.0001$; Meadow: $t_{153} = 5.1$, $P < 0.0001$; Figure 4A). Relative pollen receipt and seed production per flower increased with flower production on the focal inflorescences in the meadow, but not in the forest (Table 3). In both sites, relative seed production per plant also increased with flower production (Table 3).

Blooming date had weak effects on seed production (Table 3). In the forest, plants that produced seeds bloomed three days later than plants that did not produce seeds ($t_{136} = 2.2$, $P = 0.03$; Figure 4B). In the meadow, blooming date did not differ between plants that produced seeds and those that did not ($t_{153} = 0.6$, $P = 0.5$; Figure 4B). While relative pollen receipt per flower increased with blooming date in both sites, blooming date did not affect relative seeds per flower or seeds per plant (Table 3).

Spur length had weak and variable effects on seed production (Table 3). In the forest, plants that produced seeds had marginally (3%) longer spurs than those that produced no seeds ($t_{136} = 1.8$, $P = 0.075$; Figure 4C). In the meadow, there was no difference in spur length between plants that produced seeds and those that did not ($t_{153} = 1.5$, $P = 0.14$; Figure 4C). In the meadow, relative pollen receipt per flower increased with spur length, but relative seed production did not (Table 3). In contrast, in the forest, although relative pollen receipt did not increase with spur length, relative seed production

did increase with spur length (Table 3). Spur length was not associated with relative seed production per plant in either site (Table 3).

3. How do primary pollinators affect the relationship between D. barbeyi floral traits and seed production? In 2007, excluding bumble bees (and other large visitors) did not alter the relationships between floral traits and the probability of producing seeds (Table 4). Regardless of visitor treatment (control or fly only), plants with high flower production were more likely to produce seeds (Forest: $\chi^2 = 9.9$, $P = 0.003$; Meadow: $\chi^2 = 11.6$, $P = 0.0006$). In the forest only, later-blooming plants were marginally more likely to produce seeds ($\chi^2 = 3.0$, $P = 0.08$), irrespective of visitor treatment. Spur length did not affect the probability of producing seeds ($\chi^2 = 0.3$, $P = 0.6$).

In 2007, flower production was 32-37% higher in plants that produced seeds than plants that did not produce seeds, but this effect was not significant for fly only plants in the forest (Figure 4A, Table 5). In the meadow, average blooming time was two days later for control plants that produced seeds than those that did not produce seeds. However, this effect was only marginally statistically significant ($P = 0.055$), and did not hold for plants from which primary pollinators were excluded or for forest plants (Figure 4B, Table 5). For control plants only, spur length varied marginally between plants that produced seeds and those that did not (Figure 4C, Table 5). In the forest, spurs from control plants that produced seeds were 5.7% longer than those that did not produce seeds ($P = 0.07$, Figure 4C). In the meadow, spurs from control plants that produced seeds were 4.1% shorter than plants that did not produce seeds, but this effect was not statistically significant ($P = 0.18$, Figure 4C).

DISCUSSION

Pollinators can have strong effects on plant reproduction and selection on floral traits, but the relative effects of primary pollinators may depend on the availability and efficiency of alternative secondary pollinators (Aigner 2001, Stanton 2003). We found that excluding *Delphinium barbeyi*'s primary pollinators had different effects on seed production and selection on floral traits in different sites, and site-specific effects varied between years. In 2006, secondary pollinators compensated for primary pollinator exclusion in the forest site, but not in the meadow site. In contrast, in 2007, secondary pollinators did not compensate for primary pollinator exclusion in the forest; and in the meadow, most flowers produced no seeds, regardless of visitor treatment. In 2007, in the meadow (where many plants produced no seeds regardless of visitor treatment), excluding primary bumble bee pollinators masked selection on blooming date. In contrast, in the forest, excluding primary bumble bee pollinators dampened selection on flower production and on spur length. This study demonstrates strong spatial and temporal variability in the effects of different pollinators in mediating seed production and selection on floral traits through female reproduction in a perennial wildflower.

Variation in the contributions of pollinator species to seed set have been found using pollinator exclosures in other studies. For example, by excluding hummingbirds from *Fouquieria splendens* flowers, Waser (1979) found that carpenter bees, which extract nectar by piercing the side of *F. splendens*'s red tubular flowers, can also effectively pollinate the flowers. Also, in some geographic areas, carpenter bees are the only common pollinator visitors of *F. splendens* (Scott 1989). Excluding non-native

honey bees from *Centaurea solstitialis* flowers, and only allowing visits by smaller native bees, demonstrated that honey bees were important for promoting seed set in the invasive plant in most areas, but not on an island site where native bees could provide adequate pollination services in the absence of honey bees (Barthell et al. 2001). By excluding visitors at different times of the day from *Lavandula latifolia* flowers, Herrera (2000) demonstrated the importance of mid-day pollination services by butterflies and small bees on seeds set and seedling recruitment. Our study also demonstrates spatial and temporal variability in the contributions of secondary fly visitors to *D. barbeyi* pollination and seed set.

Several hypotheses could explain why secondary (fly) pollinator effects varied between sites and years. (1) The flies may have contributed most strongly to seed production where they were more abundant. If the adult flies that visited and pollinated flowers were also the flies that laid their eggs on immature carpels, then an increase in fly pollinator abundance should be positively correlated with an increase in seed predation (Ferdy et al. 2002). As in other systems, seed predation had weak effects on seed production, and all results were qualitatively the same for total seeds developed versus seeds surviving seed predation (Thompson and Pellmyr 1992). If higher fly abundance facilitated seed set in the forest in 2006, then we should have found higher fly seed predation in the forest than in the meadow, but we found no evidence for either of these scenarios (Appendix III). (2) Flies might avoid fly only plants if they sensed that the flowers had not been pollinated and thus, would not produce sufficient seeds for their offspring (Brody and Waser 1995, Fenner et al. 2002, Cariveau et al. 2004). If flies were more abundant in 2006, then they might have only visited the bagged plants because the

open plants were already saturated with eggs or depleted of the resource they were collecting (e.g., pollen). Similarly, if flowers were more abundant in 2007 (e.g., flower production per inflorescence was higher in 2007, Figure 3), then flies may have avoided bagged plants because they had alternative open flowers on which to oviposit. (3) The bags themselves could also be a deterrent to flies, although in 2006, flies did not avoid bagged plants. In another study, small bees in the Halictidae are not deterred by bagged *Centaurea solstitialis* flowers (Barthell et al. 2001), but we are not aware of other studies that have measured fly responses to bags. Future work in this system should consider which factors influence the behavior and population growth of the secondary fly pollinators/seed predators, such as resource availability for flies, larval survival rates, competition for oviposition sites, or avoidance of bagged or low-pollinated plants.

