

**BOTTOM-UP EFFECTS OF NUTRIENT ENRICHMENT ON PLANTS,
POLLINATORS, AND THEIR INTERACTIONS**

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ABSTRACT

Nutrients play fundamental roles in biological systems, affecting plant growth and quality, community structure, and species interactions. Although the effects of nutrients on primary and secondary production have been well documented, their effects on mutualistic consumers have rarely been addressed. Nutrient addition could alter pollinator behavior via changes in floral reward quality and quantity. Moreover, if nutrients strongly affect characters important for pollination, nutrient enrichment may influence not only plant fitness and species composition but also the performance of consumers that rely on nectar and pollen to provision their offspring. Using flowering plants in subalpine meadows and their pollinators, I tested the degree to which nutrient addition affected producers, consumers, their interactions, and the mechanisms by which those responses occurred.

At the individual plant level, one year of fertilization affected floral traits, pollen receipt, and reproduction of individual plants. Plant life-history may influence responses to nutrient additions, with delayed effects in iteroparous perennials and immediate responses in monocarps. At the community level, three years of nitrogen addition to plant assemblages showed that nitrogen increased plant productivity but did not always enhance the reproduction of flowering plants. High levels of nitrogen enrichment favored growth and reproduction of grasses, while low levels of nitrogen addition enhanced biomass, flower production, pollinator visitation, and reproduction of forbs. In both experiments, there was limited evidence of pollen limitation of female plant reproduction, and the direct effects of nitrogen addition on reproduction were relatively stronger than the indirect effects associated with changes in pollination.

In a food web context, annual variation in network structure and interactions, possibly due to fluctuations in pollinator populations, was greater than effects of nitrogen enrichment. Given that nitrogen addition can affect floral resource quality and quantity, I tested the degree to which solitary bee larvae are sugar limited to determine how nutrients might scale up to affect pollinator consumers. I found that nectar-sugar concentration limited larval growth. Together, this work suggests that nutrient enrichment can have bottom-up effects on plants and their mutualist pollinators; however, the direct effects of nutrients on plant reproduction outweighed the indirect effects via pollination.

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CHAPTER 1: INTRODUCTION

Background

Nutrients play fundamental roles in all biological systems, providing the building blocks necessary for the production of biomolecules (e.g., proteins, ATP, fatty acids) and subsequent growth and development of organisms. The effects of nutrients can scale up through ecosystems, limiting productivity and affecting plant quality, community structure, and species interactions (Peterson et al. 1993; Vitousek et al. 1997). In particular, nitrogen (N) and phosphorus (P) can limit plant growth in both terrestrial and aquatic systems (Elser et al. 2007), and the stoichiometric ratio of available nutrients can influence the growth and foraging preferences of higher trophic levels (Sternner and Elser 2002). Although the effects of nutrients on primary and secondary production have been well documented (e.g., Stiling and Rossi 1997; Forkner and Hunter 2000), their effects on mutualistic consumers and host-mutualist interactions have rarely been addressed (but see Johnson et al. 1997; Lehtonen et al. 2005). For example, nutrient enrichment could alter plant traits, such as floral, nectar and pollen quality and quantity, which are important for pollinator attraction. If nutrients strongly affect characters important for pollination and consequently change pollinator behavior and patterns of visitation, nutrient enrichment may influence not only plant fitness and plant population dynamics but also the performance of pollinating consumers that rely on nectar and pollen to provision their offspring. Here, I review the effects of nitrogen enrichment on plant individuals and communities and subsequent effects on herbivorous consumers. I then extend this

framework to pollination mutualisms and address how flowering plants, pollinators, and their interactions may be affected by nitrogen addition.

Individual and community plant responses to nitrogen addition and implications for herbivores and food web structure

Nitrogen is one of the major macronutrients, in addition to phosphorus and potassium, which is essential for plant growth and differentiation. Across many systems, N addition can increase plant biomass (Elser et al. 2007). There can be variation in N limitation of productivity based on at least two interrelated factors involved in plant resource allocation, life history and defense, which may influence the strength and timing of plant response to N addition. For instance, fast-growing annuals or monocarpic perennials may respond immediately to N addition, with plants allocating less for defense and more for growth, reproduction and differentiation (e.g., Bazzaz et al. 1987). Short-lived annuals may shunt some nitrogenous resources to defense, particularly of reproductive structures (McKey 1979). In slower-growing polycarpic perennials, plant quality may increase due to elevated nitrogen concentrations relative to carbon content (Bryant et al. 1987), or plants may store N for future use in growth and reproduction (Bollmark et al. 1999; Novotny et al. 2007). Some long-lived perennials may instead allocate a substantial portion of available N to defensive compounds, decreasing plant quality to herbivores (Coley et al. 1985).

Exploring how nitrogen enrichment affects plant traits is essential to our understanding of trophic interactions, as plants form a basal resource for higher trophic levels. The responses of individual organisms to the quality and quantity of the resource

supply can play a major role in structuring communities, affecting the abundance and interactions of species at higher trophic levels (Hunter and Price 1992; Price 1992; Bukovinszky et al. 2008). The growth and reproduction of herbivores can be N limited, in part, because of the high requirements of herbivores for N compared to typical concentrations of N in their plant forage (White 1993). Thus, depending on plant response to N addition, especially effects on plant quality and quantity, the preference and performance of herbivores may also be affected. Through the enhancement of plant food quality (via decreases in carbon-based defenses and increases in foliar N), nitrogen addition increases insect herbivore preference and performance in some (Awmack and Leather 2002) but not all cases (e.g., Fischer and Fiedler 2000). In particular, host plant quality has been shown to increase the growth, survival, and reproduction of insect herbivores (e.g., Mattson 1980; Scribner and Slansky 1981; Hemmi and Jormalainen 2002; Chen et al. 2004). However, nitrogen addition to plants may decrease herbivore preference and performance if concentrations of N-based defense compounds increase in plant tissues (e.g., Bryant et al. 1983). Thus, the bottom-up effects of nitrogen form a template upon which top-down consumer-resource interactions are structured.

At the plant community level, nitrogen enrichment not only increases aboveground net primary productivity (ANPP) but also influences species composition. When a limiting resource, such as nitrogen, is added to a system, fewer primary producers typically can coexist because of the loss of niche dimensionality due to decreased niche and life-history trade-off opportunities, altered stoichiometry, and/or increased resource homogeneity (Harpole and Tilman 2007). This pattern of loss of species richness in areas with chronic or high levels of N addition is commonly observed

(reviewed in Ditommaso and Aarssen 1989; Rajaniemi 2003; Clark and Tilman 2008) and may be influenced by plant functional group (Sebastia 2007). Nitrogen addition often results in enhanced grass productivity or dominance, possibly due to the strong competitive ability of grasses in high-N environments (e.g., Shaver and Chapin 1986; Huenneke et al. 1990; Fynn and O'Connor 2005). For example, the tundra of the Colorado Front Range responds to N fertilization, with shifts from forb-dominated to grass-dominated communities (Bowman et al. 1993). Nitrogen-fixing plants often decline in abundance under N addition, likely because legumes lose their competitive advantage over other species when N is no longer limiting (Ganade and Brown 1997; Suding et al. 2005).

The trophic interactions that occur among plants, herbivores, and higher trophic levels in all or part of a community can be integrated and illustrated with food webs. The study of food webs has enhanced our understanding of the importance of particular species or feeding links to population and community dynamics (Paine 1980; Polis 1994). Bottom-up forces play a central role in structuring trophic interactions (Hunter and Price 1992), and striking changes in food webs can occur with alterations in the resource base (Oksanen et al. 1981; Polis et al. 1997; Wallace et al. 1997; Bukovinszky et al. 2008). Resource availability can affect plant quality and subsequent interactions at higher trophic levels (e.g., Stiling and Rossi 1997; Forkner and Hunter 2000), with resource addition often increasing the abundance of consumers in terrestrial and aquatic systems (Peterson et al. 1993; Polis et al. 1998). The diet breadth of species within food webs may also be affected by the resource supply, expanding or contracting depending on the availability of preferred food items (MacArthur and Pianka 1966) and the strength and

evenness of competition (Gause 1934). These changes in diet breadth are reflected in food web structure through alterations in the identity, frequency, and strength of trophic interactions. Food webs that do not contain measures of interaction strength cannot reliably be used to make predictions and draw conclusions about key species that contribute disproportionately to community structure and dynamics (e.g., Paine 1992; Polis 1994).

Potential for bottom-up effects of nitrogen enrichment to influence floral traits, plant-pollinator interactions, and plant and pollinator reproduction

Although it is well known that N enrichment can affect plant traits important for mediating plant-herbivore interactions, the effects of N addition on floral traits important to pollinator attraction are not as well understood, and plant life history and plant defense theories do not explicitly address resource allocation to floral traits and subsequent pollinator and pollination responses. Flowering plants that benefit from high N availability will likely allocate some of these resources towards reproduction (i.e., production of more ovules, provisioning of seeds) but not necessarily towards floral traits that may indirectly benefit plants via pollinator attraction. Nitrogen addition may both contribute to enhanced biomass and plant quality as well as providing the nutrients necessary for allocation to more or higher quality floral rewards. For example, nutrient enrichment can increase flower production per plant, nectar and pollen production per flower, nectar quality (amino acid and sugar concentrations), and pollen quality (percent nitrogen) (Campbell and Halama 1993; Lau and Stephenson 1993; Petanidou et al. 1999; Gardener and Gillman 2001; Munoz et al. 2005). Thus, soil N addition may affect

individual plant fitness directly by providing resources to manufacture flowers, fruits, and seeds (Drenovsky and Richards 2005) and indirectly via changes in pollinator visitation. The relative strength of these direct and indirect effects is unknown in most systems, but likely depends on at least four factors: (1) the degree to which plants and flowers respond to nitrogen addition (Pennings et al. 2005), (2) the abundance and competitive ability of plant species for nitrogen and pollinator resources in the community (Seastedt et al. 1991), (3) the mating system and life history characters of the plants (Suding et al. 2005), and (4) the degree of pollen limitation and with whom the plants share pollinators (Ashman et al. 2004).

Due to species-specific plant responses to N addition via floral rewards and abundance, community-level effects on pollinator attraction and subsequent effects on plant reproduction are difficult to predict (Wootton 1994). Patterns of pollinator visitation are products of community-level presentation of floral traits, and thus the response of neighboring species to N addition could indirectly affect a focal plant's fitness via competition or facilitation for pollinators. If variation in N availability affects community-level reward structure of nectar and pollen, such variation may affect not only plant-pollinator interactions and the quality of pollination services but also selection on floral traits and levels of mutualistic feedback (Elle and Hare 2002).

As foragers on plant nectar and pollen, pollinators can be considered herbivores and may be similarly affected by the quality, quantity and distribution of floral food resources in the environment (Williams 2003). Many pollinators are capable of perceiving differences in the quality and quantity of floral rewards. For example, some bumble bees and honey bees perceive differences in pollen quality and prefer to forage on

high protein pollen (Fewell and Winston 1992; Rasheed and Harder 1997; Waddington et al. 1998; Cook et al. 2003). In addition, bumble bee, honey bee, and butterfly pollinators can detect differences in nectar resources and some have been shown to prefer high-quality (high amino acid content) nectar (Pleasants 1981; Alm et al. 1990). However, the perception of changes in floral traits by pollinators is not a universal pattern, and pollinators may sample floral rewards without attention to their quality. The implications of variation in the quality and quantity of floral rewards are just beginning to be understood in the context of performance and reproduction of pollinator consumers. High pollen quality or abundance can increase the larval growth and mass of some pollinators, enhancing survival, adult body size, and future fecundity in some cases (Kim 1997; Kim and Thorp 2001; Genissel et al. 2002; Roulston and Cane 2002). Similarly, nectar rich in amino acids can increase the fecundity of butterfly pollinators (Mevi-Schutz and Erhardt 2005). The availability of floral resources can also influence the number and sex ratio of solitary bee offspring, with more and larger offspring (greater proportion female) produced when floral resources are high (Kim 1999). It seems likely that pollinators will respond to changes in the quality and quantity of floral rewards that result from N fertilization of plant assemblages, affecting their visitation patterns and possibly their reproduction, just as herbivores generally show these same responses.

Like predator-prey interactions, mutualisms also can be considered consumer-resource interactions (Holland et al. 2005), and many mutualistic interactions, such as plant-pollinator interactions, are web-like in structure (e.g., Jordano 1987; Waser et al. 1996; Memmott 1999; Jordano et al. 2003b). Given that nutrient addition can affect flower production and floral rewards, I predict that plant-pollinator interaction networks

will be influenced by resource addition, with pollinators altering their frequency of interactions with certain plant species. The diet breadth of foraging pollinators will likely expand or contract depending on morphological constraints of plants and pollinators (e.g., corolla tube depths and tongue lengths), competition among pollinators for floral resources, and the effects of nitrogen addition on species composition and on species-specific production of floral rewards. Plant-pollinator networks, like food webs, are emergent properties of systems (e.g., Jordano et al. 2003a), and the degree to which the nutrient resource supply affects the structure of mutualistic networks remains unexplored. One caveat is that plant-pollinator networks do not contain the strengths of the interactions, and it is well known from studies of food webs that networks may provide poor representations of quantitative food webs (e.g., Polis 1994; Paine 1980). Nonetheless, pollination networks represent a step towards understanding the structure and properties of mutualistic interactions at the community level (Bascompte and Jordano 2006), and no quantitative pollination web (or any other mutualistic web) has been produced to date due to the difficulties in estimating per-visit pollinator efficiency and conducting manipulations in large pollination networks. Nonetheless, concepts and methods from food web ecology used to understand trophic interactions may provide initial steps towards investigating how changes in resource supply can scale up to affect the performance of plants and pollinators and their interactions.

Dissertation Overview

The goal of this dissertation was to determine the degree to which nutrient addition (nitrogen addition, in particular) affected producers, consumers, their

interactions, and the mechanisms by which those responses occurred. Using flowering plants in a subalpine system and their pollinators (consumers of both nectar and pollen), I measured the effects of nitrogen on plant and floral traits and determined how nitrogen affected productivity and fitness estimates in plants. Given the effects of nitrogen on floral rewards, I also investigated how nectar-sugar addition influenced pollinator performance. By using a trait-based approach focusing on N effects on plant and floral traits, this work provided insight into the mechanisms that influenced plant and pollinator responses to N addition.

The primary questions that I addressed were:

Chapter 2: At the individual plant level, how does nutrient addition directly and indirectly (via pollination) affect fitness estimates of two plant species that differ in life-history?

Chapter 3: At the community level, how does nitrogen addition affect plant productivity and reproduction, mediated through changes in floral traits and pollination?

Chapter 4: From a consumer-resource network perspective, how does plant-pollinator network structure vary among nitrogen treatments and years?

Chapter 5: From the pollinator perspective, how does nectar-sugar addition affect the performance of solitary bee larvae?

Study System and Experimental Approach

I manipulated nutrient availability in subalpine meadows near the Rocky Mountain Biological Laboratory (RMBL), Gunnison County, Colorado, USA (elev. 2900 m). Mountain ecosystems often have low nutrient supply, and productivity in these

systems can be limited by soil N (Bowman et al. 1993; Theodose and Bowman 1997, but see Cross and Harte 2007). Nitrogen deposition rates are low around the RMBL (0.4 g NO_3^- and $0.06 \text{ g NH}_4^+ \text{ m}^{-2} \text{ yr}^{-1}$, NADP 2006) compared to other areas in the Rocky Mountain west (e.g., Baron et al. 2000, Fenn et al. 2003, NADP 2006), so the RMBL serves as an appropriate baseline for investigating the potential effects of changes in resource availability on plants and higher trophic levels. Average annual precipitation at the RMBL is $52.1 \pm 8.3 \text{ cm}$ (NADP, 1999-2006), falling primarily as snow in the winter. Summer temperatures (June-August, 2000-2007) average $4.7 \pm 2.6^\circ\text{C}$ (daily lows) and $23.0 \pm 3.8^\circ\text{C}$ (daily highs).

The study system is characterized by a diversity of mostly perennial flowering plants that grow, bloom, and reproduce during the short growing season. Based on flower abundance, the dominant flowering plants include *Delphinium nuttallianum* (Ranunculaceae), *Erigeron speciosus* (Asteraceae), *Helianthella quinquenervis* (Asteraceae), *Heliomeris multiflora* (Asteraceae), *Ipomopsis aggregata* (Polemoniaceae), and *Potentilla pulcherrima* (Rosaceae). Many of these plants are self-incompatible and require pollinators, such as flies, solitary bees, bumble bees, hummingbirds, and/or butterflies, to confer pollination services for seed set (Price and Waser 1979; Waser and Price 1990; Kearns and Inouye 1994).

In addition to community-level manipulations of nutrient availability, I also focused on three flowering plant species, *Ipomopsis aggregata*, *Linum lewisii* (Linaceae), and *Potentilla pulcherrima*, for individual-level experiments. *Ipomopsis aggregata* blooms in mid-summer around the RMBL (early July to late-August). *Ipomopsis aggregata* is a shallow-rooted monocarp, spending 2-7 years as a rosette before sending

up a bolting stalk, flowering, and dying. Thus, lifetime reproduction can be measured in one flowering season, and increased seed set generally translates into increased seedling and juvenile recruitment (Waser et al. 2000). The trumpet-shaped, red flowers are hermaphroditic, protandrous, and last for 3-5 days (Pleasants 1983). Nectar production averages 1-5 $\mu\text{L flower}^{-1} \text{ day}^{-1}$, with a sugar concentration of 20-25% (Pleasants 1983). Plants are self-incompatible and pollinated primarily by broad-tailed (*Selasphorus platycercus*) and rufous (*S. rufus*) hummingbirds around the RMBL (Waser 1978; Price et al. 2005). In some years and sites, *I. aggregata* is pollen limited for seed set (e.g., Hainsworth et al. 1985; Campbell & Halama 1993; Juenger and Bergelson 1997; Irwin 2006).

Linum lewisii is a perennial pollinated by a variety of small bees and flies (Kearns 1992). The flowers, which have five blue petals in an open morphology, remain open for one day (Kearns and Inouye 1994). Although *L. lewisii* is self-compatible, insects are required to transfer even self-pollen from anthers to stigmas for seed set, and *L. lewisii* may be pollinator limited, especially at high elevations (Kearns and Inouye 1994). Plants have large taproots from which new stalks emerge each season, but the average lifespan of an individual is unknown.

Potentilla pulcherrima, a shallow-rooted perennial, flowers from mid-June to late-August, producing about one third of the total flowers available across the flowering season. The flowers, which have five yellow petals in an open morphology, are visited by a wide variety of pollinator species, including bees and flies. *Potentilla pulcherrima* is self-compatible but cannot autogamously self-pollinate, thus requiring pollinators for

seed set (Stinson 2004). Plants can reproduce vegetatively through the production of additional stalks emerging near the base of the parent plant.

Dissertation Outline

The remaining chapters of this dissertation discuss how individuals or assemblages of flowering plants responded to nutrient enrichment and the degree to which pollinators influenced these responses. By measuring plant and floral traits, pollination, and estimates of plant fitness under different nutrient regimes, this work provides insight into the structure and function of plant-pollinator communities in an ecosystem context.

Chapter 2 discusses the effects of nutrient (NPK) addition on the floral traits, pollination, and reproduction of *Ipomopsis aggregata* and *Linum lewisii* individuals. I hypothesized that fertilization would increase the pollination and reproduction of both species, with plant life history influencing the timing of responses. I found immediate, positive effects of fertilization on flower and nectar production, flower size, aboveground biomass, pollen receipt, and female reproduction in *I. aggregata*, a monocarp. In contrast, fertilization did not have any immediate effects on *L. lewisii*, a perennial, but delayed positive effects of fertilization on aboveground biomass and female reproduction were observed. In both species, the direct effects of fertilization on reproduction were stronger than the indirect effects associated with changes in pollination.