The decrease in meadow seed set across all treatments in 2007 could have resulted from changes in the biotic or abiotic environment. (1) In other systems, variation in bee pollinator composition and abundance can influence spatial and temporal variation in seed production (Price et al. 2005, Stone and Jenkins 2008). However, a decrease in visitation rates to *D. barbeyi* flowers in 2007 probably did not cause the decrease in seed set since visitation rates were comparable to previous years in the focal meadow when seed set was high (Appendix IV). (2) If pollinator visitation rates had been important for seed production, the earlier blooming in 2007 could have also had a negative effect on seed production because pollinator visitation rates are generally lower earlier in *D. barbeyi*'s blooming period (see Chapter 1 and 3). However, in 2007, meadow plants that bloomed earlier were more likely to produce seeds than later blooming plants (Figure 4). (3) The meadow seed set decline may have been influenced by late spring low

temperatures that occurred 10-20 days before the first study plants began to bloom (Appendix V). A late spring frost may have damaged the sexual organs of developing flowers, while still allowing them to bloom and attract pollinators (Julian et al. 2007). At high elevations, plants may be particularly susceptible to damaging frost events (Hoye et al. 2007). (4) An earlier snow melt in 2007 (12 days earlier than in 2006, Rocky Mountain Biological Laboratory 2008), correlated with earlier blooming (blooming time in 2007 versus 2006: $t_{79} = 7.4$, $P < 0.0001$, Figure 3) may have also influenced *D. barbeyi* susceptibility to frost damage (Inouye et al. 2002). Therefore, plants in 2007 may have started to develop before frost danger had past, while plants in the forest may have been protected from frost damage by later blooming and the tree canopy.

Although flower production was the strongest predictor of seed production out of the three floral traits measured, it was also the least consistent within plants from 2006 to 2007 (Appendix II) and could be linked to abiotic factors. In *D. barbeyi*, as in other species, the benefit of high flower production could be linked to higher ovule number, regardless of the effects of flower production on pollinators or seed predators (Cariveau et al. 2004). For example, in 2006, only meadow plants had higher per-flower pollen receipt and seed production with higher flower production, which suggests that, at least in the forest, pollinators were not preferentially visiting plants with high flower production. However, in 2007, excluding bumble bees (and other large visitors) from forest plants partially reduced the benefit of high flower production for seed production. Instead of flower number per inflorescence, pollinator behavior may be more sensitive to the number of inflorescences per plant (Williams et al. 2001), which only explains part of the variation in flowers produced per inflorescence (Appendix II). The correlations between

flower production and seed production could be spurious if available abiotic resources vary among plants and if abiotic resources affect floral traits, plant fitness, and the relationships between floral traits and plant fitness (Caruso et al. 2003, Caruso et al. 2005, Caruso 2006). For example, patchiness in snowpack between years (and possibility within years) may also influence the variability in *D. barbeyi* flower production (Inouye et al. 2002). Therefore, future work should investigate whether within- and between-site variation in *D. barbeyi* flower production and selection on flower production is explained, at least in part, by variation in water and nutrients.

The weak and variable effects of blooming time and spur length on seed production could have been influenced by conflicting selection pressures, indirect selection via correlations with other traits, and the inability of these traits to influence pollinator behavior (Gross and Werner 1983, Pellmyr and Thompson 1996, Brody 1997, Wright and Meagher 2003, Lavergne et al. 2005, Hirao et al. 2006). Because later-blooming plants produced fewer flowers, selection on flower production could indirectly lead to negative selection on blooming date. For example, meadow seed production increased with earlier blooming, and this trend was not mediated by bumble bee pollinators. In 2006, later blooming plants received more pollen, possibly because pollinator visitation rates to *D. barbeyi* generally increase later in the season (see Chapter 3). Similarly, meadow plants with longer spurs received more pollen, suggesting that longer spurs may facilitate more or higher quality pollinator visits. However, in both cases, the increase in pollen receipt with earlier blooming or with longer spurs did not translate into an increase in seed production, possibly because abiotic resources limited seed production (Williams 2001, see Chapter 3).

Pollinators may not only affect seed production, but also seedling recruitment and male reproductive success. For example, over longer time periods, fluctuations in abiotic and biotic factors (e.g., frost events and primary and secondary pollinator abundance) might influence the relative fitness of plants in different habitats (Ashman et al. 2004, Maron and Kauffman 2006). In perennial plants such as *D. barbeyi*, pollinator effects could be masked by species interactions at later life history stages or by costs of current reproduction on future survival and reproduction (Calvo and Horvitz 1990, Ehrlén 2002). In addition, the relationships we measured between floral traits and seed set could be enhanced or reversed by selection via male fitness (Stanton et al. 1991). In particular, early blooming may benefit pollen receipt and female fitness (higher male-to-female ratios, see Chapter 3), while late blooming might benefit pollen export and male fitness.

In general, we found very different effects of pollinators on seed set and selection on floral traits in the two sites and years. The sites were notably different in that one was in forest clearings and the other was in an open meadow, and replicating the study across many forest and meadow habitats may provide further insights into the degree to which pollinator effects are contingent on habitat type. In general, meadow sites may be favorable in terms of attracting more pollinators and having sufficient heat and sunlight for photosynthesis. For example, *Campanulastrum americanum* plants growing in sunlight have larger floral displays and receive seven times more insect pollinator visits than plants in the shade (Kilkenny and Galloway 2008). However, the numerous flowers in meadows may also compete with each other for pollinators, which could affect the degree of pollen limitation of seed set between forest versus meadow habitats (Hansen and Totland 2006). The open meadows could also expose flowers to damaging frost

events (Yamada and Takahashi 2004). Resource allocation to male versus female flowers may also vary between shady and exposed habitats (Zhao et al. 2007).

In conclusion, we found that excluding primary pollinators (mostly bumble bees) can greatly reduce seed production. However, secondary pollinators (flies) can sometimes compensate for lost primary pollinator services. Similarly, floral traits were associated with seed production, but the degree to which pollinators mediated these relationships varied between sites. Within the same system, the effects of excluding primary pollinators on seed production and natural selection on floral traits can vary greatly between nearby populations. Future studies should test how specific habitat characteristics might alter the nature of the relationships between primary and secondary pollinators and their host plants. Seemingly unimportant visitors (e.g., those that visit flowers infrequently or are known mostly as seed predators) may have a net benefit on plant reproduction in the absence of primary pollinators.

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Table 1. ANOVA testing the effects of year (2006 or 2007), site (forest or meadow), and visitor treatment (control, flies only, or all visitors excluded), and 2-way interactions with visitor treatment (VT) on \log_{10} -transformed *Delphinium barbeyi* surviving seeds per inflorescence. Plant is the unit of replication with one focal inflorescence sampled per plant (n = 21-62 plants per treatment for a total n = 436 plants; full model $F_{11,424} = 30.3$, $p < 0.0001$).

Source	DF	SS	MS	F	P
Year	1,2	49.8	49.8	2.79	0.2
Site(Year)	2,424	35.9	17.9	34.54	< 0.0001
Visitor treatment	2,2	63.0	31.5	9.85	0.09
Year*VT	2,4	6.4	3.2	0.71	0.5
Site(Year)*VT	4,424	18.1	4.5	8.69	< 0.0001
Error	1,2	220.1	0.5		

Table 2. Correlations among *Delphinium barbeyi* floral traits (flowers produced per inflorescence (Flowers), date of first bloom (Date), and spur length) in two years (2006, 2007) and two sites within each year (forest, meadow).

	2006				2007			
	Forest		Meadow		Forest		Meadow	
	Flowers	Date	Flowers	Date	Flowers	Date	Flowers	Date
Date of first bloom	-0.25 *		0.06		-0.22 *		-0.19 *	
Spur length	0.07	0.15	0.06	0.44 *	0.01	0.06	0.04	0.01

* correlations significant at $\alpha = 0.05$

Table 3. Relationship between *Delphinium barbeyi* relative seed production (developed (DEV) and surviving (SURV) seeds per plant (= seeds per focal inflorescence * number of inflorescences per plant), pollen grains per flower, and developed and surviving seeds per flower) and standardized floral traits (flowers produced per inflorescence, and date of first bloom, average spur length), measured in 2006. Selection gradients, with 95% confidence intervals in parentheses, are bolded if significant at $\alpha = 0.05$.; n = 129 (forest) and n = 145 (meadow) plants per site.

	Forest			Meadow		
PER PLANT	DEV	SURV	DEV	SURV	DEV	SURV
Flowers per inflorescence	0.53 (0.26,0.81)	0.41 (0.15,0.68)	1.15 (0.94,1.37)	1.12 (0.92,1.32)		
Date of first bloom	-0.13 (-0.41,0.15)	-0.17 (-0.43,0.10)	0.08 (-0.14,0.30)	0.02 (-0.19,0.23)		
Spur length	0.08 (-0.18,0.35)	0.09 (-0.17,0.34)	-0.04 (-0.27,0.20)	-0.09 (-0.31,0.14)		
PER FLOWER	POLLEN	DEV	SURV	POLLEN	DEV	SURV
Flowers per inflorescence	0.04 (-0.08,0.17)	-0.04 (-0.19,0.10)	0.04 (-0.23,0.32)	0.16 (0.06,0.26)	0.25 (0.16,0.35)	0.65 (0.44,0.87)
Date of first bloom	0.22 (0.10,0.35)	0.06 (-0.09,0.20)	0.11 (-0.17,0.39)	0.11 (0.00,0.21)	0.01 (-0.10,0.11)	0.09 (-0.13,0.31)
Spur length	0.02 (-0.10,0.14)	0.13 (0.00,0.27)	0.27 (0.00,0.53)	0.13 (0.01,0.24)	0.06 (-0.05,0.17)	0.20 (-0.04,0.44)

Table 4. Multiple logistic regression testing the effects of visitor treatment (control versus fly only), floral traits (flower production, blooming date, and floral spur length), and their interactions on the probability of producing seeds in *Delphinium barbeyi* in two sites (forest: n = 93 plants; meadow: n = 121 plants) in 2007.

Source	DF	Forest		Meadow	
		Wald χ^2	P	Wald χ^2	P
Visitors	2	6.8	0.03	1.9	0.4
Flower production	1	7.0	0.008	7.0	0.008
Blooming date	1	3.0	0.08	0.6	0.4
Spur length	1	0.3	0.6	1.7	0.2
Bees x Flower production	2	3.7	0.16	0.5	0.8
Bees x Blooming date	2	1.9	0.4	0.7	0.7
Bees x Spur length	2	3.1	0.2	0.04	0.98

Table 5. ANOVA testing whether *Delphinium barbeyi* floral traits in 2007 differed between plants that produced seeds and plants that did not produce seeds, visitor treatment (control or fly only), and their interaction, separating analyses by site (forest or meadow). T-test comparing traits between plants that produced seeds and plants that did not produce seeds, separating analyses by experimental treatment and site. P-values for associated F- and t-statistics are in parentheses.

	Flower production	Blooming date	Spur length
Seeds:			
$F_{\text{forest},58} =$	13.3 (0.0006)	1.0 (0.3)	0.5 (0.5)
$F_{\text{meadow},77} =$	30.7 (< 0.0001)	3.5 (0.065)	2.1 (0.15)
Visitors:			
$F_{\text{forest},58} =$	1.9 (0.2)	1.1 (0.3)	0.03 (0.9)
$F_{\text{meadow},77} =$	1.7 (0.2)	0.2 (0.6)	0.04 (0.8)
Seeds*Visitors:			
$F_{\text{forest},58} =$	3.3 (0.076)	0.1 (0.8)	3.5 (0.065)
$F_{\text{meadow},77} =$	0.2 (0.6)	0.7 (0.4)	0.03 (0.9)
Seeds, control plants:			
$t_{\text{forest}27} =$	-3.5 (0.002)	-1.0 (0.3)	1.9 (0.073)
$t_{\text{meadow}31} =$	-4.2 (0.0001)	2.0 (0.055)	-1.4 (0.18)
Seeds, bee excluded plants:			
$t_{\text{forest}27} =$	-1.4 (0.16)	-0.5 (0.6)	-0.8 (0.4)
$t_{\text{meadow}31} =$	-3.7 (0.001)	0.7 (0.5)	-0.8 (0.4)

Figure Legends

Figure 1. Proportion of *Delphinium barbeyi* plants that produced seeds in two visitor treatments (control or bee excluded) in two years (2006 and 2007) and two sites (A. forest and B. meadow). Sample sizes for each treatment-site-year combination at the base of bars. One inflorescence was measured per plant.