Chapter 3 investigates how three years of nitrogen addition to plant assemblages affected primary productivity, plant species composition, and plant fitness, as well as how floral traits and plant-pollinator interactions mediated plant-fitness responses. I

hypothesized that N enrichment would increase the productivity and reproduction of plants, with indirect effects of N addition on reproduction playing an important role, assuming that plants are pollen limited for seed set. I found that high-N addition favored the growth and reproduction of grasses, while low-N addition promoted forb growth and had variable effects on forb reproduction. Mechanistically, although N addition influenced floral traits and pollinator visitation at the community level, per-flower pollinator visitation rate remained unchanged among N treatments and evidence of pollen limitation of seed set was absent from most components of female reproduction of forb species. Thus, contrary to my initial hypothesis, the direct effects of N addition on floral traits and plant reproduction were stronger than the indirect effects mediated through changes in pollination.

Chapter 4 explores the effects of nitrogen enrichment on plant-pollinator network structure in relation to interannual variation in structure and interactions. I hypothesized that the bottom-up effects of nitrogen enrichment would strongly affect the structure of plant-pollinator networks via changes in the identity and frequency of interactions. I found that N addition altered floral resources, pollinator visitation rate to plants, and the nested position of individual plants and pollinators in the network. But N addition did not change the overall structure of plant-pollinator networks or the degree of network nestedness (i.e., the same plants and pollinator interacted with each other and with similar frequencies across N treatments). Annual variation in network structure, possibly due to factors influencing pollinator populations, was greater than the effects due to N enrichment. The presence of large interannual variation in interactions is interesting and

important because it suggests that pollination syndromes, such as a solely bumble bee pollinated plant, rarely exist in nature, and most interactions are generalized across time.

Finally, given that nitrogen addition can affect the quality and quantity of floral resources, I also tested the degree to which solitary bee larvae are nectar sugar limited to determine how nutrients might scale up to affect pollinator consumers (Chapter 5). I hypothesized that nectar-sugar addition would enhance components of solitary bee larval performance, including development time, mass, and survival. I found that larval growth increased with high nectar-sugar addition, and larval development time decreased with low nectar-sugar addition, possibly representing resource allocation trade-offs under different nectar-sugar scenarios. Larval survival was not affected by any nectar-sugar treatment. These results suggest that there are bottom-up effects of floral resources on pollinator performance. More specifically, nectar quality, in addition to pollen, is important for solitary bee larval performance.

Conclusions

Taken together, this dissertation presents how bottom-up nutrient enrichment can alter plant productivity, floral traits, and fitness estimates and the degree to which pollinators can mediate plant responses through changes in their behavioral visitation patterns. This research contributes to our understanding of plant-pollinator mutualisms as consumer-resource interactions by combining concepts and techniques from food web ecology and network theory with pollination ecology. Community-level studies of environmental effects are often limited to impacts on individual plants or species composition (Callaway et al. 2002). This work provides an important next step in

furthering the field of community ecology by extending the current framework to include producers, consumers, their mutualistic interactions, and the mechanisms by which those responses occur.

Interestingly, this work shows that the responses of plant, pollinators, and their interactions to bottom-up effects of nitrogen addition parallel those of typical consumer-resource interactions in many ways. Nitrogen enrichment affected floral traits that influenced pollinator behavior and possibly their reproduction as well. However, pollination mutualisms strayed from this consumer-resource framework in terms of the lack of pollinator mediated effects of nitrogen on plant reproduction and the lack of effects of nitrogen on network structure. Changes in pollinator behavior did not feed back to affect plant reproduction and the abundances of certain players in the system were much more important than the bottom-up effects of nitrogen. These trends are similar to the lack of effect of herbivory on overall plant biomass (Halaj and Wise 2001), but there are variable effects of herbivory on plant reproduction (e.g., Mutikainen and Delph 1996; Agrawal 1998; Poveda et al. 2003), which can be affected by soil fertility (Meyer and Root 1993).

In general, I found direct effects of nitrogen enrichment on plant reproduction to be stronger than indirect effects via changes in species interactions. These weak indirect effects were due to weak levels of pollen limitation of female plant reproduction and pollinators foraging optimally by distributing themselves evenly over the available resources. Although both pollen limitation of reproduction and optimal foraging by pollinators has been documented repeatedly (e.g., Ashman et al. 2004; Zimmerman 1983), the generality of weak indirect effects of nutrient supply on species interactions in

other plant-pollinator communities remains to be tested. In some systems, the direct effects of nitrogen addition on ecosystem function appears to be stronger than the indirect effects due to shifts in species composition (Manning et al. 2006), and in other systems, both direct and indirect effects of nutrient supply strongly affect trophic interactions (Bukovinszky et al. 2008). Thus far, there is no universal trend in the strength of direct vs. indirect effects of bottom-up manipulations of resources across systems.

The next step is to look across broader spatial scales, the scales at which humans are influencing nitrogen availability, to determine whether the patterns observed in this study are universal. Manipulating nitrogen at the scale of a watershed eliminates the possibility of pollinator choice in foraging among plant assemblages with different nitrogen supplies and could reveal stronger indirect effects of pollination and stronger effects on plant-pollinator network structure. Additional experiments that manipulate environmental resources and explore the fitness costs and benefits to both plants and pollinators are needed to more fully understand context dependency in plant-pollinator mutualisms and the potential effects of environmental change on mutualistic interactions and the species involved.

**CHAPTER 2: THE EFFECTS OF NUTRIENT ADDITION ON FLORAL
CHARACTERS AND POLLINATION IN TWO SUBALPINE PLANTS,
IPOMOPSIS AGGREGATA AND *LINUM LEWISII***

Summary

The availability of soil and pollination resources are main determinants of fitness in many flowering plants, but the degree to which each is limiting and how they interact to affect plant fitness is unknown for many species. I performed resource (water and nutrients) and pollination treatments on two species of flowering plants, *Ipomopsis aggregata* and *Linum lewisii*, that differed in life-history, and I measured how resource addition affected floral characters, pollinator visitation, and reproduction. I separated the direct effects of resources vs. indirect effects via changes in pollination using a factorial experiment. Resource addition affected *I. aggregata* and *L. lewisii* differently. *Ipomopsis aggregata*, a monocarp, responded immediately to fertilization in the year of bloom, increasing flower production, bloom duration, corolla width, nectar production, aboveground biomass, and pollen receipt relative to control plants. Fertilization also increased total seed production per plant, and hand-pollination increased seeds per fruit in *I. aggregata*, indicating some degree of pollen limitation of seed production. In contrast, fertilization had no immediate effect on growth or reproductive output in the year of treatment on *L. lewisii*, a perennial, except that fertilization increased bloom duration. However, delayed effects of fertilization were seen in the year following treatment, with fertilized plants having greater aboveground biomass, seeds per fruit, and seeds per plant than control plants. In both species, the direct effects of fertilization on reproduction were relatively stronger

than the indirect effects via changes in pollination. My results also suggest that plant life-history traits may play an important role in determining the reproductive responses to soil nutrient and pollen additions, and should be considered when interpreting or predicting the effects of abiotic and biotic factors on plant reproduction.

Introduction

Mutualisms influence the composition of ecological communities (Bronstein 2001), population dynamics (Bronstein et al. 2003), ecosystem structure and services (Lundberg and Moberg 2003; Buchmann and Nabhan 1996), and coevolutionary relationships (Gomulkiewicz et al. 2003). The outcomes of mutualistic interactions, however, can be context dependent (Bronstein 1994). Soil nutrient availability can alter the nature of interactions in plant-mycorrhizae and plant-endophyte associations (Johnson et al. 1997; Lehtonen et al. 2005). The composition and strength of plant-pollinator mutualisms are also likely dependent upon their biotic and abiotic setting. A great deal is known about plant-pollinator mutualisms in the biotic context of floral evolution (e.g., Galen 1996; Galen 1996, Johnson et al. 1998, Chittka et al. 1999, Schemske and Bradshaw 1999), competition among pollinators for floral resources and among plants for pollination (e.g., Inouye 1978; Waser 1978; Pyke 1982), and plant-pollinator community structure (e.g., Heithaus 1974; Jordano et al. 2003a; Potts et al. 2003). Yet, we lack a synthetic understanding of how plant-pollinator mutualisms are affected by abiotic resources and of the relative strength of the direct effects of abiotic resources compared to their indirect effects mediated through changes in floral characters and pollinator

behavior. Here, I investigated how soil resource additions affected floral characters and pollination of two plant species that differed in life-histories and pollinator types.

Nutrients, which limit primary productivity in many terrestrial systems (e.g., Tilman 1987; Vitousek and Howarth 1991; Elser et al. 2007), can be patchy from micro-environmental to regional scales (Stark 1994; Sogbedji et al. 2001). Soil nutrient heterogeneity can result in differences in growth, reproduction, and herbivory among plants within a few meters (Scott-Wendt et al. 1988). Plant allocation and life-history theories generate testable predictions about how plants should respond to nutrient addition. For example, in fast-growing annuals or monocarpic perennials, nutrient addition may invoke immediate responses, with plants allocating less for defense and more for growth, reproduction and differentiation (e.g., Bazzaz et al. 1987). In contrast, in slower-growing polycarpic perennials, plant food quality may increase due to elevated nutrient concentrations relative to carbon content, or plants may store nutrients for future use.

Plant allocation and life-history theories and their predictions, however, do not explicitly take plant-pollinator interactions into account, and thus, we have a less complete understanding of how nutrient availability affects mutualisms between plants and pollinators (but see Ryle 1954; Young and Stanton 1990; Campbell and Halama 1993; Sperens 1997; Munoz et al. 2005). For example, any plant that can capitalize on high nutrient availability will likely shunt some of these resources towards reproduction but not necessarily towards floral traits that may indirectly benefit plants via pollinator attraction. Nutrients may directly affect plant reproduction by providing resources to manufacture flowers, fruits, and seeds. However, indirect effects of nutrients on

reproduction may also occur through the alteration of floral rewards, such as flower production (Munoz et al. 2005) or pollen quality and quantity (Lau and Stephenson 1993), and subsequent pollinator attraction, pollination, and seed set, assuming that pollinators can cue in on changes in floral traits and that plants are pollinator-limited for seed set. Pollinator attraction to plants with increased nutrient content is not unlike herbivores that are attracted to and prefer feeding on plants with high nutrient content (Mattson 1980), due to the high carbon to nutrient ratios in plant compared to herbivore tissues, and herbivores are often nutrient limited in their growth and survival (Awmack and Leather 2002). Although some studies suggest that nutrient addition may have positive effects on floral rewards and pollination, nutrient addition may instead alter the stoichiometric nutrient balances (e.g., Sterner and Elser 2002) important to flowering plants and their floral traits. By integrating the direct and indirect effects of nutrients on plant reproduction and how these effects might be influenced by plant life-history and pollinator type, this study provides a more complete understanding of how abiotic resources affect plant-pollinator mutualisms.

Many studies of plant-pollinator interactions assume the existence of variation in the quality, quantity, and distribution of floral traits and rewards without consideration of the origin of this variation, an approach that limits our understanding of the links between environmental variation, plant growth, and fitness. Knowledge of these links is a first step towards understanding the importance of nutrient availability on the population dynamics of plants and pollinators, natural selection on floral traits, and evolution of mutualisms. The goals of this study were to determine how soil resource addition (water and nutrients) affected floral characters of two wildflower species, *Ipomopsis aggregata*

(Polemoniaceae) and *Linum lewisii* (Linaceae), that differed in pollinator types (hummingbirds vs. bees and flies) and plant life-history (semelparous vs. iteroparous) as well as to investigate the relative importance of soil resources and pollination for the reproduction of these plants. I made three predictions: (1) Based on differences in their life-history characters, and given that many floral characters can plastically respond to environmental conditions, I predicted that both species would respond to nutrient addition but that the effects would be more immediate in the semelparous compared to the iteroparous species. (2) Also based on life-history, I predicted that any direct and indirect effects of nutrients on reproduction would be stronger in the semelparous species compared to the iteroparous perennial, assuming pollen and resource limitation of seed set. (3) Finally, based on pollinator type, I predicted that pollinators that are more energetically limited, such as hummingbirds (Gass and Sutherland 1985), would cue in on differences in floral traits (driven in this case by nutrient addition) compared to pollinators that may be less energetically limited (such as some bees and flies; Morgan and Heinrich 1987).

Methods

Study System

Fieldwork was conducted at the Rocky Mountain Biological Laboratory (RMBL), Gothic, Colorado, USA (latitude: 38°57'29" N, longitude: 106°59'06" W, altitude: 2,900 m). Mountain ecosystems often have low nutrient soils, and the productivity and abundance of some plant species are limited by both N and P in some moisture regimes (Bowman et al. 1993; Theodose and Bowman 1997). Nitrogen deposition around the

RMBL is low (mean = 0.4 g NO_3^- and $0.06 \text{ g NH}_4^+ \text{ m}^{-2}\text{yr}^{-1}$, NADP) compared to other areas in the Rocky Mountain west (e.g., Baron et al. 2000; Fenn et al. 2003; NADP 2006), so the RMBL serves as a control for experimentally investigating the potential effects of changes in resource availability. I focused on the effects of resource addition on two forbs, *Ipomopsis aggregata* and *Linum lewisii*, that varied in life histories and pollination biology.

Ipomopsis aggregata (Pursh) V.E. Grant blooms in mid-summer around the RMBL (early July to late-August). *Ipomopsis aggregata* is monocarpic, spending 2-7 years as a rosette before sending up a bolting stalk, flowering (mean $\pm 1 \text{ SD} = 85 \pm 66$ flowers produced; Campbell 1989), and dying. Thus, lifetime reproduction can be measured in one flowering season. The trumpet-shaped red flowers are hermaphroditic, protandrous, and last for 3-5 days. Plants are self-incompatible and pollinated primarily by broad-tailed (*Selasphorus platycercus*) and rufous (*S. rufus*) hummingbirds around the RMBL (Waser 1978), although some insects also visit *I. aggregata* flowers and act as effective pollinators (Price et al. 2005). In some years and sites, *I. aggregata* is pollen limited for seed set (e.g., Hainsworth et al. 1985; Campbell & Halama 1993; Juenger and Bergelson 1997; Irwin 2006). In addition, *I. aggregata* responds to nutrient addition. For example, nutrient fertilization of *I. aggregata* (using a 20:20:20 NPK fertilizer) has direct, positive effects on nectar volume and flower production (Campbell & Halama 1993), and hummingbird pollinators often select plants with increased nectar or flowers (Mitchell 1994). However, only fertilization in combination with hand-pollination increased the number of seeds produced per flower and the total number of seeds produced per plant (Campbell and Halama 1993), suggesting minimal indirect effects of

nutrients on seed production mediated through changes in pollinator behavior. The degree to which these effects of pollen and nutrient supplementation to *I. aggregata* are consistent in other sites and years is unknown.

Linum lewisii Pursh is a perennial pollinated by a variety of small bees and flies (Kearns 1992). The flowers, which have five blue petals in an open morphology, remain open for one day (Kearns and Inouye 1994). Although *L. lewisii* is self-compatible, insects are required to transfer self-pollen from anthers to stigmas for seed set, and *L. lewisii* may be pollinator limited, especially at high elevations (Kearns and Inouye 1994). Plants have large taproots from which new stalks emerge each season, but the average lifespan of an individual is unknown. One season of water addition had no effect on flowering or seed set (Kearns and Inouye 1994). The combined effects of water, nutrient, and pollen addition for *L. lewisii* are unknown.

Field methods

Within one site (~75 m by 25 m) between June-August, 2004, 72 individuals of each species with flower buds were randomly chosen and assigned treatments representing a factorial cross of three resource levels (control [no additions], water added, or water plus fertilizer added) by two pollination levels (control [natural pollination] or supplemental hand pollination). The factorial design allowed me to investigate the individual and combined effects of resources and pollination on plant reproduction as well as the direct and indirect (pollinator-mediated) effects of resource additions. Drip emitters attached with tubing to plastic cups were used to administer water (400 mL) to the base of individual plants four times per week from pre-flowering through fruit

collection in the water- and fertilizer-addition treatments of both species. Once per week, the fertilized plants also received an addition of 20-20-20 NPK fertilizer (Peters Professional[®]) in dosages recommended by the manufacturer (4 mL/L in 400 mL water = 1.61 g N, 1.51 g P, and 2.92 g K per plant per season). Plants in the hand-pollination treatment received supplemental pollen to all pistillate-phase flowers three days per week for *I. aggregata* and six days per week for *L. lewisii* throughout the blooming period, so that most flowers on both species were hand-pollinated. Hand pollination enhanced conspecific stigma pollen receipt by 26% in *I. aggregata* ($t_{67} = 3.46$, $p = 0.0009$) and 61% in *L. lewisii* ($t_{45} = 3.5$, $p = 0.001$) compared to open pollination.

Throughout the summer, I measured three classes of floral characters: flower production, components of flower size, and nectar. I counted the number of open flowers three days per week in *I. aggregata* and six days per week in *L. lewisii* and measured bloom duration for each species as the number of days that plants were in bloom. I measured components of flower size (corolla length and width in *I. aggregata* and petal length and width in *L. lewisii*) on a maximum of three flowers per plant using digital calipers to the nearest 0.01 mm. On a maximum of four flowers per plant, I measured nectar production on bagged, newly opened flowers over 48 hours and nectar sugar concentration (using a hand-held refractometer) in sucrose equivalents. I was unable to measure the sugar concentration of *L. lewisii* nectar due to low nectar production rates. Measures of flower size and nectar production are repeatable across flowers within plants for *I. aggregata* and other species (Campbell et al. 1991; Mitchell and Shaw 1993; Wolf and Campbell 1995; Irwin et al. 2004). I measured these flower, morphological, and nectar characters because they are involved in pollinator attraction in these and other

systems (e.g., Pleasants 1981; Mitchell 1994; Galen 1999; Biernaskie and Cartar 2004) and may be influenced by nutrient availability (e.g., Campbell & Halama 1993; Sperens 1997; Munoz et al. 2005).

To estimate pollinator visitation, I used stigma pollen loads. Because neither *I. aggregata* nor *L. lewisii* autogamously self-pollinate, pollen receipt can be used as a proxy for pollinator visitation (Engel and Irwin 2003; Price et al. 2005). To estimate pollen receipt, I collected up to three stigmas from each plant once per week throughout the summer. Stigmas were collected once the corolla or petals had fallen off to ensure that all flowers went through female phase and to avoid affecting seed set (e.g., Waser and Price 1991). I stained stigmas in basic fuchsin (Kearns and Inouye 1993) and counted the number of conspecific and heterospecific pollen grains on each stigma under a compound microscope. The presence of conspecific pollen on stigmas indicated the potential for ovule fertilization (Engel and Irwin 2003), while heterospecific pollen represented the possibility of stigma clogging and reduced seed set (reviewed in Wilcock and Neiland 2002).

Because plant nutrient concentration and pollen supplementation can affect plant susceptibility to herbivores and seed predators (Mattson 1980; Herrera 2000), which could obscure any effects of nutrient or pollen addition, I also recorded herbivory and seed predation. Mule deer, *Odocoileus hemionus*, are the primary herbivores of *I. aggregata*, consuming portions of individuals during their reproductive phase (Paige and Whitham 1987; Sharaf and Price 2004). Herbivory was assessed in *I. aggregata* by whether or not each plant was browsed. Aphids were the main herbivores of *L. lewisii* in the year of study. I censused aphid herbivory once per week as the proportion of stalks of

each plant with aphids present. I measured pre-dispersal seed predation as the number of fruits (fruit collection described below) destroyed by fly seed predators (*Hylemya* sp., Diptera: Anthomyiidae) of *I. aggregata* and unknown lepidopteran larvae of *L. lewisii*. Measuring pre-dispersal seed predation as the number of fruits destroyed takes into account both oviposition and larval survival (Brody and Waser 1995; Brody 1992; Cariveau et al. 2004).

I estimated plant reproductive success through male and female components. I estimated male reproduction using pollen production per flower and pollen quality. Pollen production is positively correlated with seeds sired in some, but not all, species (reviewed in Stanton et al. 1992). In terms of pollen quality, soil P addition can increase percent P in pollen, and soil N and P addition can increase male siring ability (Lau and Stephenson 1993; Lau and Stephenson 1994), suggesting that the concentration of pollen N and P may indicate pollen quality in some systems. Here, I focused on measuring the concentration of pollen N only. For all plants, pollen production per flower was measured by collecting anthers from up to three enlarged buds once per week and storing them in ethanol. Samples were sonicated for 10 min to ensure that all pollen had dehisced from the anthers, and grains were counted on a hemacytometer under a dissecting microscope (Snow and Lewis 1993). After pollen production was measured, all pollen samples from each plant were pooled, and pollen quality was estimated as percent N using a CarloErba elemental analyzer (Roulston et al. 2000).

To estimate female reproduction, I collected all of the fruits from each plant at the end of the summer, and I counted all of the seeds. I also weighed the seeds of each plant, as seed mass is an estimate of quality, such as potential germination, growth and

flowering, in some species (Stanton 1984). I calculated female reproduction per plant as percentage fruit set (number of successful fruits divided by total flowers), mean seeds per fruit (total seeds divided by successful fruits), total seeds, and mean mass per seed.

At the end of the season, I collected and dried plants and measured dry biomass to assess if plant growth was affected by resource addition. Because *I. aggregata* die after flowering, I measured both the shoot and root biomass of each plant in 2004. Because *L. lewisii* are perennial, I measured only their aboveground dry biomass in 2004. I measured seed set and plant biomass of the surviving *L. lewisii* in the following three years (2005-2007) to determine if resource addition could have delayed effects, as storage of resources for future growth and reproduction may be common in perennials (Munoz et al. 2005).

Statistical analyses

All statistical analyses were performed in SAS 8.2 unless otherwise noted. Multiple comparisons were used where appropriate to evaluate pairwise comparisons between resource treatments. Although the experiment was designed with an equal number of plants per treatment, due to herbivory, unbalanced sample sizes were common, so Type III SS from PROC GLM were used for F-tests, and LSMEANS were used to estimate averages and standard errors.

Effects of resource treatments on floral and plant characters. For *I. aggregata*, I determined how resource treatments affected total flower production per plant, bloom duration (total flowering period), mean flower size (length and width), mean nectar (volume and concentration), and biomass (above- and below-ground) using a MANOVA

(PROC GLM). I performed the same MANOVA for *L. lewisii*, but nectar concentration and belowground biomass were excluded from the analysis because they were not measured (see above). Based on life-history, I expected that effects of resource treatments on floral and plant characters would be more immediate in *I. aggregata* compared to *L. lewisii*. By using MANOVA (here and in many analyses below), I reduced the probability of inflating the Type I error rate (Rencher 1995) and could test how my treatments affected several interrelated measures of floral characters. A significant MANOVA was followed by univariate ANOVAs for each response variable (Scheiner 1993).

Pollination and herbivore responses to resource and pollen treatments. I analyzed the effects of resource treatments on mean stigma pollen receipt per flower per plant (number of conspecific and heterospecific grains) of *I. aggregata* and *L. lewisii* using MANOVAs. Based on pollinator type, I expected that the hummingbird pollinators of *I. aggregata* would be more likely to cue in on changes in floral characters that occurred as a result of resource treatments and increase their visitation to more rewarding plants, resulting in increased pollen receipt in these plants, as compared to the bee and fly pollinators of *L. lewisii*. Mule deer responses (presence or absence of herbivory) to *I. aggregata* and aphid responses (proportion of stalks infested) to *L. lewisii* in different resource and pollen treatments were assessed using logistic regression (JMP 4.0) and two-way ANOVA, respectively. Responses of seed predators (percent of fruits destroyed) to nutrient and pollen treatments in *I. aggregata* and *L. lewisii* were evaluated using two-way ANOVAs.

Effects of resource treatments on male plant reproduction. For both *I. aggregata* and *L. lewisii*, I analyzed the effects of resource treatments on mean pollen production

per flower per plant and pollen quality (percent N) using MANOVAs. One *L. lewisii* data point was excluded because it was an extreme outlier in percent N (5.3% below the mean). Based on life-history, I expected the effects of resource treatments on pollen production per flower and pollen N concentration would be stronger and more immediate in *I. aggregata* compared to *L. lewisii*.

Effects of resource and pollen treatments on female plant reproduction. I analyzed the effects of resource and pollen treatments on female reproduction per plant (percent fruit set, mean seeds per fruit, total seeds, mean mass per seed) in *I. aggregata* and *L. lewisii* using MANOVAs (PROC GLM) with two fixed factors (resource and pollination treatment) and their interaction. Significant effects of resources and pollination would indicate direct effects of these factors on female reproduction. A significant interaction would indicate that resource additions affect the level of pollen limitation. Based on life-history, I expected that the direct and indirect effects of resource treatments on female reproduction would be stronger in *I. aggregata* compared to *L. lewisii*, assuming that I also found evidence of pollen limitation of seed set.

Delayed effects of resource treatments on L. lewisii. The effects of resource treatments on *L. lewisii* female reproduction (percent fruit set, mean seeds per fruit, total seeds, mean weight per seed) in 2005 and 2006 were assessed using a MANOVA for each year. The effects of resource treatments on *L. lewisii* aboveground biomass in 2005, 2006 and 2007 were evaluated using a one-way ANOVA for each year. I could not use MANOVAs to investigate the overall effect of resource treatments on female reproduction and biomass because individual plants often had biomass values but did not reproduce (which would bias my results towards representing only those plants that both

grew and reproduced), and repeated-measures analyses were not possible because fewer individuals were measured in each successive year due to plant mortality. Approximately equal numbers of plants from each resource treatment were lost every year due to mortality, so resource treatment did not affect survival. Furthermore, correlations of aboveground biomass and seed production of plants between years were positive (aboveground biomass 2004 vs. 2005: $r = 0.69$, $P < 0.0001$, $N = 59$; 2005 vs. 2006: $r = 0.62$, $P < 0.0001$, $N = 49$; 2006 vs. 2007: $r = 0.56$, $P = 0.0018$, $N = 28$; seed production 2004 vs. 2005: $r = 0.68$, $P < 0.0001$, $N = 36$; 2005 vs. 2006: $r = 0.30$, $P = 0.078$, $N = 35$), suggesting that there were no delayed costs of reproduction. Although female reproduction of *L. lewisii* was also measured in 2007, only three of the 29 remaining individuals produced fruit in that year and were not included in the analyses.

Results

Effects of resource treatments on floral and plant characters

Resource addition affected floral and plants characters in both *I. aggregata* (MANOVA: Wilks' $\lambda = 0.52$, $F_{16,116} = 2.81$, $P = 0.0007$) and *L. lewisii* (MANOVA: Wilks' $\lambda = 0.54$, $F_{12,76} = 2.30$, $P = 0.015$), but the responses were species specific. For *I. aggregata*, fertilized plants produced 91% more flowers than control plants and 105% more flowers than watered plants (Fig. 1a, $F_{2,69} = 10.39$, $P < 0.0001$). One aspect of flower size, corolla width, in *I. aggregata* was also affected by resource addition. Fertilized plants produced flowers with 11% wider corollas than controls [$F_{2,68} = 5.2$, $P = 0.0078$; means $\pm$ SE = 4.02 ± 0.097 (control), 4.30 ± 0.095 (water), 4.46 ± 0.095 (fertilized)]. Resource addition affected *I. aggregata* nectar, with fertilized plants

producing 38% more nectar per flower over 48 hr (Fig. 1b, $F_{2,67} = 3.74$, $P = 0.03$), but of moderately lower concentration ($F_{2,67} = 2.84$, $P = 0.065$), than control plants. Both fertilized and watered *I. aggregata* bloomed an average of 12 days longer ($F_{2,69} = 6.80$, $P = 0.002$) than control plants. Resource addition affected the biomass of *I. aggregata*, with fertilized individuals having 64% and 53% more aboveground biomass than control and watered plants, respectively (Fig. 1c, $F_{2,68} = 5.74$, $P = 0.005$), with no effect on belowground biomass ($F_{2,67} = 0.56$, $P = 0.57$).

For *L. lewisii*, flower production (Fig. 1A, $F_{2,66} = 0.16$, $P = 0.85$), flower size (MANOVA: Wilks' $\lambda = 0.88$, $F_{4,102} = 1.61$, $P = 0.18$), and nectar production (Fig. 1B, $F_{2,49} = 1.13$, $P = 0.33$) were not affected by resource treatments. Fertilized *L. lewisii* bloomed 9 days longer on average ($F_{2,66} = 4.46$, $P = 0.02$) than control plants. The aboveground biomass of *L. lewisii* was not affected by resource treatment in the year of addition (Fig. 1C, $F_{2,69} = 0.86$, $P = 0.43$). However, resource addition had a delayed effect on *L. lewisii*, with fertilized plants producing 50% and 83% higher aboveground biomass than control and watered plants (respectively) in the year following treatments (2005, Fig. 4a, $F_{2,56} = 3.54$, $p = 0.036$). This effect was temporary, however, and was not evident two years (2006: $F_{2,48} = 1.28$, $p = 0.29$) and three years (2007: $F_{2,26} = 1.38$, $p = 0.27$) after treatments.

Pollination and herbivore responses to resource and pollen treatments

Pollinators responded to resource treatments in *I. aggregata* and *L. lewisii* differently. In *I. aggregata*, resource addition affected stigma pollen receipt, an estimate of pollinator visitation (MANOVA: Wilks' $\lambda = 0.86$, $F_{4,132} = 2.63$, $P = 0.037$), with

fertilized plants receiving 26% more conspecific pollen grains per stigma ($F_{2,67} = 4.17$, $P = 0.02$) than control plants [means $\pm$ SE = 131.0 ± 8.75 (control), 148.0 ± 8.56 (water), 165.6 ± 8.21 (fertilizer)]. There was no effect of resource treatments on *I. aggregata* stigma receipt of heterospecific pollen ($F_{2,67} = 1.17$, $P = 0.32$). In contrast, resource treatments did not affect stigma pollen receipt in *L. lewisii* (MANOVA: Wilks' $\lambda = 0.93$; $F_{4,70} = 0.65$, $P = 0.63$). Overall, heterospecific pollen represented 6% of the average total pollen receipt in *I. aggregata* and 34% in *L. lewisii*.

Trends in herbivore response to nutrient and pollen treatments also differed with plant species. In *I. aggregata*, I found no effect of nutrient or pollen treatment on incidence of herbivory by mule deer ($\chi^2 = 3.91$, $P = 0.27$). In contrast, watered *L. lewisii* harbored 62% and 31% more aphid-infected stalks than control and fertilized plants, respectively, although this trend was not statistically significant ($F_{3,68} = 2.12$, $P = 0.11$). Treatments did not affect the proportion of fruits destroyed by seed predation in either species (*I. aggregata*: $F_{3,66} = 0.27$, $P = 0.85$; *L. lewisii*: $F_{3,66} = 0.69$, $P = 0.56$). Seed predation was higher overall in *I. aggregata* than *L. lewisii*, with 13% vs. 0.8% of fruits destroyed, respectively.

Effects of resource treatments on estimates of male reproduction

Pollen quality and quantity in *I. aggregata* were marginally affected by resource addition (MANOVA: Wilks' $\lambda = 0.88$, $F_{4,124} = 2.30$, $P = 0.06$), but univariate tests of pollen production per flower ($F_{2,67} = 0.59$, $P = 0.59$) and pollen percent N ($F_{2,63} = 2.02$, $P = 0.14$) were not statistically significant, possibly because pollen production and pollen percent N were strongly positively correlated ($r = 0.57$, $P < 0.0001$, $N = 66$). In *L. lewisii*,

resource addition had a marginally significant effect on pollen quality and quantity (MANOVA: Wilks' $\lambda = 0.76$, $F_{4,60} = 2.19$, $P = 0.08$). Pollen production per flower in *L. lewisii* ($F_{2,59} = 0.66$, $P = 0.52$) was not affected by resource addition, but pollen percent N was 15% higher in control plants than fertilized plants [$F_{2,31} = 4.74$, $P = 0.016$; means $\pm$ SE = $8.16 \pm 0.28\%$ (control), $7.52 \pm 0.25\%$ (water), $7.08 \pm 0.22\%$ (fertilized)].

Effects of resource and pollen treatments on estimates of female reproduction

Resource and pollen treatments affected the female reproduction of *I. aggregata* and *L. lewisii* differently. Female reproduction in *I. aggregata* was affected by resource and pollen additions (MANOVA: Wilks' $\lambda = 0.51$, $F_{20,203} = 2.25$, $P = 0.002$). There was a significant effect of resource treatment on total seeds per plant (Fig. 2c, $F_{2,64} = 5.56$, $P = 0.006$), with fertilized plants producing 94% more seeds than control plants across pollen treatments. The effects of resource and pollination treatments on seeds per fruit were marginally significant (Fig. 2b, $F_{5,64} = 2.19$, $P = 0.066$), with hand pollinated plants having 27% more seeds per fruit than open-pollinated controls across resource treatments. There was no effect of either treatment on percent fruit set (Fig. 2a, resource treatment: $F_{2,64} = 0.56$, $P = 0.58$; pollination treatment: $F_{1,64} = 0.38$, $P = 0.54$) or mean mass per seed (Fig. 2d, resource treatment: $F_{2,64} = 0.13$, $P = 0.88$; pollination treatment: $F_{1,64} = 1.48$, $P = 0.23$). In addition, there were no significant interactions between resource and pollination treatment for any of these response variables ($P > 0.05$ in all cases), suggesting that resource additions did not affect the level of pollen limitation.

In contrast, there were no main effects of resource and pollen treatments or their interaction on any measures of female reproduction in *L. lewisii* (Fig. 3, MANOVA:

Wilks' $\lambda = 0.77$, $F_{20,203} = 0.85$, $P = 0.65$) in the year of treatment (2004). However, resource addition had a delayed effect on female reproduction in *L. lewisii* (MANOVA: Wilks' $\lambda = 0.65$, $F_{8,98} = 2.92$, $P = 0.006$), with fertilized plants producing 129% and 195% more seeds than control and watered plants, respectively, in 2005 (Fig. 4c, $F_{2,52} = 5.24$, $P = 0.0085$). Fertilized and watered *L. lewisii* also had 22% and 19% more seeds per fruit than controls in 2005 (Fig. 4b, $F_{2,52} = 5.08$, $P = 0.0096$). Resource treatments had no effect on percent fruit set ($F_{2,52} = 1.75$, $P = 0.18$) or mass per seed ($F_{2,52} = 1.22$, $P = 0.3$) in 2005. Again, these effects of resource treatments on female reproduction were not sustained, and no effects were observed in 2006, two years after treatment application (MANOVA: Wilks' $\lambda = 0.96$, $F_{8,62} = 0.17$, $P = 0.3$).

Discussion

Plant ecologists have long been interested in resource limitation and its consequences for plant growth and reproduction and community dynamics (e.g., Tilman 1987; Tilman and Wedin 1991; Vitousek and Howarth 1991). To maximize reproduction in many flowering plants, both nutrient and pollination resources are required. These resources are not independent, as belowground resources can indirectly contribute to plant reproductive success through changes in floral traits and pollination (e.g., interaction-modification indirect effects; Wootton 1993; Abrams et al. 1996). Here, I used resource and pollen treatments on two flowering plant species and showed that the direct effects of nutrients, largely through increased flower production, dominate in their effects on reproduction compared to the indirect effects mediated via changes in pollination. However, the timing of these effects on plant reproductive success was

influenced by the life-history of the plant: a monocarp responded immediately to fertilization, while a perennial showed delayed effects. Given that I investigated only two plant species in this study, I could not fully separate the influence of life-history vs. pollinator type (e.g., hummingbirds vs. small bees and flies) on the effects of resource additions. Further demonstrations of the importance of these factors in influencing plant response to nutrient and pollen addition in other systems is necessary to solidify the generality of these findings.

Both nutrient and pollen resources have been found to limit plant reproduction in a number of other systems (e.g., Dimling 1992; Mattila and Kuitunen 2000; Asikainen and Mutikainen 2005; Pina et al. 2007). However, there are some species in which nutrients are more important than pollen availability in determining reproductive success (Ne'eman et al. 2006; *L. lewisii*, this study), and vice versa (Mattila and Kuitunen 2000). Furthermore, nutrient and pollination limitation can vary among populations (Eppley 2005) and by year (Vaughton 1991). It is likely that plant life-history plays a role in determining the importance of nutrient vs. pollen limitation. The pathways by which nutrient addition affect reproduction have not been tested in many systems, but increased flower production in most cases plays an important role (e.g., Sperens 1997; Munoz et al. 2005, Perner et al. 2007). Additional studies that simultaneously manipulate nutrients and pollen in multiple populations and years are needed to determine general patterns in their effects, including delayed effects (Munoz et al. 2005). Nutrient limitation of growth and reproduction by both nitrogen and phosphorus is important in terrestrial systems (Elser et al. 2007), and knowledge of their independent and synergistic effects with pollination

will increase our understanding of the patterns and mechanisms of limitation across systems.

In *I. aggregata*, nutrient addition affected floral traits, such as flower production, corolla width, nectar production and bloom duration, which are important for plant attraction of pollinators, as well as pollinator behavior and plant reproduction. In particular, corolla width in *I. aggregata* is a floral trait important to male reproductive success, with more pollen produced and removed from flowers with wider corollas (Campbell et al. 1996). In addition, hummingbird pollinators preferentially visit plants and flowers with higher nectar availability (Mitchell 1994), and increased pollinator visitation often results in increased pollen deposition (Engel and Irwin 2003). I must note, however, that the increase in pollen receipt that I observed in fertilized plants may represent both an increase in pollinator per-flower visitation (Engel & Irwin 2003) and an increase in pollinator efficiency (Campbell et al. 1991). Separating these alternatives further would require estimates of pollinator visitation and pollen deposition per visit. Aside from increasing bloom duration, watering did not affect floral traits, pollen receipt, or any reproductive measures. Lack of watering effects in *I. aggregata* was also observed by Campbell and Halama (1993), suggesting that water does not typically limit reproduction even in a dry montane habitat. Further investigation of water effects is warranted, however, given the few years of examination relative to the frequency of droughts (e.g., ENSO cycles; Philander 1990).

Nutrient and pollen availability affected female plant reproduction in *I. aggregata*. I showed experimentally that one component of female reproduction, total seeds per plant, was nutrient limited, and this appears to be due in large part to increased

flower production (and thus total ovules available for potential fertilization) with nutrient addition. Hummingbird pollinators also respond positively to increased floral display in *I. aggregata* (Brody and Mitchell 1997; Mitchell 1994) as well as in other plant species (Rodriguez-Robles et al. 1992; Podolsky 1992). My experimental hand pollination marginally enhanced seed production per fruit, indicating some degree of pollen limitation. The lack of interaction between nutrient and pollen treatment, however, suggested that the effects of nutrient addition on female reproduction per plant via changes in pollinator behavior were relatively weak in comparison to the direct benefits.

This research demonstrates that plant species respond differently to nutrient and pollen addition, and plant life-history traits may play a role in determining this response. I found immediate effects of nutrient and pollen treatments on the monocarp *I. aggregata*. In the perennial *L. lewisii*, there was a time lag in the responses of growth and female reproductive output to nutrient addition. The enhanced biomass and female reproduction of *L. lewisii* in 2005, the year following fertilization, was likely a result of nutrient storage or preformation of vegetative and reproductive meristems in 2004. These effects of nutrient addition on growth and aboveground biomass support a wide body of plant physiological and ecological research (e.g., Chapin et al. 1986; Tilman and Wedin 1991) as well as corroborate the important role of plant life-history on plant response to environmental resources, with immediate effects of nutrient addition evident in annuals and monocarps and little or delayed effects in iteroparous perennials (e.g., Chiarucci et al. 1999; Paschke et al. 2000; Monaco et al. 2003; Chalmers et al. 2005). Further signs of nutrient storage and delayed use were absent in subsequent years, possibly because these individuals were nearing the end of their lifespan. The lifespan of *L. lewisii* individuals is

unknown, but the majority of the plants studied in 2004, regardless of fertilization or other treatments, were dead by 2007 (either due to natural mortality or gophers). In addition, I observed no effects of pollen treatment on female plant reproduction in *L. lewisii*, indicating a lack of pollen limitation in the year of study. The open floral morphology of *L. lewisii* allows many different pollinators access to pollen and nectar rewards (Kearns and Inouye 1994). Thus, pollen limitation of female reproduction might be rare in this species, assuming a plant receives many visits. Furthermore, the bee and fly pollinators of *L. lewisii* might have been less likely to cue in on changes in floral traits compared to the energetically-limited hummingbird pollinators of *I. aggregata*. Male plant fitness may be relatively more pollen limited than female fitness if this diversity of pollinators results in pollen removal that is rarely deposited on conspecifics (Aizen and Harder 2007). Indeed, I observed high deposition of heterospecific pollen in *L. lewisii*.