Figure 2. *Delphinium barbeyi* seeds produced per inflorescence in 2006 and 2007 in two sites (A. forest and B. meadow) from control plants, fly only plants, and plants from which all visitors were excluded. Box plots represent median (solid line in box), mean (dotted line), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers), and points outside the 10th and 90th percentiles (circles) for seeds per inflorescence from n = 21-62 (sample size above each distribution) plants per treatment, with one representative inflorescence sampled per plant. Letters signify treatments with significantly different seed production (at $\alpha = 0.05$), testing each site-year combination separately.

Figure 3. Distribution of *Delphinium barbeyi* floral traits for two sites (forest and meadow) measured in two years (2006, 2007) with sample sizes (number of plants, one representative inflorescence sampled per plant) below each year-site combination: flowers produced per inflorescence (A), Julian date of first bloom (B), and nectar spur length in mm (C). Box plots as in Figure 2.

Figure 4. Comparison of *Delphinium barbeyi* floral traits (A. flowers per inflorescence, B. Julian date of first bloom, and C. spur length (mm)) between plants that did not

produce any seeds (white) versus those that did (gray). Control plants have solid bars; plants with fly visitors only have hatched bars. One inflorescence was sampled per plant from plants growing in one of two sites, (forest or meadow) in two years (2006: control plants only, 2007: control plants and fly only plants)

Figure 1.

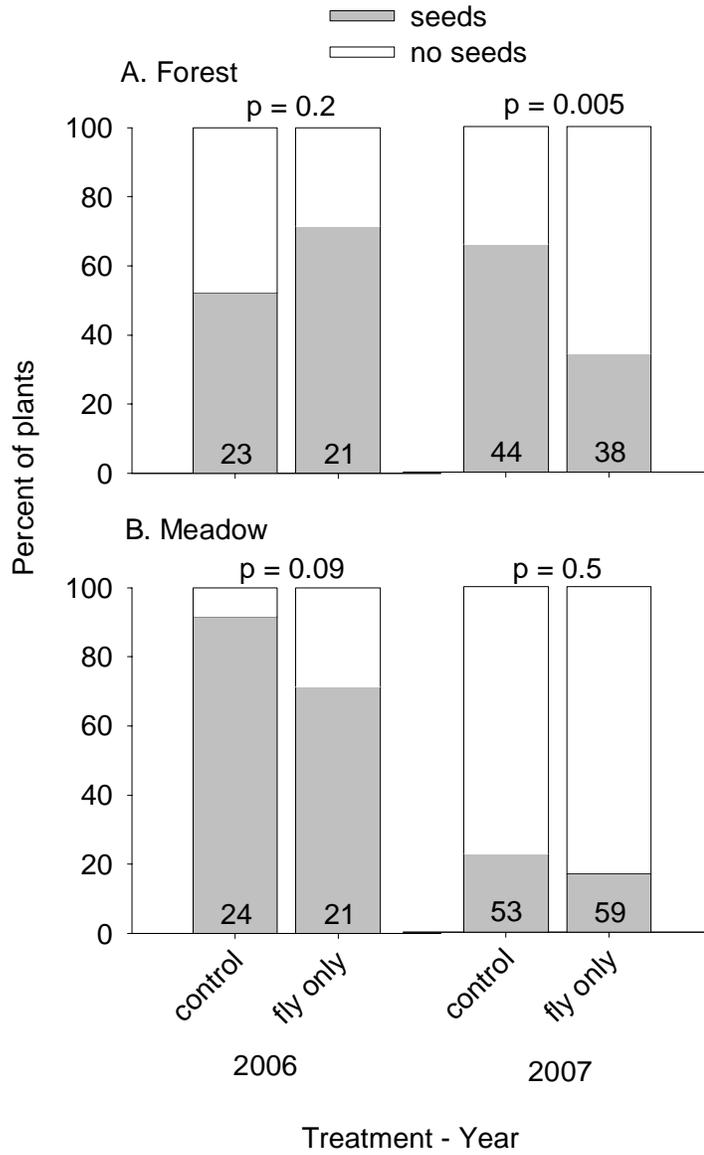


Figure 2.