In this study, I assessed plant reproduction through both male and female components. I estimated male reproduction on a per-flower basis. Although I found no effect of nutrient addition on pollen production per flower and a decrease in pollen N concentration (in *L. lewisii*), soil nutrient addition does have the potential to alter male reproductive success on a per-plant basis through increased flower production (Devlin et al. 1992; Strauss et al. 2001; Holland et al. 2004). Pollen production per plant in *I. aggregata* was enhanced by nutrient addition through increased flower production, with fertilized plants producing twice as many flowers and presumably twice as much pollen per plant on average than controls, assuming that per-flower estimates of pollen production can be scaled up to the plant level. To determine the potential male fitness

benefits of increased flower (and whole plant pollen production), it would be necessary to measure realized seeds sired.

In conclusion, the direct effects of fertilization on plant fitness estimates were stronger than the indirect effects via changes in pollination, but the timing and extent of those responses depended in part on plant life-history. By broadening research on plant competition and coexistence to include the effects of resources on plants via pollination mutualisms, we will enhance our understanding of the role of abiotic factors in context dependent outcomes of interactions among species. The next step is to extend the scale of this work through community-level experiments of resource additions to allow for complex interactions, such as competition and facilitation among plants for pollinators, which may be under- or over-estimated at the individual plant level.

Figure 2.1. Effects of resource treatments (C = control, W = water, W+F = fertilizer) on (a) floral display, (b) floral resources, and (c) biomass in *Ipomopsis aggregata* (black bars) and *Linum lewisii* (gray bars) in the treatment year, 2004. Error bars are ± 1 SE. Letters above bars indicate significant treatment differences within species at $P < 0.05$.

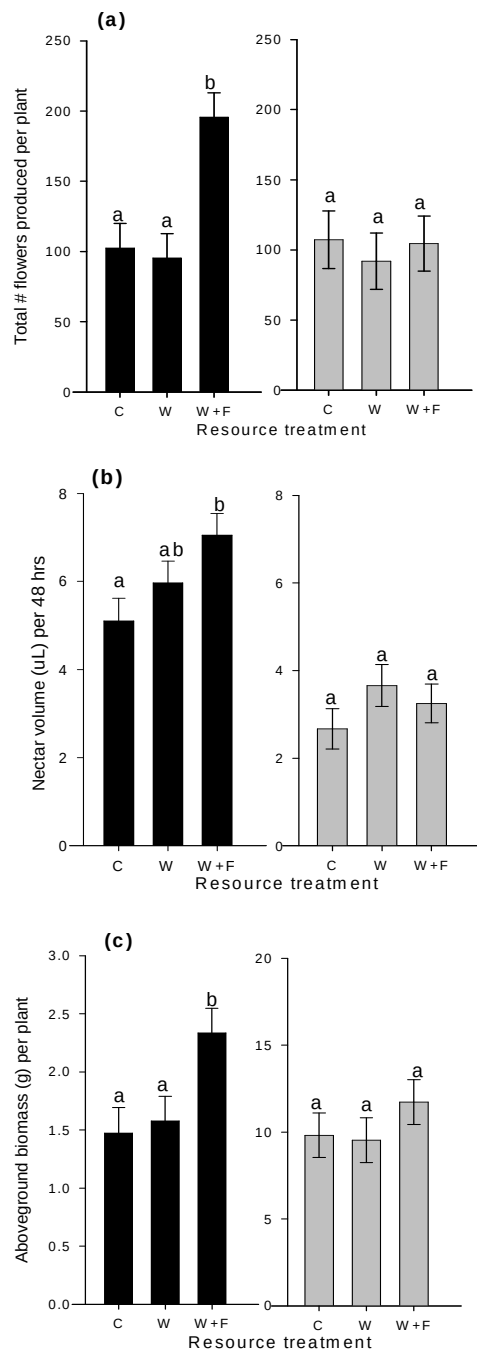


Figure 2.2. Effects of nutrient (C = control, W = water, W+F = fertilizer) and pollen treatments (open = black, hand = gray) on (a) percent fruit set, (b) seeds per fruit, (c) seeds per plant, and (d) mass per seed of *Ipomopsis aggregata* in the treatment year, 2004. Error bars are ± 1 SE.

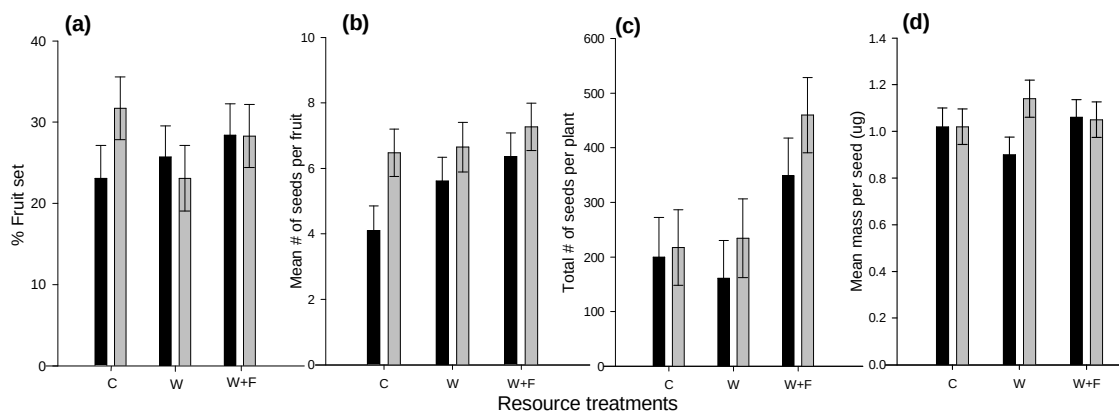


Figure 2.3. Effects of resource treatments (C = control, W = water, W+F = fertilizer) and pollen treatments (natural pollination = black, hand pollination = gray) on (a) percent fruit set, (b) seeds per fruit, (c) seeds per plant, and (d) mass per seed of *Linum lewisii* in the treatment year, 2004. Error bars are ± 1 SE.

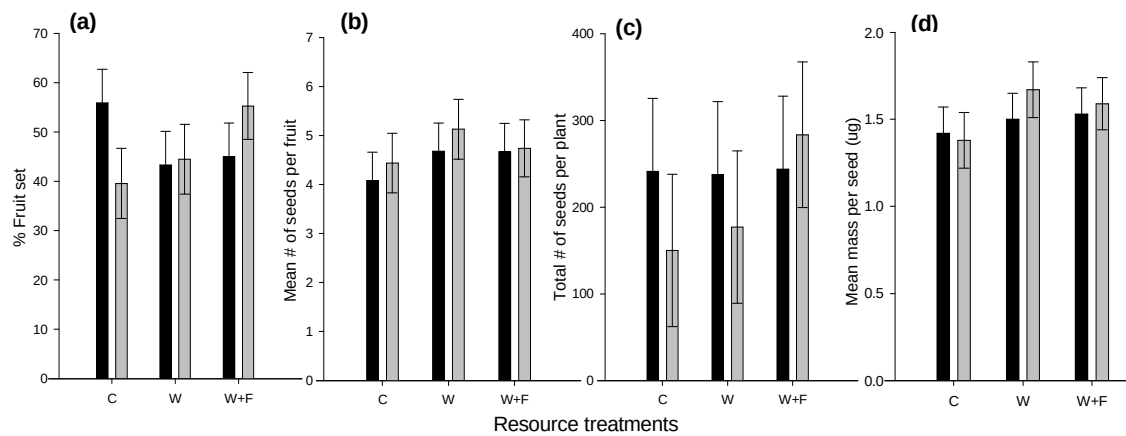
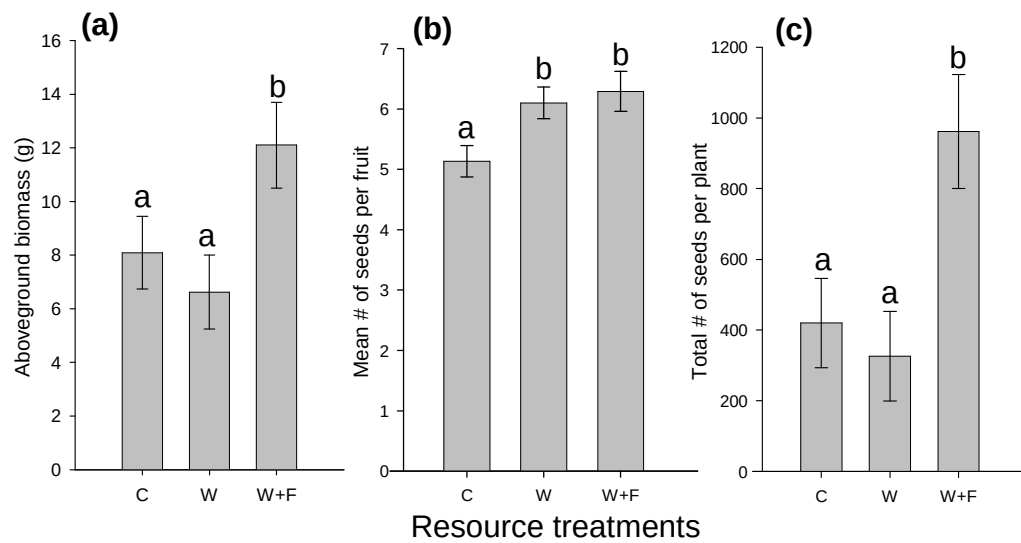


Figure 2.4. Delayed effects of resource treatments (C = control, W = water, W+F = fertilizer) in 2004 on (a) aboveground biomass, (b) seeds set per fruit, and (c) seeds per plant of *Linum lewisii* in 2005. Error bars are ± 1 SE. Letters above bars indicate significant treatment differences at $P < 0.05$.



CHAPTER 3: LINKING POPULATION AND ECOSYSTEM RESPONSES TO NITROGEN ADDITION THROUGH PLANT-POLLINATOR INTERACTIONS

Summary

Nitrogen (N) limits primary productivity in many systems, but plant allocation to biomass may not predict plant community dynamics if plant fitness is not positively correlated with productivity. The fitness of many plants depends on both soil N and pollination, and N may affect floral traits, such as floral, nectar and pollen quality and quantity, that are important to pollinator attraction. Thus, nitrogen may influence plant biomass and fitness directly as well as indirectly via changes in pollination, which may dominate or overturn the direct effects of N addition. For three years, I tested how N addition to subalpine plant assemblages in Colorado, USA affected primary productivity, plant species composition, and plant fitness, as well as how floral traits and plant-pollinator interactions mediated plant-fitness responses. I found that increased productivity due to N addition was not always indicative of increased fitness estimates of forb species. High-N addition favored the growth and reproduction of grasses, while low-N addition promoted forb growth and resulted in variable effects on forb reproduction. I predicted that the differences in forb reproduction were driven by variation in the indirect effects of N addition on pollen limitation. Using a pollen supplementation experiment, I found no evidence of pollen limitation for most components of female reproduction, and nitrogen addition did not indirectly influence the level of pollen limitation via changes in pollination. Although N addition influenced floral traits and subsequent patterns of pollinator visitation, pollinators distributed themselves evenly across floral resources such that per-flower

visitation rate did not differ among N treatments. Understanding how species interactions influence population and ecosystem responses to abiotic resources may provide insight to the dominant forces structuring systems and is especially important in the context of predicting the effects of environmental change. In this case, despite large changes in floral traits that affected pollinator behavior, the direct effects of N addition on plant reproduction were stronger than indirect effects mediated through plant-pollinator interactions.

Introduction

Community and ecosystem ecologists are fundamentally interested in how nutrient limitation affects primary productivity and species composition (e.g., Tilman 1987; Elser et al. 2007). Nitrogen in particular limits primary productivity in terrestrial as well as aquatic systems across a diversity of habitats (Elser et al. 2007), given the essential role of nitrogen in photosynthesis. Understanding the effects of nitrogen on primary productivity has important implications for secondary production and food web structure (e.g., Oksanen et al. 1981; Polis et al. 1997; Wallace et al. 1997); however, plant allocation to biomass alone may not provide insight into long-term plant community dynamics. If biomass production does not correlate with estimates of plant fitness, such as seed production, primary production may not be linked to future population size or species composition. Depending on plant life-history and allocation to growth versus reproduction, it is easy to envision scenarios where nitrogen addition may result in increased primary productivity but not seed production, and vice versa. While a wealth of studies have experimentally measured how nitrogen enrichment affects primary

productivity and species composition (e.g., Tilman 1987; Ditommaso and Aarssen 1989; Stevens et al. 2004), relatively few studies have coupled these measurements with an exploration of estimates of plant fitness, representing a critical gap in our predictive understanding of how nitrogen affects populations and communities. The goal of this study was to test how nitrogen enrichment to subalpine habitats affected estimates of primary productivity, plant species composition and fitness, and some of the mechanisms involved.

The fitness of many terrestrial plants is strongly influenced not only by access to soil nitrogen (e.g., Drenovsky and Richards 2005; Munoz et al. 2005) but also by pollination services (reviewed in Ashman et al. 2004). Up to 90% of flowering plants rely on insects or other animals for pollination and subsequent seed production (Kremen et al. 2007). Nitrogen and pollination limitation, however, are intertwined because within a plant, nitrogen may be allocated to biomass and seed production as well as to traits that are important to pollinator attraction, such as flower production, floral morphology, and nectar and pollen rewards (e.g., Pleasants 1981; Mitchell 1994; Galen 1999). Thus, nitrogen can influence plant biomass and fitness directly as well as indirectly via changes in floral traits and subsequent species interactions. The direct effects of nitrogen may be overridden or even reversed by indirect effects of species interactions. Trait-mediated indirect interactions are increasingly recognized as important drivers of trophic cascades (e.g., Wootton 1994, Schmitz et al. 1997; Trussell et al. 2006) and may also be important drivers of how nutrients affect plant fitness through changes in floral traits and mutualisms (e.g., Poveda et al. 2005; Wolfe et al. 2005).

The addition of nitrogen to individual plant species has provided insight into how pollinators and plants respond at the individual level (Munoz et al. 2005); however, patterns of pollinator visitation and plant fitness are also products of community-level presentation of floral traits (Potts et al. 2003). The vast majority of plant-pollinator interactions are generalized, with plants relying on multiple pollinator species that consume rewards from multiple plant species (e.g., Waser et al. 1996; Jordano et al. 2006). Thus, the response of neighboring species to nitrogen addition could indirectly affect a focal plant's fitness via competition or facilitation for pollinators (e.g., Levin and Anderson 1970; Moeller 2004). For example, nitrogen addition may have weak direct effects on the biomass of some plants but may disproportionately benefit or disadvantage the seed production of these plants through increased pollinator facilitation or competition in flower-rich plots (Feinsinger 1987). It is likely that not all plant species or plant-pollinator interactions will respond in the same direction or with equal magnitude to nitrogen addition to communities. These community-level indirect effects are difficult to predict from experiments of nitrogen addition to individual plant species given that they are emergent properties of ecosystems (Wootton 1993).

In this study, using a three year nitrogen (N) enrichment, I tested how N addition affected primary productivity, plant species composition, and plant fitness, as well as if and how floral traits and plant-pollinator interactions mediated plant-fitness responses. I focused on the effects of N addition in subalpine meadows, an area susceptible to global environmental change (Krajick 2004). Primary productivity in subalpine meadows is often N limited (e.g., Brancaloni et al. 2007), and estimates of female plant fitness are often pollinator limited (Ashman et al. 2004); thus, subalpine systems provide an ideal

habitat for comparing the affects of N addition on primary productivity, estimates of plant fitness, and the mechanisms involved. Specifically, I asked the following questions: (1) Does nitrogen addition affect primary productivity, species diversity, and estimates of plant fitness? I estimated plant fitness using seed production and compared productivity and plant fitness estimates at the whole-plot level, for plant functional groups, and for individual dominant species. Finding that there were species-specific effects of nitrogen addition on the productivity vs. reproductive success of forbs and that forb responses differed from those of grasses, I predicted that these differences were driven by plant-pollinator interactions and pollination. Using a pollen supplementation experiment to a subset of plant species within N treatments, I asked: (2) Does nitrogen addition affect plant fitness directly and indirectly via pollination? Finding no indirect effects of N treatments on pollen limitation, I predicted that, mechanistically, this result may be due to a lack of an effect of N addition on floral traits and pollinator behavior. Thus, I asked: (3) How does nitrogen addition affect floral traits and plant-pollinator interactions? By combining concepts and methods from studies of pollination and ecosystem ecology, this work provides insight into the importance of the direct and indirect effects of nitrogen addition on plant populations and communities.

Methods

Study system

I explored the effects of nitrogen on plant communities in subalpine meadows near the Rocky Mountain Biological Laboratory (RMBL), in western Colorado, USA (latitude: 38°57'29" N, longitude: 106°59'06" W, altitude: 2900 m). Mountain ecosystems

often have low nutrient supply (Bowman and Fisk 2001), and aboveground net primary productivity (ANPP) in these systems can be limited by soil N (Bowman et al. 1993, Theodose and Bowman 1997, but see Cross and Harte 2007). Nitrogen deposition rates are low around the RMBL [mean = 0.4 g nitrate (NO_3^-) and 0.06 g ammonium (NH_4^+) $\text{m}^{-2}\text{yr}^{-1}$, NADP 2006] compared to other areas in the Rocky Mountain west (e.g., Baron et al. 2000, Fenn et al. 2003, NADP 2006), so the RMBL serves as an appropriate baseline for investigating the potential effects of changes in N availability. Average annual precipitation at the RMBL is 52.1 ± 8.3 cm (NADP, 1999-2006), falling primarily as snow in the winter. Summer temperatures (June-August, 2000-2007) average $4.7 \pm 2.6^\circ\text{C}$ (daily lows) and $23.0 \pm 3.8^\circ\text{C}$ (daily highs).

Based on flower abundance, the dominant forbs in this system include *Delphinium nuttallianum* (Ranunculaceae), *Erigeron speciosus* (Asteraceae), *Helianthella quinquenervis* (Asteraceae), *Heliomeris multiflora* (Asteraceae), *Ipomopsis aggregata* (Polemoniaceae), and *Potentilla pulcherrima* (Rosaceae) (Appendix 1). There are also two other plant functional groups: grasses (including *Bromus*, *Elymus*, *Festuca*, *Melica*, *Poa*, and *Trisetum*) and N-fixers (primarily *Lathyrus leucanthus* and *Vicia americana*). The effects of abiotic resources at the individual level have been studied for some of these species near the RMBL. For example, *D. nuttallianum*, a perennial pollinated by hummingbirds and queen bumble bees, increases nectar production per flower, pollinator visitation, and seed set with water addition (Zimmerman 1983). *Erigeron speciosus*, a perennial pollinated by bees and flies, increases biomass, flowering, and inflorescence number with watering, and increases biomass with N addition (de Valpine and Harte 2001). *Helianthella quinquenervis*, perennial pollinated

by bees and flies, increases flowering shoots with water plus N addition (de Valpine and Harte 2001). It is important to note that all of these responses can vary among years, and the treatments are dependent upon background N levels.

To understand the effects of N addition on ANPP, plant reproduction, and pollination, I focused measures on the community level, on plant functional groups (forbs, grasses, N-fixers), and on dominant plants (described above), with particular focus on *Ipomopsis aggregata* and *Potentilla pulcherrima* (hereafter referred to by genus). *Potentilla* is the dominant floral resource in this system across the flowering season, providing the greatest number of flowers (about one third of the flowers available, L. A. Burkle, *unpub. data*). *Ipomopsis* and *Potentilla* differ in life-history and pollinator types, and by studying these two species in depth, I could gain insight into how community-level N addition affected the pollination of plants with two classes of floral traits and pollinator types.

Ipomopsis, a shallow-rooted monocarp, blooms in mid-summer (early July to late-August). *Ipomopsis* remains as a rosette for 2-7 years before flowering (mean $\pm$ 1 SD = 85 ± 66 flowers produced; Campbell 1989) during one season and then dieing. Thus, I could estimate lifetime seed set in one season, and increased seed set generally translates into increased seedling and juvenile recruitment (Waser et al. 2000). *Ipomopsis* is pollinated primarily by broad-tailed (*Selasphorus platycercus*) and rufous (*S. rufus*) hummingbirds around the RMBL (Price et al. 2005). The red, trumpet-shaped flowers are hermaphroditic, protandrous, and bloom for 3-5 days (Pleasants 1983). Nectar production averages $1\text{-}5 \mu\text{L flower}^{-1} \text{ day}^{-1}$, with a sugar concentration of 20-25% (Pleasants 1983). *Ipomopsis* is self-incompatible, requiring pollinators for seed set, and is pollen limited in

some years (Hainsworth et al. 1985; Campbell and Halama 1993; Juenger and Bergelson 1997; Irwin 2006). Nutrient fertilization of *Ipomopsis* (using a 20:20:20 NPK fertilizer) has direct, positive effects on floral rewards and seed production, but minimal indirect effects of nutrients on seed production mediated through changes in pollinator behavior were detected (Campbell and Halama 1993). The effects of N addition alone on *Ipomopsis* are unknown.

Potentilla, a shallow-rooted perennial, blooms from mid-June to late-August. The flowers, which have five yellow petals in an open morphology, are visited by a wide variety of pollinator species, including bees and flies. *Potentilla* is self-compatible but cannot autogamously self-pollinate, thus requiring pollinators for seed set (Stinson 2004). Plants can reproduce vegetatively through the production of additional stalks emerging near the base of the parent plant (L. A. Burkle, *pers. obs.*). The effects of N addition on *Potentilla* are unexplored.

Field manipulations

Nitrogen treatments. In 2005, I identified 24 plots (4 by 4 m each) containing similar densities of wildflower species but covering a diversity of slopes, aspects, and elevations. Plots were grouped into blocks of three based on proximity, and each plot within a block was randomly assigned one of three N treatments (applied for three consecutive summers, 2005-2007): control, “low” N addition ($1\text{ g N m}^{-2}\text{yr}^{-1}$), and “high” N addition ($20\text{ g N m}^{-2}\text{yr}^{-1}$). Treatment plots within blocks were at least 6 m apart from each other, and blocks were up to 2.7 km apart. I applied N in the form of ammonium nitrate (NH_4NO_3) in one dose per week for 10 weeks during each growing season. Each

week, the ammonium nitrate was dissolved in 7.57 L of water, and the control plots received a similar amount of water. The low-N treatment was similar to atmospheric N deposition in the Front Range of the Colorado Rocky Mountains, USA (Sievering et al. 1996). In the high-N treatment, N should have been abundant to plants even after chemical and microbial immobilization (Eviner et al. 2000). These N treatments translated into expected increases in soil N availability [nitrate and nitrite ($\text{NO}_3 + \text{NO}_2$) and ammonium (NH_4), measured using ion-exchange resin bags (Binkley 1984); MANOVA, $\lambda = 0.48$, $F_{4,40} = 4.41$, $P = 0.005$], with high-N plots having greater N availability than low-N plots ($\text{NO}_3 + \text{NO}_2$: $t_{14} = 3.0$, $P = 0.005$; NH_4 : $t_{14} = 2.2$, $P = 0.024$) and low-N plots having higher N availability than controls ($\text{NO}_3 + \text{NO}_2$: $t_{14} = 1.84$, $P = 0.044$; NH_4 : $t_{14} = 2.1$, $P = 0.027$). Experimental plot size was chosen for two reasons, first, to maximize observation of insect visitors (described below) and to reflect the scale at which pollinators make foraging decisions once inside a meadow (Klinkhamer et al. 2001). Second, a previous study found that soil N availability varied naturally at this spatial scale in this system (Dunne 2000). A 1 m border of vegetation around each plot was clipped at the beginning of each season to distinguish the plots to foraging pollinators.

From 2005-2007 in each plot, I marked focal *Ipomopsis* and *Potentilla* (approx. eight per species per plot) to investigate individual plant reproduction (see below). Because *Ipomopsis* is monocarpic, new focal plants were marked in each plot at the beginning of each season. *Potentilla* is perennial, and thus, plants were marked at the end of each season with aluminum tags for relocation the following year. About 8 focal

Potentilla across all plots were missing each year (likely due to herbivory or gopher disturbance); missing plants were replaced with new plants when necessary.

1) *Does nitrogen addition affect primary productivity, species diversity, and estimates of plant fitness?*

To assess if N addition affected ANPP, I collected, separated by species, dried, and weighed aboveground plant biomass of three randomly placed quadrats (0.0625 m²) per plot at the end of each growing season. I measured the effects of N treatments on total ANPP, on individual functional groups (grasses, forbs, and N-fixers), and on the marked individuals of the two focal species, *Ipomopsis* and *Potentilla*. I only collected aboveground biomass of *Potentilla* because they are perennial, but I collected both above- and belowground biomass of *Ipomopsis* because these plants die after flowering. To estimate species diversity (richness and evenness), I used the dried biomass collected and separated by species.

To understand how N addition affected estimates of female plant fitness of forbs, I used measures of per-flower and per-plant seed production in each year. At the end of each season, I collected all of the fruits from each *Ipomopsis* and *Potentilla* focal plant and counted all of the seeds. I calculated per-plant female reproduction as percent fruit set (number of successful fruits divided by total number of flowers), mean seeds per successful fruit, total seeds per plant, and mean mass per seed. Seed mass is an estimate of quality in some species, with heavier seeds positively correlated with the probability of germination, seedling growth, and adult flower production (Stanton 1984). In 2007, I also randomly collected up to 10 fruits per plot from seven other plant species (*Agoseris*

aurantiaca, *Arabis hirsuta*, *Campanula rotundifolia*, *Delphinium nuttallianum*, *Erigeron speciosus*, *Helianthella quinquenervis*, *Heliomeris multiflora*), counted seeds per fruit, and weighed the seeds. These seven forbs were chosen because of their presence in many of the plots and because they spanned a diversity of flower forms (Appendix 1). For *Arabis*, in addition to counting and weighing seeds, I also weighed each silique and counted the number of siliques per plant, a common method for measuring reproductive output for plants in the *Brassicaceae* (e.g., Spataro and Negri 2008). I calculated per-flower female reproduction of each species as the mean seeds per fruit and mass per seed per plot. In 2007, I divided grass biomass (see ANPP above) into vegetative (grass blades) and reproductive (seeds) components and weighed these separately to estimate female grass reproduction at the plot level. No estimates of forb reproduction were made at the plot level, so any inferences made about the effects of N on the reproduction of whole plant communities are limited to the per-flower or per-plant scale.

Statistical analyses. For productivity, I tested the effects of N treatment (control, low, high) on total ANPP, on individual functional group productivity (grasses, forbs, and N-fixers), and on per-plant *Ipomopsis* and *Potentilla* productivity using repeated-measures (rm) ANOVAs with year as the repeated factor. Significant year effects in the rm-ANOVAs were followed by individual tests to determine which year(s) was driving the response (here and in rm-ANOVAs below). I did not include block in these analyses because differences among most blocks were not statistically significant ($P > 0.22$), and including block reduced power to test for the effect of N treatment (Gotelli and Ellison 2004). I tested the effects of N addition on species richness and evenness using rm-

ANOVAs. There was no qualitative difference in the results when I rarefied species richness and evenness (Gotelli and Entsminger 2004), so I reported the original data.

For female reproduction, for focal *Ipomopsis* and *Potentilla* I tested the effects of N treatment, year, and their interaction on mean female reproduction (percent fruit set, mean seeds per successful fruit, total seeds per plant, and mean mass per seed) per plot using a MANOVA for each species. I was unable to perform rm-ANOVAs to assess the effects of N treatment on female reproduction because I would have lost replicates when *Ipomopsis* was not present in a plot in all three years (here and below). Thus, I included plot (nested within N treatment) as a factor to account for repeated sampling of the plots (Quinn and Keough 2002). There were no significant N treatment*year interactions for any of the response variables for *Ipomopsis* or *Potentilla*, so I removed the interaction from the model. For female reproduction of non-focal flowering species in 2007, the effects of N treatment on female reproduction (mean seeds per fruit and mass per seed) were tested using a MANOVA for each species. For *Arabis*, I also included mass per silique and siliques per plant in the MANOVA. For grass reproduction, I tested the effect of N treatment on seed mass per m² using an ANOVA. Throughout, significant MANOVAs were followed by appropriate univariate tests (Scheiner 1993). I predicted that both total ANPP and total seed production would respond positively to N addition (e.g., Bowman et al. 1993; Drenovsky and Richards 2005), but that the response would be moderated by plant functional group and life history given that high N availability typically enhances grass relative to forb productivity (e.g., Bowman et al. 1993; Fynn and O'Connor 2005) and annuals typically respond to N addition more immediately than perennials (e.g., Chiarucci et al. 1999; Paschke et al. 2000; Monaco et al. 2003).

To compare the *relative* responses of ANPP vs. seed production to N addition, I calculated effect sizes. For each block (low- and high-N additions compared to controls), I calculated mean log-response ratios (Hedges et al. 1999; Hillebrand et al. 2007) for ANPP and female reproductive success of all plants, functional groups (grasses and forbs), and species (*Ipomopsis* and *Potentilla*) over the three years of treatment. Using the mean effect size provides an integrated view of effects over the course of the experiment and buffers against small sample sizes. Data for reproduction of individual functional groups (and thus calculation of total plant reproduction) was only available for 2007. I used a random-effects model, including both sampling error and random variation between blocks, for calculating effect sizes (Rosenberg et al. 2000). I calculated 95% confidence intervals with bias-corrected bootstrapping using MetaWin (Rosenberg et al. 2000). If the confidence intervals did not overlap zero, effect sizes were considered statistically significant (Gurevitch and Hedges 2001). I compared these effect sizes to determine whether the magnitude and direction of plant responses to N treatments differed between productivity and fitness estimates.

(2) *Does nitrogen addition directly and indirectly affect plant fitness via pollination?*

Because I found that N addition differentially affected the productivity vs. reproductive success of some forbs, that forbs responded differently than grasses, and that not all forb species responded similarly (see *Results*), I predicted that these differences were driven by variation in the indirect effects of N addition on pollen limitation of seed production, given that many forbs are limited by both soil N and biotic pollination. Thus, I used pollen supplementation treatments to tease apart the direct effects of N addition on

plant fitness vs. the indirect effects mediated through changes in pollination for the two dominant plants *Ipomopsis* and *Potentilla*. *Ipomopsis* reproduction was positively affected by low-N addition, and I predicted that low-N addition would ameliorate pollen limitation, potentially due to N-induced changes in floral traits associated with pollination. Due to the lack of response of *Potentilla* reproduction to N addition relative to controls (see *Results*), I hypothesized that *Potentilla* would be pollen limited for reproduction but that the degree of pollen limitation would not be moderated by N addition.

Supplemental pollen treatments. One-half of the focal *Ipomopsis* and *Potentilla* in each plot were assigned to pollen-supplementation and control treatments. Pollen supplementations were performed every 2-3 days throughout the flowering season by brushing dehiscing anthers on receptive stigmas. Anthers were collected from outside each plot, approximately 5-10 m away. All fruits and seeds were collected and counted at the end of the flowering season. I tested for the effects of pollen supplementation on female reproduction (percent fruit set, seeds per fruit, total seeds per plant, and mass per seed) with a MANOVA with N treatment (control, low, high), pollen-supplementation treatment (supplemented or control), year, plot (nested within N treatment), and the interaction between N and pollen supplementation treatment. A significant interaction between N and pollen supplementation treatment would suggest that N addition alters the level of pollen limitation.

(3) How does nitrogen addition affect floral traits and pollination?

Given that I found no evidence that N treatment altered pollen limitation in *Ipomopsis* or *Potentilla* (see *Results*), I investigated two mechanisms that may explain this lack of effect, namely the effects of N addition on (1) floral traits and (2) pollinator visitation. I focused on these mechanisms because I hypothesized that floral traits and/or pollinators did not respond to the nitrogen treatments. Herbivory and seed predation did not vary among N treatments, suggesting that they did not confound direct or indirect effects of N addition (Appendix 2).

Floral traits. In each year, I measured four floral traits (flower production, flowering phenology, flower size, and nectar production and concentration) to provide a mechanistic understanding of the direct effects of N treatments on characters important to pollination (e.g., Galen 1999; Biernaskie and Cartar 2004; Pleasants 1981). I measured flower production as the total number of open flowers approximately every three days throughout the blooming season for all forb species in all plots (see Appendix 1). To estimate flowering phenology, I measured bloom duration (flowering start and end dates) of each forb species per plot. I measured flower size only for plants of the focal species, *Ipomopsis* and *Potentilla*, using digital calipers to the nearest 0.01 mm. For *Ipomopsis*, I measured corolla length and width (as in Campbell 1996); for *Potentilla*, I measured the length and width of one haphazardly chosen petal. Measurements were made on up to three flowers per focal plant. These components of flower size were chosen because they can contribute to the attractiveness of flowers to pollinators and per-flower pollen removal and deposition (e.g., Galen 1999; Campbell et al. 1996). I estimated nectar

rewards in *Ipomopsis* by measuring nectar production rate (over 48 hr on bagged flowers) and sugar concentration in sucrose equivalents (using a hand-held refractometer) on a maximum of four flowers per focal plant. Nectar measurements are highly repeatable within *Ipomopsis* (Irwin et al. 2004). Both volume and concentration of nectar are important to hummingbird pollinators, who often feed from flowers with copious, dilute nectar (e.g., Baker 1975; Bolten and Feinsinger 1978; Mitchell 1993). I was unable to measure nectar traits in other species due to the small quantities of nectar they produced compared to measurement precision.

I tested the effects of N addition on total flower production per m⁻² and on flower production per plot of nine common forb species present in the majority of the plots (*Agoseris*, *Arabis*, *Campanula*, *Erigeron*, *Helianthella*, *Heliomeris*, *Ipomopsis*, *Lathyrus*, and *Potentilla*) using rm-ANOVAs for each year with N treatment as a fixed effect and sampling date as the repeated factor. To understand whether effects of N addition on flower production per plot were due to differences in flower production per stalk versus the number of flowering stalks per plot, I tested the effects of N addition on the number of flowering stalks per plot over the three years of treatment using a rm-ANOVA for each species. The effects of N addition on bloom duration were tested on the forb species using ANCOVAs with year and plot (nested within N treatment) included as covariates. For all *Ipomopsis* and *Potentilla* focal plants, I tested the effects of N addition on average date of first flower, flower size, and nectar production and concentration (*Ipomopsis* only) per plot using separate rm-ANOVAs with N treatment as a fixed factor and year as the repeated factor. If there were any effects of N treatment on floral traits, I predicted that I would observe increases in floral density, bloom duration, flower size, and nectar

production in the monocarp (*Ipomopsis*) but no effects in the perennials (Chiarucci et al. 1999).

Pollinator visitation. Throughout each flowering season, I observed plant-pollinator interactions in each plot for approximately one hr per week during peak insect activity (0900–1600). I observed plots for a total of 126 hours in 2005, 178 hours in 2006, and 168 hours in 2007. All N treatments were observed equally within a given summer ($F_{2,21} < 0.99$, $P > 39$). I followed visitors from the time they entered the plot until they left, recording the identity of the plants (to species) and pollinators (to species, genus or family; see below) involved in each interaction and the duration of each flower visit. I only recorded visitors that contacted the sexual organs of flowers; thus, it is likely that my estimates of visitation are for effective pollinators. Because I wanted to observe pollinator behavior in the plots, including the number of plants and flowers probed and time spent per flower, I did not collect visitors for identification to species. Instead, I visually identified visitors on the wing to the lowest taxonomic unit possible (to species for bumble bees and hummingbirds and to genus or family for solitary bees, butterflies, moths, and flies).

I calculated four pollinator visitation metrics that were most relevant for understanding overall pollinator visitation to these plots as a function of N treatment. For each plot, I calculated the average (1) time spent per flower, (2) number of flowers visited per pollinator foraging bout, (3) plant visitation rate, and (4) per-flower visitation rate. I tested the effects of N addition on plot averages within years and across all pollinators (all pollinators combined for all tests) for each of the four metrics using separate rm-ANOVAs with year as the repeated factor. Finding that N addition affected

plant visitation rate by pollinators in some years (see *Results*), I repeated this test, including mean number of flowers per plot as a covariate, to determine if N addition *per se* or if the effects of N addition via community-level changes in flower production were associated with the result. I predicted that the main effect of N addition on pollinator visitation would be through changes in community-level flower production.

I also investigated plant-pollinator interactions for the focal plants, *Ipomopsis* and *Potentilla*. For *Potentilla*, I calculated the four pollinator visitation metrics per plot. As above, I tested the effects of N addition on these metrics using separate rm-ANOVAs with years as the repeated factor. I also repeated the test for *Potentilla* plant visitation rate, including the mean number of *Potentilla* flowers per plot and the mean number of total flowers per plot as covariates to determine if N addition or if one or both community-level properties of flower production were involved in pollinator visitation rate to *Potentilla*.

Ipomopsis was pollinated primarily by hummingbirds, which visit infrequently (Campbell et al. 1991) and may be deterred by the presence of a human observer. Thus, I estimated pollinator visitation using stigma pollen loads. Because *Ipomopsis* does not autogamously self-pollinate, pollen receipt is a proxy for pollinator visitation (i.e., increased hummingbird pollinator visits result in increased pollen deposition per stigma; Engel and Irwin 2003). To measure pollen receipt, I collected up to three stigmas from each focal plant once per week throughout each summer. Stigmas were collected once the corollas abscised, ensuring that all flowers went through full female phase and that stigma collection did not affect seed set (Waser and Fugate 1986). Stigmas were stained in basic fuchsin dye (Kearns and Inouye 1993), and the number of conspecific and

heterospecific pollen grains on each stigma were counted under a compound microscope. The presence of conspecific pollen on stigmas indicated the potential for ovule fertilization, while heterospecific pollen represented the possibility of stigma clogging and reduced seed set (reviewed in Wilcock and Neiland 2002). I tested the effects of N addition on average conspecific and heterospecific pollen receipt (mean per stigma per plant) of focal *Ipomopsis* per plot in each year using separate rm-ANOVAs with year as the repeated factor. I also repeated the tests for each year, including mean number of *Ipomopsis* flowers per plot and the mean number of total flowers per plot as covariates to determine if N addition or if one or both community-level properties of flower production were involved in *Ipomopsis* stigma pollen receipt.

Results

1) Does nitrogen addition affect primary productivity, species diversity, and estimates of plant fitness?

ANPP and species diversity. Nitrogen addition affected total ANPP ($F_{2,21} = 14.94$, $P < 0.0001$; Fig. 1). Although there was no effect of N addition on total ANPP in 2005, ANPP increased with N addition in 2006, with double the biomass in the low-N treatment and almost triple the biomass in the high-N treatment relative to the control (Appendix 3). In 2007, both the low-N and high-N treatments supported double the ANPP compared to the control (Appendix 3). Nitrogen addition affected plant functional groups differently (Fig. 1). The ANPP of both forbs ($F_{2,21} = 16.34$, $P < 0.0001$) and grasses ($F_{2,21} = 23.5$, $P < 0.0001$) were affected by N addition, but the ANPP of nitrogen-fixing legumes was not ($F_{2,14} = 0.76$, $P = 0.48$). These effects on forbs and grasses were

delayed, with no effects evident in 2005, but with twice the forb ANPP in the low-N plots and eight times higher grass ANPP in the high-N plots in 2006 and 2007 compared to controls (Appendix 3). Species richness was not affected by N addition ($F_{2,21} = 0.71$, $P = 0.50$), but species evenness reflected the differential effects of N addition on plant functional groups, with 10% to 40% greater evenness in controls compared to low-N and high-N treatments in 2006 and 2007 ($F_{2,21} = 5.93$, $P = 0.009$; Appendix 4). Both forbs (in the low-N plots) and grasses (in the high-N plots) are driving these differences in species evenness. For individual focal plants, there were no effects of N addition on *Ipomopsis* shoot ($F_{2,8} = 0.68$, $P = 0.54$) or root ($F_{2,8} = 0.58$, $P = 0.58$) biomass or on *Potentilla* shoot biomass ($F_{2,18} = 2.2$, $P = 0.13$).