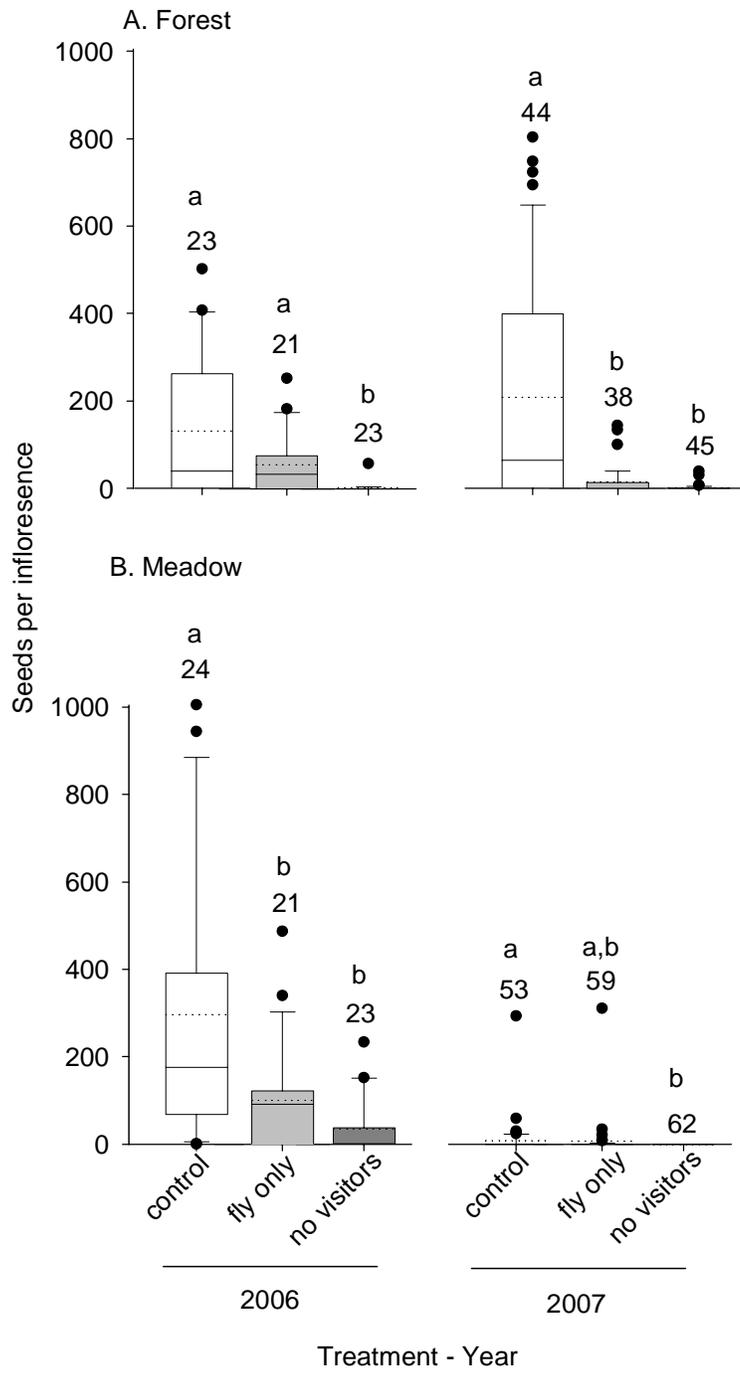


Figure 3.

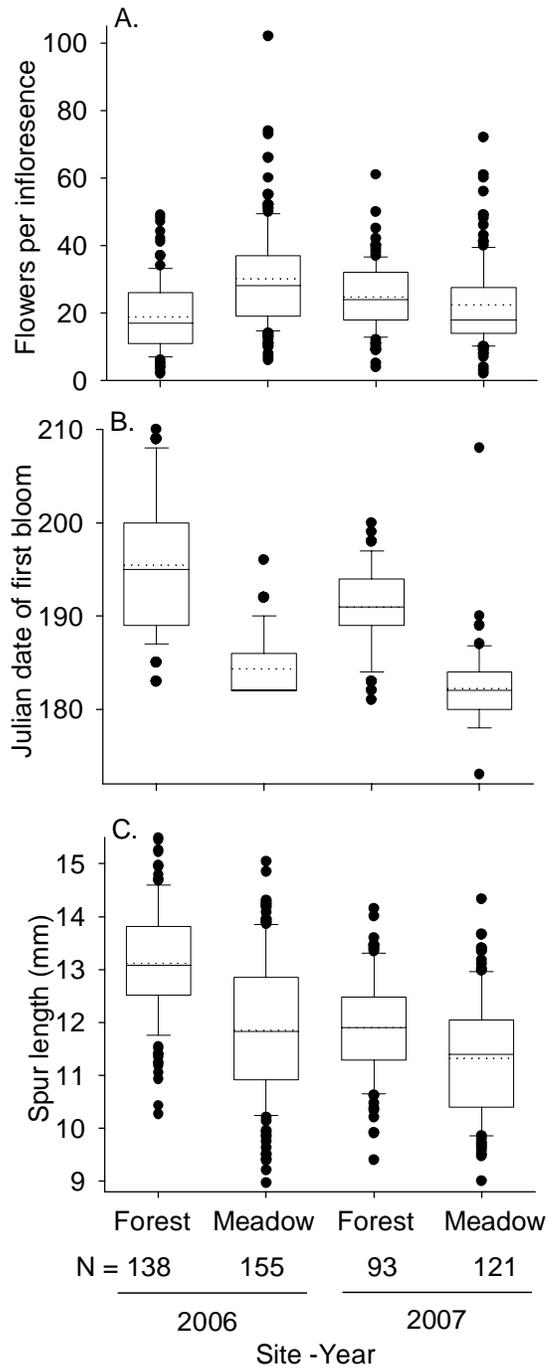
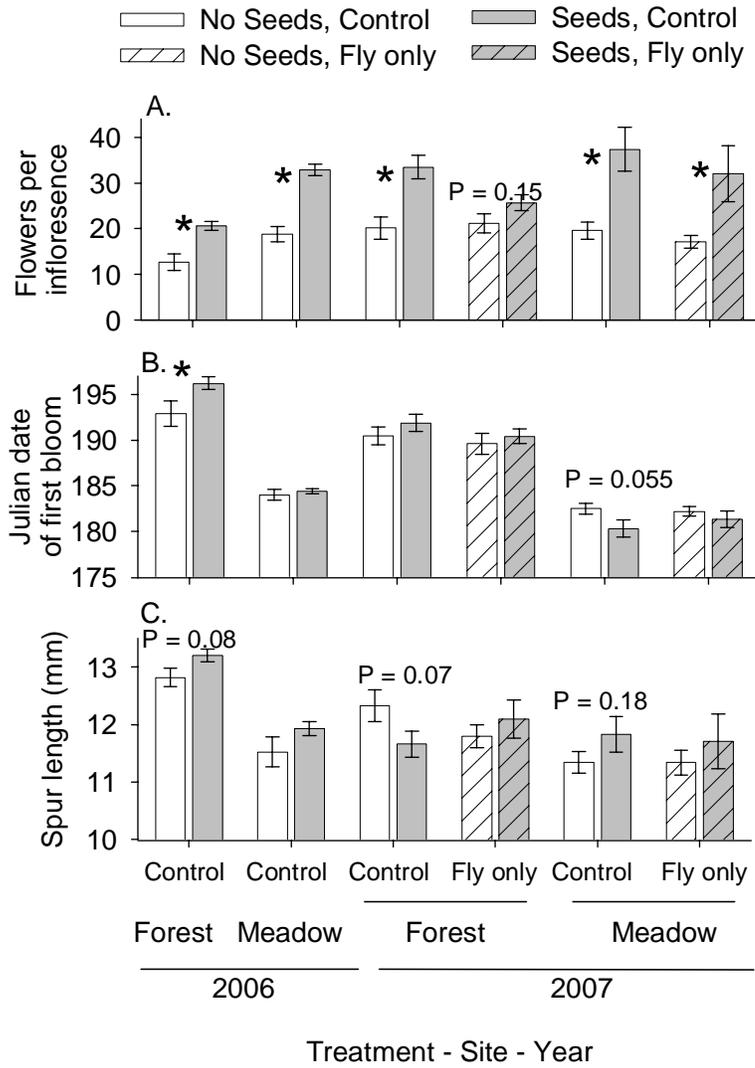


Figure 4.