Estimates of female plant fitness. For *Ipomopsis* focal plants, N treatment affected some components of female reproduction (Table 1). Seed set per fruit was 43% higher in control and low-N plots compared to high-N plots ($F_{2,24} = 4.67$, $P = 0.019$). Mass per seed was 32% and 37% greater in control and low-N plots compared to high-N plots ($F_{2,24} = 6.31$, $P = 0.006$). Percent fruit set was not affected by N treatment ($F_{15,24} = 1.5$, $P = 0.17$). Although there was a trend for total seeds per plant to be highest in low-N plots, the trend was not statistically significant ($F_{2,24} = 2.23$, $P = 0.13$). Most components of *Ipomopsis* reproduction varied among years (Appendix 5), with lowest overall reproductive success in 2007.

For *Potentilla* focal plants, N treatment affected some components of female reproduction (Table 1). Percent fruit set was 13% higher in low-N compared to high-N plots ($F_{2,40} = 3.71$, $P = 0.033$). Total seeds per plant and mass per seed were both 16% higher in control and low-N than in high-N plots (total seeds: $F_{2,40} = 6.69$, $P = 0.003$;

mass per seed: $F_{2,40} = 7.42$, $P = 0.002$). There was no effect of N treatment on seeds per fruit ($F_{2,40} = 1.98$, $P = 0.24$). All components of *Potentilla* reproduction varied among years, but there was no one year in which all components were most successful (Table 1).

For non-focal forb species and grasses in the plots in 2007, N addition had species-specific effects on female reproduction. For *Arabis*, N addition affected female reproduction ($\lambda = 0.049$, $F_{8,18} = 3.50$, $P = 0.048$). *Arabis* siliques weighed 200% more in low-N plots compared to controls, but there was no effect of N addition on seeds per fruit, weight per seed, or siliques per plant (Table 1). For the other forbs I measured (*Agoseris*, *Campanula*, *Delphinium*, *Erigeron*, *Helianthella*, or *Heliomeris*), I found no effect of N addition on per-flower female reproduction for any species (Table 1). For grasses, I found that N addition increased grass seed biomass per m^2 ($F_{2,21} = 7.1$, $P = 0.0044$), with 13 and 3 times greater grass seed mass in high-N than control or low-N plots, respectively.

Comparison of the relative responses of ANPP vs. seed production. Some measures of ANPP and estimates of plant fitness responded differently in magnitude and direction to N addition (Fig. 2). The effect sizes of N addition depended on the scale (plot-level, plant functional group, or plant species) and the allocation measure (biomass vs. reproduction) under observation. Grasses and forbs responded differently to N addition. High-N addition strongly increased both ANPP and reproduction of grasses, while low-N addition moderately increased total forb ANPP but not total forb reproduction. Nitrogen treatments did not affect all forb species similarly and differentially affected their productivity vs. fitness. *Ipomopsis* exhibited moderately increased reproduction, but not biomass, in the low-N treatment. Although there was a

tendency for high-N addition to decrease *Potentilla* biomass and reproduction, there were no significant effects of N treatments relative to controls. The high-N treatment had moderate, positive effects on both total ANPP and total reproductive success, driven by the strong effects of N on grass ANPP and reproduction.

(2) Does nitrogen addition directly and indirectly affect plant fitness via pollination?

Based on the results above, I predicted that low-N addition would ameliorate pollen limitation in *Ipomopsis*. For *Potentilla*, I predicted that reproduction would be pollen limited but that there would be no indirect effects of N addition on the level of pollen limitation. These predictions were partially supported for *Potentilla*, but not for *Ipomopsis*. For both species, there were no significant interactions between the N and pollen treatments for any measures of reproduction, indicating that N addition did not influence the degree of pollen limitation (Table 2). In *Ipomopsis*, the supplemental-pollen treatment had no effect on any measurement of female reproduction ($F_{4,225} = 0.24$, $P = 0.92$), suggesting that increased seed set per fruit, when found, was likely driven by increased resources to provision seeds or possibly increased pollen quality received to sire seeds. In *Potentilla*, there was an effect of pollen supplementation on total seeds across N treatments ($F_{1,413} = 10.39$, $P = 0.001$), with 15% higher total seeds in hand-pollinated plants compared to open-pollinated controls in 2007 ($F_{1,112} = 8.00$, $P = 0.006$).

(3) How does nitrogen addition affect floral traits and pollination?

Flower production. Despite my prediction, I found that at the plot level, nitrogen addition affected total flower production, with greater flower production in the low-N

plots compared to control or high-N plots (Fig. 3). These effects were delayed, present only in 2006 ($F_{2,21} = 5.59$, $P = 0.011$) and 2007 ($F_{2,21} = 9.3$, $P = 0.0013$). This pattern held for individual forb species. *Ipomopsis*, *Potentilla*, *Agoseris*, and *Erigeron* all had greater floral densities in the low-N plots compared to the other treatments in 2006 and 2007 (Appendix 5). However, *Lathyrus*, a nitrogen-fixing legume, had greater flower density in control plots compared to either N addition treatment in 2006 ($F_{2,16} = 4.93$, $P = 0.022$) and 2007 ($F_{2,11} = 4.18$, $P = 0.045$). *Helianthella* was the only forb species with greater floral density in high-N plots compared to control or low-N plots (2007; $F_{2,14} = 9.86$, $P = 0.0021$). Nitrogen treatment did not affect the flower production at the plot level of *Arabis*, *Campanula*, or *Heliomeris* in any year (Appendix 5). Nitrogen addition did not affect the number of stalks per plot in any species (Appendix 6), suggesting that effects of N on flower production per plot were driven by differences in flower production per stalk and not by changes in the number of flowering stalks present.

Flowering phenology. At the plot level, nitrogen addition affected the bloom duration of several plant species. *Potentilla* bloomed 8 days longer in 2005 and 6 days longer in 2006 in low-N plots compared to control and high-N plots, but bloom duration was unaffected by N addition in 2007 (Appendix 7). *Agoseris* bloomed 20 days and 31 days longer in low-N plots than control and high-N plots, respectively, in both 2006 and 2007 (Appendix 7). The bloom duration of *Helianthella* was unaffected by N addition in 2005 or 2006, but bloomed 12 days longer in high-N plots compared to control or low-N plots in 2007 (Appendix 7). Although there was no effect of N addition on *Lathyrus* bloom duration in 2005 or 2006, *Lathyrus* bloomed 13 days longer in control plots compared to low-N or high-N plots in 2007 (Appendix 7). There were no effects of N

addition on bloom duration in *Ipomopsis*, *Arabis*, *Campanula*, *Erigeron*, or *Heliomeris* in any year (Appendix 7). For individual focal plants, N addition did not affect the date of first flower of *Ipomopsis* ($F_{2,9} = 0.20$, $P = 0.82$) or *Potentilla* ($F_{2,18} = 0.20$, $P = 0.82$).

Flower size. There were effects of N addition on flower size in both *Ipomopsis* and *Potentilla* focal plants (Appendix 8). I also found a significant effect of year, such that the effects of N addition on flower size were delayed. For *Ipomopsis*, although I saw no effects of N addition on components of flower size in 2005, I found that flowers of plants in low-N and control plots had at least 10% longer and wider corollas than flowers in high-N plots in 2006 and 2007 (Appendix 8). For *Potentilla*, there were no effects of N addition on flower size in 2005 or 2006, but plants in the low-N plots had at least 20% longer and wider petals than those in control or high-N plots in 2007 (Appendix 8).

Nectar production and concentration. Nitrogen addition affected nectar production ($F_{2,7} = 13.89$, $P = 0.004$) but not nectar sugar concentration ($F_{2,7} = 0.94$, $P = 0.44$) in *Ipomopsis* focal plants. In each year, N addition affected nectar production, with plants in the low-N plots producing 64% more nectar on average (Appendix 9).

Pollinator visitation. Nitrogen addition affected plant visitation rate by pollinators (Fig. 4, rm-ANOVA, $F_{2,21} = 3.7$, $P = 0.042$), with low-N plots having a higher rate of visitation than control or high-N plots. This effect was driven by flower production and not N addition *per se* (Appendix 10); when included as a covariate, flower production was statistically significant (2006: $F_{1,20} = 7.41$, $P = 0.013$; 2007: $F_{1,20} = 13.73$, $P = 0.0014$), but N addition was not (2006: $F_{2,20} = 0.72$, $P = 0.50$; 2007: $F_{2,20} = 0.59$, $P = 0.56$). This result suggested that community-level effects of N addition on floral traits were important in influencing pollinator behavior. I also found a significant effect of

year, such that the effects of N addition on plant visitation rate were delayed (Appendix 10). In support of my prediction, however, there were no effects of N addition on the number of flowers visited per foraging bout, the number of seconds spent per flower, or the per-flower visitation rate (Fig. 4) in any year (Appendix 10). Thus, increased flowers enhanced visitation, but there were no benefits of these changes in pollinator behavior on a per-flower basis.

I found similar effects of N addition on pollinator visitation to *Potentilla*, as was observed for all flowers combined. Nitrogen addition affected *Potentilla* plant visitation rate (rm-ANOVA, $F_{2,18} = 7.64$, $P = 0.004$), with low-N plots having the highest visitation rate. This effect was driven by both N addition and *Potentilla* flower production per plot, but not by total flower production per plot (Appendix 10).

For focal *Ipomopsis* individuals, mean stigma receipt of conspecific (rm-ANOVA, $F_{2,8} = 1.38$, $P = 0.31$) and heterospecific pollen (rm-ANOVA, $F_{2,8} = 0.17$, $P = 0.85$) per plot was unaffected by N addition, but pollen receipt in general declined over the three years of study (conspecific: $F_{2,7} = 100.1$, $P < 0.0001$; heterospecific: $F_{2,7} = 5.02$, $P = 0.044$), with over three times higher conspecific pollen receipt in 2005 compared to 2007. Neither *Ipomopsis* flower production per plot nor total flower production per plot influenced *Ipomopsis* stigma pollen receipt in any year (ANCOVAs; 2005: $F_{4,6} = 1.28$, $P = 0.37$; 2006: $F_{4,9} = 0.77$, $P = 0.99$; $F_{4,8} = 0.37$, $P = 0.83$).

Discussion

The availability of nutrients can limit primary productivity and affect community composition in many systems (e.g., Siemann 1998; Elser et al. 2007; Clark and Tilman

2008). However, our understanding of how nutrient limitation differentially affects plant productivity versus fitness and the role that species interactions play in mediating these effects have not been thoroughly explored at the community level. Here, I built upon previous ecosystem studies by adding N and measuring biomass effects over multiple years, and, in addition, I considered how N indirectly affected plant fitness estimates through changes in functional traits and species interactions. I found that increased productivity with N addition was not always positively correlated with fitness estimates, indicating that productivity may not necessarily be linked to future population sizes or population dynamics. High levels of N addition favored grasses via increased ANPP and reproductive success. Forbs responded positively to low levels of N addition via increased ANPP, but effects of N on female reproduction were variable. Pollen-supplementation showed that most components of plant reproduction were not pollen limited and that N addition did not have indirect effects on reproduction via changes in pollination. Mechanistically, I observed that N addition affected floral traits, and pollinators responded by changing their plant visitation rate per plot, but, from the plants' perspective, there were no effects of N addition on per-flower visitation rate, likely explaining why there were no indirect effects of N addition on plant reproduction. This work emphasizes the importance of considering productivity, fitness measures, and the mechanisms by which they are affected to more fully understand the bottom-up effects of resource addition to plant communities. In this case, despite large changes in floral traits that affected pollinator behavior, the direct effects of N addition on plant reproduction were stronger than indirect effects mediated through plant-pollinator interactions.

Nitrogen addition affected ANPP at the plot level, establishing the first evidence that N can be limiting in this system (Cross and Harte 2007 found no effect of adding 6 g N m⁻² yr⁻¹, a level intermediate between my low- and high-N treatments). This result is in agreement with a host of other studies documenting N as a major limiting nutrient in terrestrial systems (Vitousek and Howarth 1991; Elser et al. 2007), including subalpine meadows (Brancaleoni et al. 2007). These effects on productivity, however, were delayed, emphasizing the important role of perennial life history in plant response to environmental conditions, with immediate effects of nutrient addition on annuals and little or delayed effects in perennials (e.g., Chiarucci et al. 1999; Paschke et al. 2000; Monaco et al. 2003). In high-N plots, increased ANPP was driven by grasses, while forb productivity increased in low-N plots. Nitrogen addition often results in enhanced grass productivity or dominance, possibly due to the strong competitive ability of grasses in high-N environments (e.g., Shaver and Chapin 1986; Huenneke et al. 1990; Fynn and O'Connor 2005). For example, the tundra of the Colorado Front Range responds to N fertilization, with shifts from forb-dominated to grass-dominated communities (Bowman et al. 1993). Moreover, the floral density and bloom duration of *Lathyrus*, an N-fixer, declined in N-addition plots relative to controls, likely because legumes lose their competitive advantage over other species when N is no longer limiting (Ganade and Brown 1997; Suding et al. 2005). These effects of N on productivity were evident in measures of species diversity. I did not observe any loss of species richness in the N-addition plots as expected (reviewed in Ditommaso and Aarssen 1989; Rajaniemi 2003), but the large changes in biomass of plant functional groups with N addition resulted in decreased evenness in these plots. These changes in evenness may forecast potential

losses of species (or functional groups) in some N treatments if the treatments had been applied for longer time periods.

In addition to altering ANPP of plant functional groups, N addition affected female reproduction of the individual focal plants, *Ipomopsis* and *Potentilla*. For each species, at least two of four components of female reproduction measured, including percent fruit set, seeds per fruit, total seeds per plant, and mass per seed, were influenced by N addition, with low-N addition generally increasing female reproductive success and high-N addition decreasing success relative to controls. However, pollen supplementation did not have widespread effects on female reproduction in either *Ipomopsis* or *Potentilla*. Only the number of total seeds per plant in *Potentilla* was increased by pollen supplementation. I did not find any interactions between N and pollen treatments, suggesting that N addition did not influence the degree of pollen limitation and providing evidence of the lack of indirect effects on reproduction associated with changes in pollination. These results parallel an ecosystem-level assessment in which the direct effects of N addition on ecosystem properties, such as soil carbon and nitrogen storage, were stronger than the indirect effects associated with changes in species composition (Manning et al. 2006). Thus, although I found that N addition altered the quality and quantity of floral traits and some subsequent aspects of plant-pollinator interactions, these changes in species interactions did not translate into effects on plant reproduction. Although pollen limitation varies among years (reviewed in Ashman et al. 2004) and there can be both nutrient and pollen limitation of reproduction in other systems (e.g., Dimling 1992; Mattila and Kuitunen 2000; Asikainen and Mutikainen 2005; Pina et al.

2007), I found that for three years, N limitation was more important for female reproduction than pollination, a common trend (e.g., Ne'eman et al. 2006).

The outcomes of mutualistic interactions can be context dependent, depending in part on the availability of resources in the environment (Bronstein 1994). For example, changes in soil nutrients can alter the nature of plant-mycorrhizae interactions, with plants reducing or even eliminating mycorrhizae when soil phosphorus is abundant, suggesting that the costs of mycorrhizal fungi exceed their benefits to the plants under nutrient-rich conditions (Johnson et al. 1997). Likewise, in plant-endophyte interactions, the benefits of endophytes to plants only exceed the costs in nutrient-rich environments (Lehtonen et al. 2005). I also expected plant-pollinator mutualisms to be influenced by nutrient availability, with nitrogen addition altering the degree of pollen limitation through pollinator responses to changes in floral rewards in different N treatments. However, pollination did not limit most components of reproduction in all years of study, and thus, surprisingly I did not reveal any conditionality of plant-pollinator mutualisms depending on soil nitrogen availability.

Two caveats should be considered when interpreting the results of the effects of N addition on plant reproduction in this study. The first caveat is the scale at which I was able to measure seed set for most species. I saw few effects of N addition on female reproduction measured as seeds per fruit and mass per seed of non-focal flowering species. This result, however, may be due to sampling individual fruits instead of quantifying whole-plant reproductive success (Reekie and Bazzaz 2005), given the widespread effect of N addition on total flower production. Future assessments of female reproduction involving per-plant and per-plot estimates of forb fitness will allow stronger

conclusions to be drawn about the effects of N across forb species. Second, the spatial scale of my N manipulations mimicked the scale at which N varies naturally in this system (Dunne 2000) but did not address the potential effects of larger-scale changes in nitrogen, such as N deposition. If nitrogen availability was manipulated at the watershed scale, many of the same mechanisms would likely explain the effects of N on plant biomass and reproduction, but pollinator choice would likely play less of a role at this larger scale.

Investigating mechanisms at different scales (e.g., individuals to plant communities) was important in understanding the effects of N addition on plant reproduction via traits and species interactions. Low-N addition increased community-level flower production, subsequently enhancing plant visitation rate per plot by pollinators. In this system during the years studied, however, the two plant species investigated were not pollen limited for seed production, and pollinators distributed themselves evenly over the available floral resources across treatments. Previous work has shown that pollinators can exhibit patterns of foraging approximating an ideal free distribution (e.g., Ishihama and Washitani 2007). Pollinators may alternatively visit proportionally fewer flowers from a large display (see Goulson 2000 for summary). Here, low levels of N addition to a flowering plant assemblage resulted in community-level facilitation of attracting pollinators to the area, but neither competitive nor facilitative effects on per-flower pollination were observed. In order to determine whether flower production itself or other effects of N addition on plant community traits were driving pollinator behavior, direct manipulations of flower abundance of different species are needed. It is likely that *Potentilla* flower production alone contributes strongly to

pollinator attraction. Indeed, only *Potentilla* flower number per plot, and not total flowers per plot, contributed to the enhanced plant visitation rate that I observed in this species. In addition, I focused the majority of my measures of reproduction on common or dominant plants, but to make more universal conclusions across species, quantifying the effects of N addition on the reproductive success of rare plants may provide additional insights.

In summary, three years of N enrichment affected productivity, floral functional traits, pollinator visitation, and female plant reproduction. The higher overall productivity in the high-N treatment was not positively correlated with fitness estimates of individual forb species. The mechanisms involved in this pattern likely include the competitive dominance of grasses over forbs in high-N environments. The direct effects of N on reproduction were stronger than the indirect effects associated with pollination. Although more pollinators were attracted to the high floral densities in the low-N plots, they appeared to be foraging optimally, such that on a per flower basis, visits were equal in number among treatments. Thus, pollinators did not drive differences in plant fitness, and bottom-up effects of N availability were more important to plant reproduction. The generality of these results in other systems remains to be tested.

Table 3.1. ANOVA table for reproduction responses of forbs and grass to nitrogen addition across the three years of treatment. These tests were used to understand the direct effects of N on components of reproductive success on different plant species and possible delayed effects. Statistically significant P-values are bolded.

Response variable	Predictor variable	df	F	P
<i>Ipomopsis</i>	MANOVA ($\lambda = 0.04$)	60,84	1.79	0.0067
Percent fruit set	ANCOVA (whole model)	15,24	1.5	0.17
	N treatment	2,24	0.72	0.50
	year	2,24	3.82	0.036
Seeds per fruit	ANCOVA (whole model)	15,24	3.42	0.0036
	N treatment	2,24	4.67	0.019
	year	2,24	4.87	0.017
Seeds per plant	ANCOVA (whole model)	15,24	4.77	0.0004
	N treatment	2,24	2.23	0.13
	year	2,24	13.98	< 0.0001
Mass per seed	ANCOVA (whole model)	15,24	3.11	0.0064
	N treatment	2,24	6.31	0.006
	year	2,24	4.32	0.025
<i>Potentilla</i>	MANOVA ($\lambda = 0.02$)	88,149	2.86	< 0.0001
Percent fruit set	ANCOVA (whole model)	22,40	3.09	0.001
	N treatment	2,40	3.71	0.033
	year	2,40	13.09	< 0.0001
Seeds per fruit	ANCOVA (whole model)	22,40	3.08	0.001

	N treatment	2,40	1.48	0.24
	year	2,40	5.84	0.0059
Seeds per plant	ANCOVA (whole model)	22,40	3.84	0.0001
	N treatment	2,40	6.69	0.0031
	year	2,40	15.25	< 0.0001
Mass per seed	ANCOVA (whole model)	22,40	3.54	0.0003
	N treatment	2,40	7.42	0.0018
	year	2,40	10.01	0.0003
<i>Arabis</i>	MANOVA ($\lambda = 0.049$)	8,18	3.50	0.048
Silique mass		2,7	5.36	0.039
Seeds per fruit		2,11	0.90	0.44
Mass per seed		2,11	2.65	0.12
Siliques per plant		2,10	2.17	0.17
<i>Agoseris</i>	MANOVA ($\lambda = 0.41$)	4,6	0.83	0.55
<i>Campanula</i>	MANOVA ($\lambda = 0.86$)	4,14	0.28	0.89
<i>Delphinium</i>	MANOVA ($\lambda = 0.76$)	4,18	0.66	0.63
<i>Erigeron</i>	MANOVA ($\lambda = 0.75$)	4,24	0.95	0.45
<i>Helianthella</i>	MANOVA ($\lambda = 0.74$)	4,20	0.81	0.53
<i>Heliomeris</i>	MANOVA ($\lambda = 0.75$)	4,16	0.62	0.65
<i>Grass</i>	ANOVA	2,21	7.1	0.0044

Table 3.2. ANOVA table for reproduction responses to supplemental pollination. Here, I tested for evidence of pollen limitation and of indirect effects of nitrogen addition on the degree of pollen limitation (nitrogen x pollen interaction). See Appendix 3 for direct effects of nitrogen addition on the reproduction of *Ipomopsis* and *Potentilla* across the three years of treatment. Statistically significant P-values are bolded.

Response variable	Predictor variable	df	F	P
<i>Ipomopsis</i>	whole model	76,889	2.50	<0.0001
	pollen treatment	4,225	0.24	0.92
	nitrogen x pollen	8,450	0.77	0.63
<i>Potentilla</i>	whole model	108,1582	5.26	<0.0001
	Percent fruit set			
	pollen treatment	1,413	1.85	0.17
	nitrogen x pollen	2,413	0.66	0.52
	Seeds per fruit			
	pollen treatment	1,402	0.46	0.50
	nitrogen x pollen	2,402	0.65	0.52
	Seeds per plant			
	pollen treatment	1,413	10.39	0.0014
	nitrogen x pollen	2,413	2.15	0.12
	mass per seed			
	pollen treatment	1,402	2.35	0.13
	nitrogen x pollen	2,402	0.23	0.80

Figure 3.1. Annual net primary productivity of grasses (black), forbs (light gray), and nitrogen fixers (dark gray) in control, low-N addition, and high-N addition plots over three years of nitrogen enrichment (2005-2007). Error bars not shown.

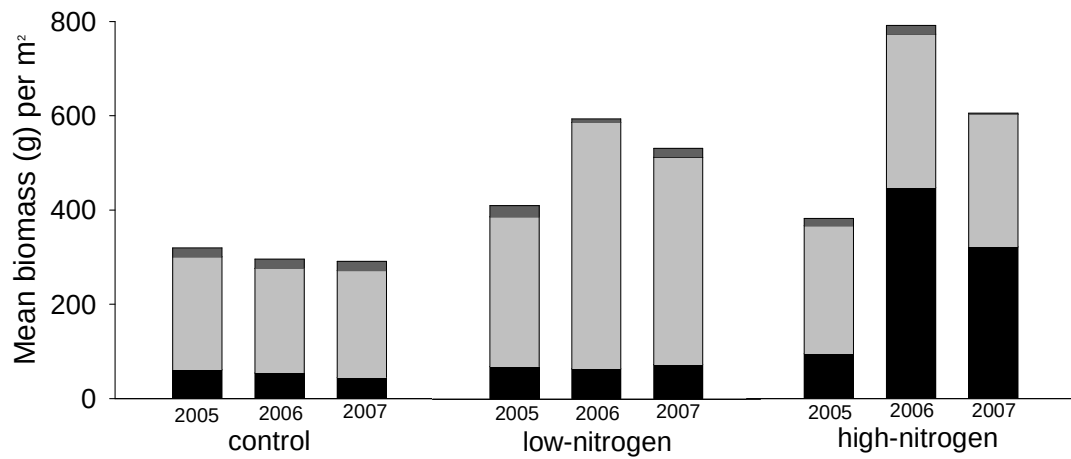


Figure 3.2. Mean effect size (log response ratio) of plant responses to low- (black circles) and high-N addition (open circles) for both aboveground net primary productivity (ANPP; panels a–e) and female fitness estimates (panels f–j). ANPP was measured as biomass per m² for total plants, grasses, and forbs, and as biomass per plant for *Ipomopsis* and *Potentilla*. Fitness estimates were measured as seeds per fruit for total plants and forbs, biomass per m² for grasses, and total seeds per plant for *Ipomopsis* and *Potentilla*. Effect sizes are means over all three years of N treatment, except for total seeds, grass seeds, and forb seeds, which were only measured in 2007. Error bars are bias-corrected 95% confidence intervals. Asterisks (*) denote significant effect sizes at $\alpha = 0.05$.

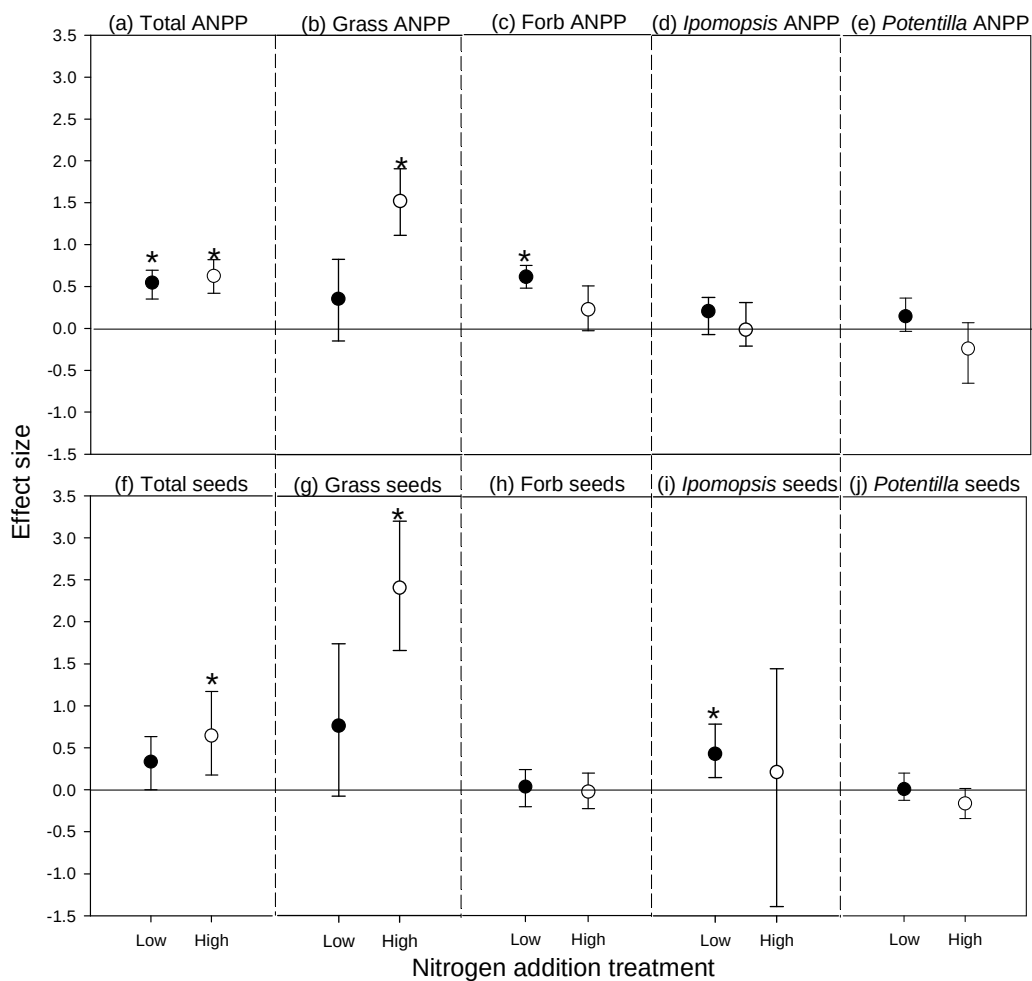


Figure 3.3. Mean number of total flowers m^{-2} in control (black circles), low-N (white circles), and high-N (black triangles) plots over the flowering season in (A) 2005, (B) 2006, and (C) 2007. Error bars not shown.

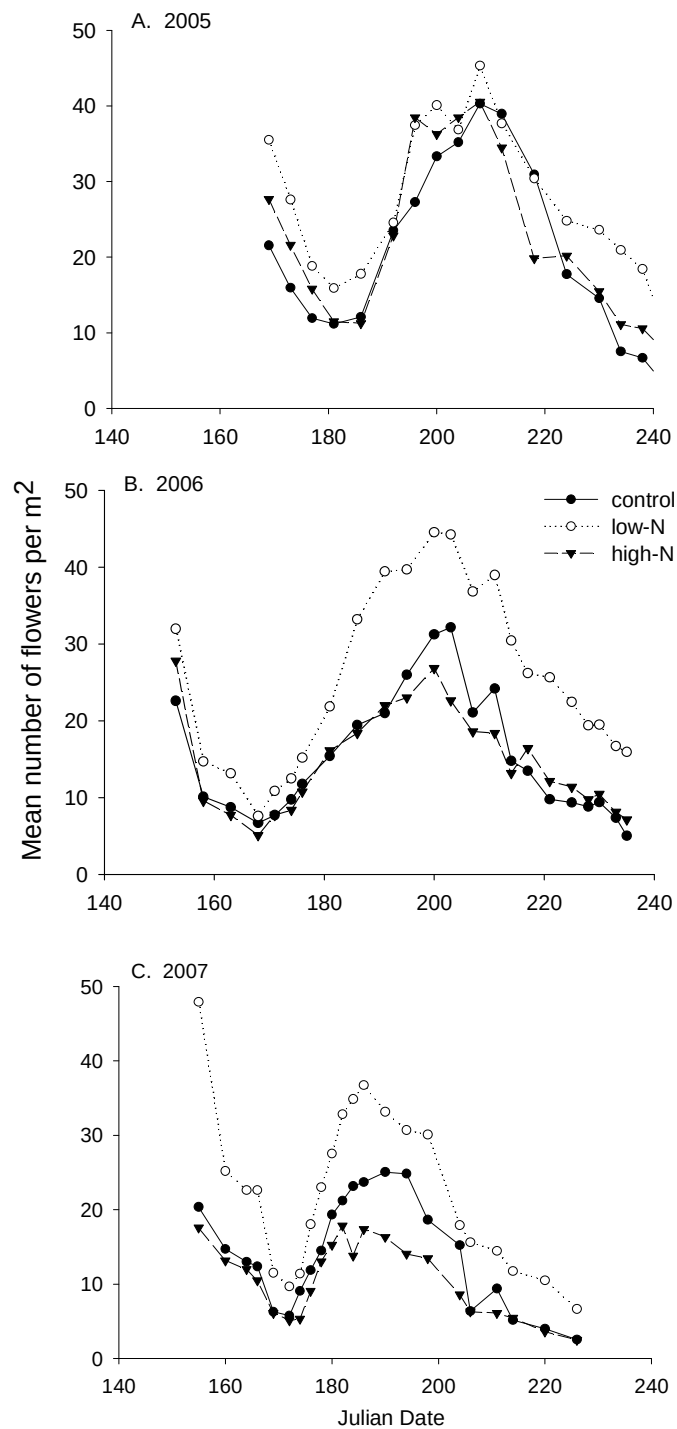
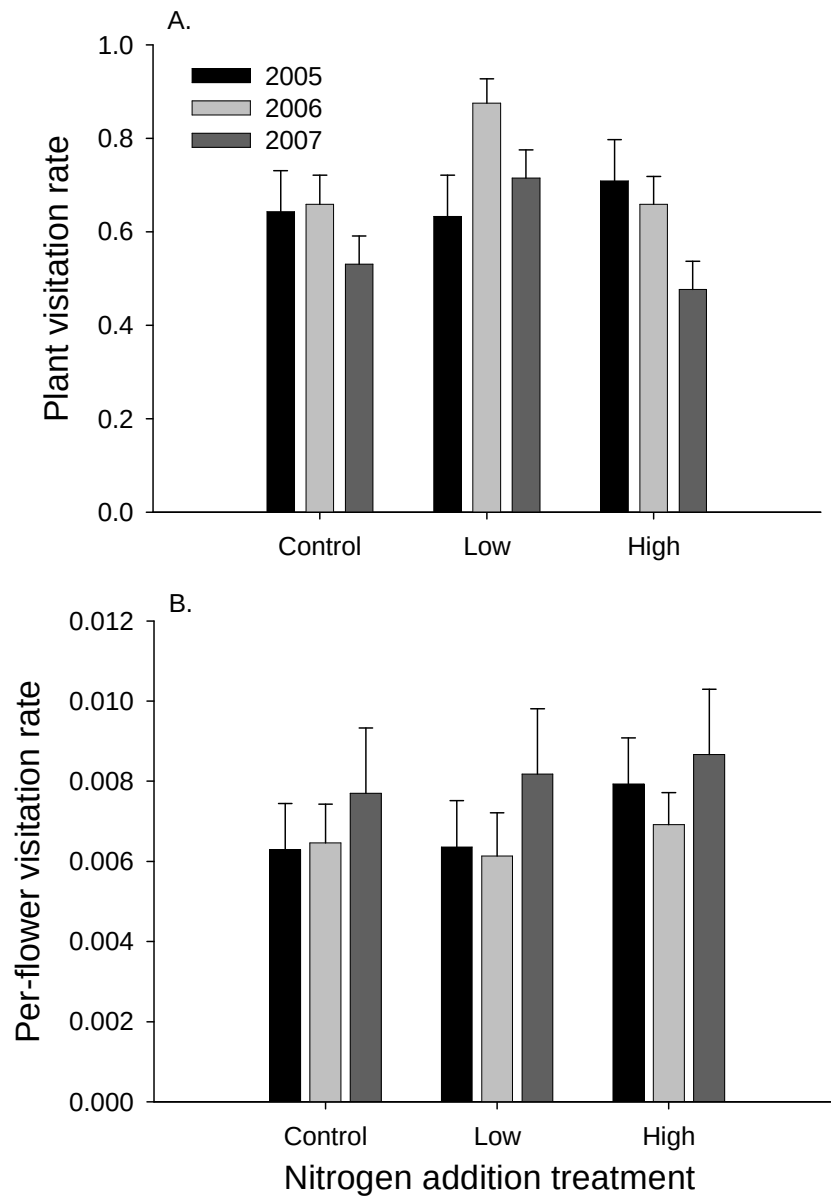


Figure 3.4. Mean plant visitation rate and per-flower visitation rate of pollinators to control, low-N addition, and high-N addition plots over the three years of treatment (2005: black, 2006: light gray, 2007: dark gray). Error bars are ± 1 SE.



CHAPTER 4: PLANT-POLLINATOR NETWORKS: LARGE INTERANNUAL VARIATION IN STRUCTURE BUT NO BOTTOM-UP EFFECTS OF NITROGEN ENRICHMENT

Summary

Striking changes in food web structure occur with alterations in the resource supply. Like predator-prey interactions, many mutualisms, such as plant-pollinator interactions, are also consumer-resource interactions, and network structure may be influenced by resource availability. However, no studies have explored how the structure of plant-pollinator networks may be affected by nutrient enrichment. For three years, I experimentally added different levels of nitrogen to subalpine meadows and measured plant-pollinator interactions. Although nitrogen enrichment affected floral rewards and individual pollinator behavior, there were no effects on plant-pollinator network structure. I found that rarefied species richness and evenness of plants used by a pollinator and of pollinators visiting a plant were not affected by nitrogen addition. Furthermore, nitrogen addition had no effect on the core group of generalist plants and pollinators or the degree of plant-pollinator network nestedness, indicating that the same plants and pollinators interacted with each other and with similar frequency across nitrogen treatments. Individual plant and pollinator taxa, however, were packed into the nested networks differently among nitrogen treatments. This result suggests that there were widespread effects of nitrogen addition on individual taxa in the community but that the effects were not strong enough at this spatial and temporal scale to fundamentally change plant-pollinator associations throughout the network. Overall, I found that,

independent of nitrogen enrichment, there were large interannual differences in network structure and interactions. These results indicate the importance of other factors, such as climate and top-down effects of predators, which may influence pollinator populations and their interactions with plants in a community.

Introduction

Understanding what factors control the diversity of species and their interactions in natural systems are major goals in ecology (van der Heijden et al. 1998; Tilman 2000; Worm et al. 2002). Trophic interactions, in particular, are of fundamental importance in communities, providing pathways for the flow of energy and resources (Lindeman 1942) and contributing to the distribution and abundance of species (Price et al. 1980; Carpenter et al. 1985; Hunter and Price 1992). Food web studies have advanced our understanding of the structure of trophic interactions and the importance of particular species or feeding links to population and community dynamics (Paine 1980; Polis 1994). Like predator-prey interactions, mutualisms also can be considered consumer-resource interactions (Holland et al. 2005), and there is growing recognition that some mutualistic interactions, such as plant-pollinator interactions, are web-like in structure (e.g., Jordano 1987; Waser et al. 1996; Memmott 1999; Jordano et al. 2003b). Thus, we may be able to apply concepts and techniques established by food web research to make predictions and formulate empirical tests to understand the community ecology of pollination mutualisms.

In many systems, bottom-up forces, particularly resource supply, can play a central role in structuring trophic interactions (Hunter and Price 1992), and dramatic

changes in food webs can occur with alterations in the resource base (Oksanen et al. 1981; Polis et al. 1997; Wallace et al. 1997; Bukovinszky et al. 2008). High resource availability can increase plant quality and subsequent interactions at higher trophic levels (e.g., Stiling and Rossi 1997; Forkner and Hunter 2000), with resource addition increasing the abundance of consumers in terrestrial and aquatic systems (Peterson et al. 1993; Polis et al. 1998). Optimal foraging and competition theories make different predictions about how resource addition should affect the diet breadth of species within food webs. Optimal foraging theory predicts that niche breadth should expand when resources are poor or limiting (MacArthur and Pianka 1966), while competition theory predicts that niche breadth can expand or contract depending on the evenness of competition among species (Gause 1934). In the context of predator-prey food webs, positive, negative, and nonlinear relationships between productivity and the number of feeding links per species (i.e., niche or diet breadth) have been found for individual consumers, but no relationship was detected between productivity and the mean number of links across predators within the food web (Townsend et al. 1998; Arim and Jaksic 2005). Thus, despite dynamic changes in individual links, network structure may be robust to changes in resource base (Arim and Jaksic 2005). The effects of resource additions on mutualistic interaction networks are unknown, but I can make predictions based on food web theory, flowering plant responses to resource additions, and consumer responses to variation in resource quantity and quality.

The addition of nutrients to flowering plants can affect the quantity and quality of the floral resource base. For example, the addition of low to moderate nitrogen or fertilizer to individual plants can increase nectar and flower production as well as flower

size (e.g., Ryle 1954; Campbell and Halama 1993; Sperens 1997; Munoz et al. 2005), while high or chronic fertilization of plant assemblages can increase the competitive dominance of some plant species, such as grasses, and reduce species richness, especially of flowering forbs (e.g., Bowman et al. 1993; Sebastia 2007). Food web theory predicts that food web structure should be affected by traits of the component species at the resource base that affect, for example, the behavior of consumers (Bukovinszky et al. 2008). Indeed, large-bodied, high energy-use pollinators, such as hummingbirds, bumble bees, and solitary bees, can behaviorally respond to variation in nectar and flower quality and quantity, typically being attracted to large flowers and floral displays and copious nectar (e.g., Pleasants 1981; Mitchell 1994; Galen 1999; Biernaskie and Cartar 2004). Thus, I might expect resource addition to decrease the niche breadth of pollinators and the structural complexity of plant-pollinator webs if pollinators forage only from highly rewarding flowering species that respond positively to resource addition.