Appendix I. Effects of bagging treatments on *Delphinium barbeyi* seed production of paired open stalks (A), blooming rate (B. percent of plants that failed to bloom), seed weight (C), and seed germination rate (D. percent of seeds that germinated).

A. ANOVA testing the effect of bagging treatments (control, fly only, or no visitors) on potential resource reallocation to paired open stalks on the same plant in two sites (forest and meadow). Mean (± 1 SE) seeds developed per inflorescence, reported for the open inflorescences that were paired with another open (control) inflorescence or with a treated (fly only or no visitors) inflorescence.

Treatment	Forest		Meadow	
	F _{2,48} = 1.1	P = 0.3	F _{2,66} = 1.7	P = 0.2
	Mean \pm 1 SE	N	Mean \pm 1 SE	N
Control	100.0 \pm 15.2	19	99.7 \pm 11.2	24
Fly Only	133.3 \pm 17.1	15	82.3 \pm 11.4	23
No visitors	111.9 \pm 16.0	17	69.9 \pm 11.6	22

B. Visitor treatments did not affect blooming rates (i.e., percent of plants that failed to bloom): control = 18% of 120 plants, fly only = 21% of 126 plants, no visitors = 17% of 130 plants; $\chi^2_{1,373} = 0.59$, P = 0.7, n = 120-130 plants per treatment.

C. ANOVA testing the effect of visitor treatment (open control, fly only, or no visitors) on seed weight. Average seed weight calculated as the average mg per seed from 10 seeds weighed from individual fruits (mean \pm 1 SE: open control = 1.48 \pm 0.04 mg per

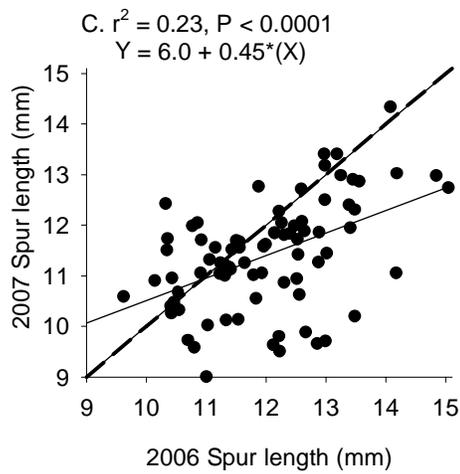
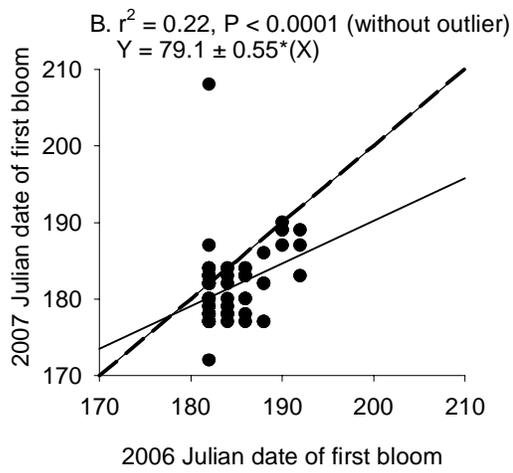
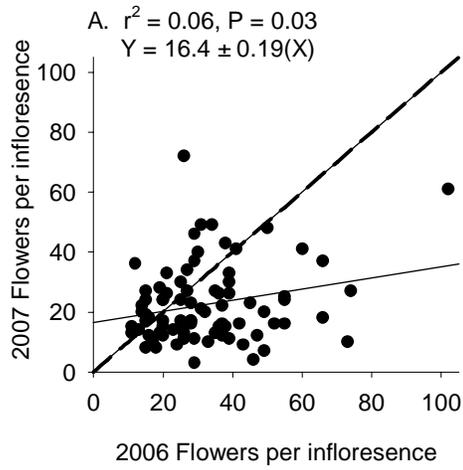
seed, n = 299 fruits; bee excluded = 1.46 ± 0.13 mg per seed, n = 27 fruits; all excluded = 1.6 ± 0.23 mg per seed, n = 9 fruits).

Source	DF	SS	MS	F	P
Visitor community	2	0.2	0.1	0.2	0.8
Error	332	155.6	0.5		
Total	334	155.8			

D. Chi-squared statistics testing the effect of visitor treatment (open control or fly only) on seed germination. Seeds were tested in groups of 10 and each group was scored as at least one seed germinated or no seeds germinated. Out of 424 control groups, 13.0% had at least one seed germinate, and out of 31 fly only groups, 12.9% had at least one seed germinate ($\chi^2_{1,453} = 0.03$, P = 0.9).

Appendix II. Variation in *Delphinium barbeyi* floral traits between years on the same individuals (A), within versus among plants (B), and in relation to plant size (C. inflorescences per plant).

A. Floral trait repeatability, quantified as the relationship between *Delphinium barbeyi* floral traits (flowers produced per inflorescence (A), Julian date of first bloom (B), and nectar spur length in mm (C)) measured in 2006 (X variable) and 2007 (Y variable) on the same 81 plants in a subalpine meadow. One-to-one lines (hatched) demonstrate consistently smaller trait values in 2007 than 2006.



B. ANOVA comparing flower production per inflorescence between sites (forest or meadow) and plants nested within site (n = 3-6 inflorescences per plant x 377 plants).

These results suggest greater within- versus among-plant variation in flower production

(Full model: $F_{85,291} = 5.6$, $P < 0.0001$).

Source	DF	SS	MS	F	P
Site	1,84	21668.1	21668.1	45.9	< 0.0001
Plant(Site)	84,291	39669.1	472.3	3.5	<.0001
Error	291	39562.5	136.0		

C. Linear regression parameter estimates and t-statistics for the relationship between

Delphinium barbeyi flower production (flowers per inflorescence) and plant size

(inflorescences per plant) in the forest site ($r^2 = 0.06$, $n = 242$ plants) and the meadow site

($r^2 = 0.05$, $n = 205$ plants).

	Forest				Meadow			
	Estimate	SE	t	P	Estimate	SE	t	P
Intercept	10.7	1.2	9.2	<.0001	20.6	2.4	8.7	<.0001
Slope	0.7	0.2	4.2	<.0001	0.5	0.1	3.4	0.0008

D. ANOVA comparing spur length of flowers between two inflorescences within plants and among plants (n = three flowers per inflorescence x two inflorescences per plant x six plants). Full model: $F_{11,24} = 11.24$, $P < 0.0001$.

Source	DF	Sum of Squares	F	P
Plant	5	64.14	23.28	<.0001
Inflorescence(Plant)	6	4.01	1.21	0.3

Appendix III. I used chi-squared tests to compare the proportion of *Delphinium barbeyi* study plants (one focal inflorescence examined per plant) with seed predation (any seeds consumed by fly larvae) and those without seed predation (no seeds consumed). In two sites and years, plants were either visited by flies only (0.6-cm mesh excluded all other visitors) or they were open to visits by any visitors (control). I report differences in seed predation where I also found differences in fly contributions to seed set (see Figure 2):

- Forest versus meadow, in 2006: In 2006, there was no difference in the likelihood of seed predation in fly only plants between sites ($\chi^2_{1,40} = 1.6$, $P = 0.2$).
- 2006 versus 2007, in the forest: Within the forest site, the probability of seed predation did not vary between years in the control treatment ($\chi^2_{1,65} = 0.02$, $P = 0.9$), but it decreased from 2006 to 2007 in the fly only treatment ($\chi^2_{1,55} = 8.6$, $P = 0.003$).

Appendix IV. Comparison of flower visitation rates to *D. barbeyi* flowers in 2005 and 2007.

In 2005, in the meadow study site, pollinator visitation rates ranged from 0.31-3.7% of flowers visited per minute from early to late in the blooming period (see Chapter 3). Early blooming flowers produced more seeds than late blooming flowers, suggesting that the lower visitation rates were sufficient to maximize seed production. In 2007, flower visitation rates at peak pollinator abundance in ten independent meadows averaging $0.40 \pm 0.19\%$ of flowers visited per minute (range = 0.19-0.77% of flowers visited per minute).

Appendix V. At the Rocky Mountain Biological Laboratory weather station, the temperature dropped to 0°C on 8 and 9 June and it dropped to 1-1.5°C on 10, 14, and 19 June (Rocky Mountain Biological Laboratory 2008).

SUMMARY CONCLUSIONS

Although mutualisms may only facilitate coupled population growth or coevolutionary relationships if reproduction or survival of both species is limited by the interaction, few studies have tested how both species in a mutualism respond to changes in each other's abundance (Cushman and Beattie 1991, Bronstein 1994b). Theoretical models often predict that coupled growth of mutualist populations should be bounded by costs associated with interactions at high partner densities (Wolin 1985, Holland et al. 2002). However, in my study of a plant-pollinator mutualism, neither species responded strongly to changes in partner density or to the resources or services provided by its partner. The lack of response was probably not because the interaction became costly at high partner densities. Instead, the reciprocal benefits exchanged between partners were probably limited by factors external to the mutualism (as in Lundberg and Ingvarsson 1998). Here, I review the results from my study, which suggest that the two focal species are relatively insensitive to changes in partner abundance. Then, I more broadly discuss the conditions that might create mutualists that are reciprocally insensitive to changes in partner densities.

Pollinator conclusions – In contrast to studies from lower elevations, I found no evidence that flowers were limiting to bumble bee density or colony reproduction (Goulson et al. 2002, Pelletier and McNeil 2003, Westphal et al. 2003). However, food additions at the nest generally increased gyne production. Therefore, although colony growth and reproduction may be food-limited, more flowers may not increase

reproduction, possibly because the subalpine colonies have too few workers to harvest extra resources.

Plant conclusions – I found no evidence that pollinator availability was limiting to plant seed production. Pollen limitation of seed production is known to vary greatly within and across systems (Kalisz and Vogler 2003, Ashman et al. 2004, Aizen and Harder 2007, Kilkenny and Galloway 2008). In this system, visitation rates suggest that all flowers should receive at least one visit, which is most likely to come from a bumble bee. The fly seed predator could have potentially diminished the benefit of high bumble bee visitation for seed set if fly larvae had consumed a larger proportion of seeds on plants with higher pollination. Instead, adult flies diminished the cost of low or no bumble bee visitation for seed set.

Mutualism stability – Many mutualisms may be stabilized by costs associated with the interactions (Bronstein 2001). For example, inefficient or costly partners can restrict the reciprocal benefits exchanged between more effective partners (Stanton 2003). Also, partners who are beneficial under one condition (e.g., a particular density, in the presence or absence of other community members, etc.) may become parasitic under other conditions (Bronstein 1994a, Johnson et al. 1997, Ferdy et al. 2002, Rudgers and Strauss 2004). However, in classic plant-pollinator mutualisms, especially those involving bee pollinators, there are no clear costs to high interaction frequencies for either the plant or the pollinator (but see Young and Young 1992). Therefore, it is difficult to envision a cost internal to the mutualism (e.g., exerted by plant or pollinator on each other) that would limit coupled population growth. Instead, classic plant-pollinator interactions may be kept in check by factors external to the mutualism, such

that changes in mutualistic partner abundance do not immediately affect reproduction and population growth.

Abundant generalist mutualists that incur no costs at high partner densities may be insensitive to partner declines and increases. For example, in this study, I examined a plant and a pollinator that were locally very abundant. The plant species was the second most abundant species according to floral abundance, and the pollinator was among the top three most abundant bumble bee species. As a result, they associated frequently with each other, but also occasionally associated with less common species. These types of abundant generalist mutualists are key players at the core of many plant-pollinator and plant-seed disperser mutualisms (Bascompte et al. 2003, Vazquez and Aizen 2004). High abundance allows a mutualistic species to provide ample resources or services for their partners. High abundance may also render the species attractive to another abundant partner, allowing less abundant species to be ignored (Sargent and Otto 2006). For example, a pollinator may forage most efficiently by learning to extract resources from the most abundant flower species (Kunin and Iwasa 1996). Similarly, plants may benefit from evolving traits that attract the most abundant, efficient, and reliable pollinators (Aigner 2001, Castellanos et al. 2004).

Less abundant mutualists may be more sensitive to partner declines. For example, pollinators may only visit less abundant plants when there are other more abundant plants present to attract pollinators via facilitation (Lamont et al. 1993, Johnson et al. 2003, Field et al. 2005, Ghazoul 2006). Similarly, relative abundance in the community can influence the strength of selection on specialist or generalist floral traits (Sargent and Otto 2006). Mutualistic communities may contain a very stable core of abundant species,

surrounded by less common species that are more sensitive to changes in partner abundance. Future work should consider how relative abundance in the community not only influences reproduction, population growth, and the evolution of floral traits (Forsyth 2003, Kalisz and Vogler 2003, Traveset et al. 2003, Morgan et al. 2005), but also how it may mediate coupled or unilateral responses to changes in partner density.

If populations of abundant mutualists are saturated with mutualistic resources, then a small increase in either partner would probably not lead to higher population growth. But how much of a decrease in partner density would lead to lower population growth? If species have more than enough mutualistic resources, then even less abundant alternative partners may be able to provide sufficient mutualistic services in the absence of primary partners. Therefore, less common species may only contribute significantly to the success of abundant partners when other abundant partners in their guild are unavailable.

Defining responses to changes in mutualistic partner density requires re-evaluation of how we classify and define mutualisms. Abrams (1987) suggested that to avoid ambiguity, interactions should be defined by the reciprocal responses (positive, neutral, or negative) of species to changes in each others populations. Some species may not respond to a change in partner abundance if that partner contributes relatively few resources or services compared to other partners at a given point in time. In this case, the mutualism may approach commensalism (Bronstein 1994a). In other situations, the partner may provide a large proportion of the resources that are necessary for reproduction or survival (e.g., > 90% of floral resources for pollinators and > 90% of flower visits for plants in this study). But an increase in either partner's density may not

lead to changes in reproduction or survival if they are limited by other resources or constraints on individual fitness. Thus, the responses to changes in partner density that can be measured are dependent not only on the degree to which partners use each other, but also on the magnitude and direction of the change in density.

The magnitude and direction of the change in partner density is likely to alter the magnitude of the effect and the interpretation of the reciprocal relationship. For example, initial tests of a species response to the presence versus the absence of a partner may demonstrate that both species benefit from each other (Cushman et al. 1994, Savage and Peterson 2007). However, mutualisms that have been surveyed across broad geographic gradients demonstrate that these interaction outcomes are highly contingent on the abiotic and biotic environment and on the genotypes of the partner species involved (Jones and Smith 2004). Such variability in mutualism outcomes has called for interaction classification based on physiological or structural characteristics rather than the requirement of an interaction outcome (Jones and Smith 2004). Although recently many studies have tried to describe functional interaction strengths between species (Wootton 2002, Vazquez et al. 2005), continued natural history observations and short-term experiments may provide more reliable descriptions of the variable effects that species have on each other (Abrams 2001).

An alternate approach to classifying the reciprocity of species interactions is to evaluate evidence of reciprocal evolutionary changes between species. For example, species-specific morphological variation among myrmecophyte plant species traits are complimentary with species-specific behavioral traits of the specialized ants that occupy their domatia (Brouat et al. 2001). Within species, reciprocal phenotypic matching

between mutualistic traits such as flower spur length and bee pollinator leg length (which facilitates oil collection from flower tubes) can be seen by comparing species traits across geographically separated populations (Steiner and Whitehead 1990). However, species trait matching across space is often variable (Thompson 2005). For example, in the antagonistic interaction between wild parsnips and parsnip webworms, phenotypic variation in plant defensive compounds and insect detoxification profiles were related in some of the populations surveyed, but the presence of alternative hosts decreased the likelihood of trait matching in other populations (Zangerl and Berenbaum 2003). Observing closely matched phenotypic traits may only occur when fitness of both species responds rapidly to changes in each other's phenotypes.

Many inter-specific interactions may be asymmetric, in that only one of the two species responds to changes in partner abundance (or changes in phenotypic traits) at any given place or time. For example, many inter-specific competition coefficients show stronger effects on one species than the other (Weiner 1990). In host-parasites systems, the availability of alternative hosts may also dampen strong reciprocal effects (Brassil and Abrams 2004). Similarly, prey heterogeneity and variation in basal resources can influence the strength of predator-prey interactions and trophic cascades (Steiner 2001). Thus, the coupled population dynamics predicted from reciprocally responsive species may be rare in antagonisms as well as in mutualisms.

Understanding the potential for coupling in mutualisms has important implications for conserving species and species interactions. For example, recent pollinator shortages have called attention to the possibility of coupled plant and pollinator declines (Buchmann and Nabham 1996, Kearns et al. 1998, Biesmeijer et al. 2006). Also,

increases in the abundance of key mutualists could facilitate the recovery of threatened partner species (Barrow et al. 1997, Grondona et al. 1997, Thornton et al. 2001) or fuel the spread of invasive partner species (Schiffman 1994, Wolfe and Klironomos 2005). Communities of mutualistic species may be more sensitive to changes in highly linked species (Memmott et al. 2004). As this study illustrates, highly linked species such as bumble bees, which are classic generalists, may themselves be insensitive to changes in the availability of their mutualistic partners. However, less abundant species may be more sensitive to changes in these abundant generalized species, which are the core of the community (Spira 2001). Therefore, perturbations to assemblages of mutualistic species may be disproportionately detrimental to less common species. Thus, the loss of mutualistic species and the breakdown of mutualisms may initially have only unidirectional effects. If pairs of species are invariably reciprocally sensitive to changes in each other's densities, then previous stochastic population fluctuations should have led to catastrophic declines and eventual coextinctions. This could explain why tight reciprocal interactions are difficult to find in nature.

Examining reciprocal mutualistic effects across environmental gradients may provide further insights into the stability of reciprocal responses across the landscape. For example, meta-analyses of plant competition and facilitation studies found that negative plant-plant interactions were less likely in more productive environments (Goldberg et al. 1999). The outcomes of plant-animal mutualisms (ranging from no responses to reciprocal positive responses to increases in each other's abundance) may also vary across productivity gradients. For example, the subalpine conditions in this study may have caused plants and pollinators to be limited by factors external to the mutualism

instead of by each other. The bumble bee colonies in this study remained small and therefore required fewer resources over their lifetime than colonies at lower elevations. The subalpine plants in this study also had to cope with harsh abiotic conditions (Körner 1999), which may lessen the degree to which pollen receipt limits reproduction. In less pristine conditions or in areas where habitat fragmentation prevents easy access to abundant mutualist partners, mutualists may be limited by each other instead of factors external to their interaction (Steffan-Dewenter et al. 2001). Examining parallel changes in food limitation of pollinator reproduction and pollen limitation of seed set across habitat gradients may provide additional insights into the stability of mutualistic interactions (Olesen and Jordano 2002).

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