Optimal foraging and competition theories can also be applied to make predictions about how changes in the floral resource supply will affect plant-pollinator network structure. For example, an increase in the nectar resource supply could allow insects to expand their niche breadth and increase niche overlap if nectar accumulation allows insects with short proboscises to gain access to typically inaccessible nectar (Inouye 1978). Or, increased floral resources may simply attract more total pollinator species (Potts et al. 2003; Hegland and Boeke 2006), especially if enhanced floral resources can support more competing pollinator species or if resource addition affects the diversity or abundance of rare floral forms that attract more specialized pollinators (Fenster et al. 2004). Alternatively, under competition theory, I may expect that increased

floral resources would decrease niche breadth of pollinators (as pollinators often forage on their preferred food items) as well as niche overlap (Tepedino 1980), assuming that enhanced floral resources alleviate competition pressure and food limitation in pollinator populations. Many of our predictions about how flowering plants and pollinators will respond to resource addition derive from manipulations of individual plants and observations of their pollinators (Munoz et al. 2005). However, plant-pollinator networks, like food webs, are emergent properties of systems, and the degree to which the nutrient resource supply affects the structure of mutualistic networks remains unexplored, representing a critical gap in the study of plant-pollinator networks.

The goals of this study were to investigate the bottom-up effects of nitrogen (N) enrichment on the structure of plant-pollinator networks and on the generalization of species (i.e., the number of other plants or pollinators with which a species interacts) within these networks. For three years, I added different levels of N (control, low-N addition, and high-N addition) to plots in subalpine meadows and observed plant-pollinator interactions. I focused on the subalpine because floral production in these ecosystems is often N-limited (de Valpine and Harte 2001), and pollinators are often energy (nectar) limited (Montgomerie and Gass 1981). In addition, other research in this system has shown that low levels of N enrichment increase the productivity of flowering plants, flower production across species, flower size, per-flower nectar production, and pollinator visitation rate to plants, while high levels of N enrichment have the opposite effect; these alterations in flowering plant abundance and floral rewards occurred without affecting plant species richness (this dissertation, Chapter 3). Thus, different levels of N enrichment may have strong and divergent effects on the floral resource supply as well as

the structure plant-pollinator networks. Over this subalpine landscape varying in resources, I investigated the existence of differences in the structure of plant-pollinator networks among N treatments and how this variation compared to the magnitude of variation I observed across years. Specifically, I asked the following questions:

(1) *How do N addition and year affect the diversity of plants used by a pollinator and of pollinators visiting a plant?* I expected the diversity of both plants and pollinators to be affected, with higher diversity in the low-N addition networks (due to enhanced floral rewards) and lower diversity in the high-N addition networks (due to reduced floral rewards associated with high chronic N addition; Fynn and O'Connor 2005) compared to the controls.

(2) *Does the structure of plant-pollinator networks vary among N treatments and years?* In other words, do the same plants and pollinators interact with each other among N treatments and do they maintain these relationships among years? I predicted that patterns of pollinator visitation, and thus network structure, would vary both among N treatments (due to changes in pollinator visitation rates and richness of visitors to plant species) and years (due to interannual variation in pollinator composition and population sizes).

(3) *Do the patterns of network nestedness vary among N treatments and years?* Nested networks are common in ecological systems (Bascompte et al. 2003; Jordano et al. 2006), containing specialists interacting with subsets of more generalist species. Because N addition to plant assemblages can influence the abundance, phenology, and morphology of flowers (Munoz et al. 2005; Chau et al. 2005; Kumar et al. 1999; this dissertation, Chapter 3), I predicted that N addition would alter the nestedness of plant-

pollinator networks. In the absence of variation in resource supply, plant phenology and the quality and quantity of floral resources produced by certain plant species can be relatively predictable (e.g., Wcislo and Cane 1996; Price and Waser 1998), so I hypothesized that plant-pollinator interactions in control plots would remain highly nested, while N addition (low and high) would disrupt the predictable presentation of floral rewards, rendering plant-pollinator interactions more random and decreasing nestedness.

(4) *Do the members of the generalist core vary among N treatments and years?* I predicted a greater richness of plants and pollinators to be categorized as members of the generalist core (i.e., those plants and pollinators with the greatest frequency and richness of interactions with other taxa) in low-N addition networks due to novel visitation by pollinators responding to increased floral rewards. Although plant and pollinator abundances may fluctuate among years, I did not expect these fluctuations to affect the identity of core generalist species.

Methods

Study System

Field work for this study was conducted near the Rocky Mountain Biological Laboratory (RMBL) in Gothic, Gunnison County, Colorado, USA (2900 m). Nitrogen deposition rates are low around the RMBL (0.4 g NO_3^- and $0.06 \text{ g NH}_4^+ \text{ m}^{-2} \text{ yr}^{-1}$, NADP 2006), so it serves as an appropriate baseline for investigating the potential effects of changes in resource availability on plants and higher trophic levels. Mountain ecosystems

often have low nutrient supply, and productivity in these systems can be limited by soil N (Bowman et al. 1993; Theodose and Bowman 1997).

The meadow habitat where this work was conducted is dominated by perennial angiosperms with a diversity of floral forms. Plants with open floral morphology include *Erigeron speciosus*, *Helianthella quinquenervis*, *Linum lewisii*, and *Potentilla pulcherrima*, and they are pollinated by a variety of bees and flies (e.g., Kearns and Inouye 1994). Plants with more complex floral morphology, such as those with tubular corollas or nectar spurs, include *Ipomopsis aggregata* and *Delphinium nuttallianum*, which are pollinated primarily by hummingbirds and queen bumble bees, respectively (Waser 1978). Hereafter, I refer to plants by their genus. The reproductive biology of these plants ranges from self-incompatible (e.g., *Ipomopsis*) to self-compatible (e.g., *Delphinium*), although many of the self-compatible species still require pollinators to transfer even self-pollen from anthers to stigmas (e.g., *Delphinium*, *Linum*, and *Potentilla*; Price and Waser 1979; Waser and Price 1990; Kearns and Inouye 1994; Stinson 2004). The plants in this system each produce tens to hundreds of flowers per plant per year, providing nectar and/or pollen to floral visitors, and flower production per plant can vary with resource addition (Campbell and Halama 1993; de Valpine and Harte 2001).

Nitrogen manipulations

In summer 2005, I identified 24 plots (16 m² each) with similar abundance and richness of plant species. Each plot received one of three N treatments over three summers (2005-2007): control, low-N addition (1g N m⁻²yr⁻¹), or high-N addition (20 g N

$\text{m}^{-2}\text{yr}^{-1}$) in 10 aqueous doses of ammonium nitrate (NH_4NO_3) applied throughout the growing season. The low-N treatment was similar to atmospheric N deposition in the Front Range of the Colorado Rocky Mountains (Sievering et al. 1996). In the high-N treatment, N should have been abundant to plants even after chemical and microbial immobilization (Chapin et al. 1986; Eviner et al. 2000). I chose the plot size for three reasons. First, plots of this size maximize observation of insect visitors and reflect the scale at which pollinators make foraging decisions once inside a meadow (Klinkhamer et al. 2001). Second, the plot size and arrangement encompassed a scale at which both nested structure and pollinator choice may be present (Summerville et al. 2002). Third, soil N availability varies naturally at this scale in this system (Dunne 2000).

Pollinator observations

Each plot was observed for approximately 1 hr per week during peak insect activity (~0900 - 1600) in good weather across the entire flowering season (June through August). The plots were observed for a total of 126 hrs in 2005, 178 hrs in 2006, and 168 hrs in 2007. The number of observation hours per N treatment did not vary significantly within summers ($F_{2,21} < 0.64$, $P > 0.39$). Rarefaction curves created from visitation data from each year (1000 sub-samples; EcoSim 7.72 used here and below; Gotelli and Entsminger 2004) did not overlap for the majority of the visitation range (Fig. 4.1), indicating that although treatment plots were observed for similar numbers of hours in each year, the number of realized plant-pollinator links were different among years. The year with the greatest number of observation hours, 2006, had an intermediate number of links, suggesting that factors other than small differences in observation hours among

years were driving differences in observed link numbers (see *Results*). Although the number of plant-pollinator links increased with time, such that the greatest numbers of links were observed in 2007, I do not consider this to be an artifact of increased proficiency at observing and identifying links. The team of observers was different in each year, and we all used the same reference insect collection and resources to confirm identifications. L.A. Burkle was the only observer present in all three years. In addition, the number of rarefied pollinator families observed reached asymptote in each year, indicating that there were simply more pollinator families (and their interactions with plants) present in 2007.

For all flower-visitor interactions observed, I recorded the identity of the plant and pollinator. I only recorded interactions in which I saw visitors actively contacting plant reproductive parts while feeding on or collecting pollen and nectar. For the analyses, I identified the plants to species and the pollinators to family (the lowest common taxonomic unit I had for all visiting pollinators; see Appendix 4.1 for flowering plants and pollinators observed). Because I did not want to disturb the pollinators during their foraging bouts (see Burkle and Irwin, *in prep*), I did not collect insects for identification to genus or species. My goal here was to investigate the effects of nitrogen addition on the structure of plant-pollinator interactions, and elsewhere, I have performed detailed analyses of visit duration and number of flower visits per foraging bout of those pollinators that were identified to species or morphospecies (Burkle and Irwin, *in prep*).

One caveat in this study is that plants and pollinators were identified to different taxonomic resolution (species vs. family, respectively), and taxonomic lumping can affect interpretation of food web properties (Paine 1988; Martinez 1993; Solow and Beet

1998). I have retained the species-level classification of plants to take advantage of this level of detail and to avoid problems associated with lumping plants. If I classified plants by family, I would lose half of my resolution, since many of the plants were in the Asteraceae, a dominant plant family in this system (Appendix 4.1). To determine if this difference in classification level between plants and pollinators influenced the outcome of my analyses (described below), I repeated all analyses with both plants and pollinators classified to family (Appendix 4.2). The results were qualitatively similar, and so I report only the analyses with plants identified to species and pollinators identified to family.

Data analyses

Pollinators could fly and choose among plots within a meadow, so for network analyses comparing N treatments (questions 2-4), all observation data from an N-treatment level were pooled across plots, and for analyses comparing years, all observation data were pooled across N treatments within a year. From the observation data, I created Pollinator x Plant matrices for each N treatment within a year and for each year, indicating the total number of times I saw each interaction (Fig. 4.1; see Appendix 4.3 for summary statistics for each matrix). All statistical analyses were conducted using JMP 4.0 (JMP 2001) unless noted otherwise.

(1) How do N addition and year affect the diversity of plants used by a pollinator and of pollinators visiting a plant? For each plot in each year, I calculated the rarefied richness and evenness of all pollinators visiting all plants and compared among N treatments, years, and their interaction using a MANOVA. Rarefied richness was used to compare among treatments and years with different numbers of visits observed. A

significant interaction in the analysis would indicate that the effect of N addition on visiting pollinator diversity depended on year. Univariate tests followed significant MANOVAs (here and below; Scheiner 1993). I also calculated the richness and evenness of all pollinators visiting each plant species and the richness and evenness of all plants visited by each pollinator family and compared among N treatments, years, and their interaction using MANOVAs. I included plant species or pollinator family identities, respectively, as a factor in the MANOVA to avoid performing separate tests for each taxa while controlling for taxa-specific differences in responses.

(2) Does the structure of plant-pollinator networks vary among N treatments and years? Here I asked if the same plants and pollinators interact with each other and with the same frequency among N treatments and if they maintain these interaction patterns among years. To determine whether the structure of the plant-pollinator networks varied among N treatments within a year and among years, I investigated the correspondence between Pollinator x Plant interaction matrices by performing Procrustes analyses using the FATHOM toolbox in MatLab v. 7.4 (Jones 2002). Procrustes analysis can be used for community-level ecological comparisons (Jackson 1995, Peres-Neto and Jackson 2001) by mapping the positions of the pollinators between two superimposed floral backgrounds (e.g., comparing Pollinator x Plant matrices between two N treatments) and minimizing the pollinator sums-of-squares distances between the two matrices (Alarcón et al., *in review*). I determined the significance of the resulting goodness-of-fit statistic (m^2) with the permutations test set to 10,000 permutations (Procrustes permutation option in MatLab; Jackson 1995, Peres-Neto and Jackson 2001). When m^2 approaches 0, there is a good fit between the two matrices and indicates similar network structures (similarity in

the identity and frequency of plant-pollinator interactions), but when m^2 approaches 1, there is a poor fit and indicates different network structures (differences in the identity or frequency of interactions). I did not use the Bonferroni method to adjust α to correct for multiple comparisons (here and below), and I report the original P-values (Gotelli and Ellison 2004). I then used the vector residuals to identify pollinator families that exhibited the greatest changes in interactions between N treatments and years (Alarcón et al., *in review*). Large vector residuals indicate large changes in position between two matrices, highlighting pollinator families that had altered patterns of visitation among N treatments or years. I predicted that network structure would vary among N treatments, with large values of m^2 for all matrix comparisons. I predicted that the bottom-up effects of N treatments would affect niche breadth, with generalist bees and flies increasing their niche breadths in low-N addition treatments compared to controls due to increased floral rewards and visitation to certain plant species, and the opposite occurring in high-N treatments. As a result, I expected to observe large vector residuals for generalist bees and flies in these analyses. In addition, I predicted that network structure would differ among years due to interannual variation in pollinator species composition and population sizes (Herrera 1988, Petanidou and Ellis 1993, Williams et al. 2001; Petanidou et al. 2008, *in press*; Alarcón et al., *in review*).

(3) *Do the patterns of network nestedness vary among N treatments and years?* A nested network structure is characterized by specialists mainly interacting with generalists. Understanding alterations in nestedness is important because the degree of network nestedness indicates how robust species and interactions will be to perturbations (e.g., Bascompte et al. 2003; Memmott et al. 2004), such as the bottom-up effects of N

addition. I used the program ANINHADO to calculate the nestedness of each Pollinator x Plant matrix (Guimaraes and Guimaraes 2006). Nestedness can be measured in units of Temperature (T), with 0° representing a perfectly nested network and 100° representing a random network (Atmar and Patterson 1993). To determine the significance of Temperature, I used a Monte Carlo randomization procedure with a null model (called CE) in which the probability of an interaction occurring is proportional to the degree (the number of interactions in which a species participates) of both the plant and the pollinator. This null model was used because it incorporates the observed pattern that the number of interactions per species is highly skewed, with some species interacting with many others and other species interacting with very few, as opposed to other null models that make the unrealistic assumption that each species has the same probability of having an interaction (Jordano et al. 2006). I also calculated the Idiosyncratic Temperature, a measure of how a species' pattern of links deviates from the pattern expected in a perfectly nested matrix, of plant and pollinator taxa seen in all N treatments within a year and also of all taxa observed in all three years. Finally, I calculated Spearman Rank correlations to determine if taxa were "packed" into the community matrix similarly in N treatments within years and in each year (Wessa 2007; Alarcón *et al.*, *in review*). Similar values of Temperature would indicate similarity in the degree of nestedness among plant-pollinator networks among N treatments and years. Positive, significant correlations of Idiosyncratic Temperatures between two matrices would indicate that each plant and pollinator taxon was occupying a similar position in community nestedness.

(4) *Do the members of the generalist core vary among N treatments and years?*

When a limiting resource is added to a system, a common pattern observed is that fewer

primary producers can coexist because of the loss of niche dimensionality due to decreased niche and life-history trade-off opportunities, altered stoichiometry, and/or increased resource homogeneity (Harpole and Tilman 2007). However, the number of species in the generalist core of consumers could theoretically increase due to expanded niche breadth. For example, adding a resource (like N) to a plant assemblage could result in greater community-level presentation of floral rewards, like nectar and pollen (Campbell and Halama 1993; Lau and Stephenson 1993; Munoz et al. 2005). With lower competition for more abundant floral resources, pollinators could expand their diet through the use of additional plant species (Fenster et al. 2004) even if there is an assemblage-wide decline in plant species richness, assuming that pollinators can perceive differences in floral rewards (Goulson 2003; Jersakova and Johnson 2006; Johnson and Morita 2006). Alternatively, pollinators could contract their diet breadth if their preferred host plant(s) offered ample floral resources, but this scenario is unlikely given the small quantities of nectar produced in this system (even with N addition) and the likelihood of nectar (energy) limitation of pollinator populations in the subalpine (Montgomerie and Gass 1981; Pyke 1982; Bowers 1985). Although pollinator access to some floral resources may be limited by morphology (e.g., combination of flower size and pollinator tongue length; Stang et al. 2007), I predicted that the plant and pollinator species comprising the generalist core would differ among N treatments. I hypothesized that more total species would be categorized as generalists in low-N addition treatments due to novel visitation by pollinators responding to increased floral rewards associated with N addition, and a loss of these same plants and pollinators from the core in high-N plots due to highly reduced visitation (Chittka and Schurkens 2001).

I used the program UCINET (version 6) to identify the core generalist group of plant and pollinator taxa with which specialists interacted (Borgatti et al. 2002; Alarcón *et al.*, *in review*). For plants and pollinators in each treatment per year and in each year, I calculated eigenvector centrality scores, which are proportional to the sum of the degree centrality scores of all of the partner taxa with which a taxon interacts, such that taxa with larger values tend to interact with more generalized taxa (Jordano et al. 2006). The core group of generalists was delineated by separating out those plants and pollinators with the highest eigenvector centrality scores that also participated in >5% of the visits and interacted with >25% of the taxa in that N treatment or year. I compared the core groups to determine if generalist members differed among N treatments and years.

Results

(1) How do N addition and year affect the diversity of plants used by a pollinator and of pollinators visiting a plant?

The rarefied richness and evenness of pollinator families visiting all plants per plot were not affected by N treatment (Wilks' $\lambda = 0.96$, $F_{4,106} = 0.62$, $P = 0.65$) but differed among years (Wilks' $\lambda = 0.25$, $F_{4,106} = 26.41$, $P < 0.0001$). There was no interaction between N treatment and year (Wilks' $\lambda = 0.91$, $F_{8,106} = 0.64$, $P = 0.74$). Rarefied richness was 79% and 30% greater in 2007 than in 2005 or 2006, respectively (Fig. 4.2a, $F_{2,60} = 84.7$, $P < 0.0001$). Evenness was 31% and 16% greater in 2007 than in 2005 or 2006, respectively (Fig. 4.2b, $F_{2,60} = 22.5$, $P < 0.0001$).

Similarly, N treatment did not affect the rarefied richness and evenness of pollinators visiting a plant species (Wilks' $\lambda = 0.99$, $F_{4,638} = 0.12$, $P = 0.98$) or of plants

used by a pollinator (Wilks' $\lambda = 0.99$, $F_{4,924} = 1.75$, $P = 0.14$), but year did (respectively, Wilks' $\lambda = 0.62$, $F_{4,638} = 43.12$, $P < 0.0001$; Wilks' $\lambda = 0.95$, $F_{4,924} = 6.19$, $P < 0.0001$). There were no interactions between N treatment and year for rarefied richness or evenness of pollinators visiting a plant species (Wilks' $\lambda = 0.99$, $F_{8,638} = 0.42$, $P = 0.91$) or of plants used by a pollinator family (Wilks' $\lambda = 0.99$, $F_{8,924} = 0.60$, $P = 0.78$). Richness and evenness of both visiting pollinators and plants used were greater in 2007 than in 2005 or 2006, as above ($F_{2,320} > 11.3$, $P < 0.0001$).

(2) Does the structure of plant-pollinator networks vary among N treatments and years?

Network structure was similar among N treatments within a year (m^2 approaching 0) but not among years (m^2 approaching 1; Table 4.1). This result indicates that the pollinators present in one year visited the plant species in different N treatments similarly; however, large differences in visitation occurred between years (Table 4.2). The changes in interactions among years were due to large fluctuations in abundances of certain pollinator families, especially pollinators in the Apidae and Anthomyiidae. Between 2005 and 2007, the total number of visits by Apidae dropped from 1,430 to 322 visits with a loss of 12 links to plant species, while the total number of visits by Anthomyiidae increased from 7 to 705 visits, with a gain of 19 links to plant species (Fig. 4.3).

(3) Do the patterns of network nestedness vary among N treatments and years?

The plant-pollinator networks were highly nested in structure in each of the three treatments and in each of the three years ($P < 0.0001$ in all cases). Overall, there was little

among-treatment or among-year variation in nestedness (Appendix 4.3). However, correlations of Idiosyncratic Temperatures indicated that plants and pollinators were not packed into N treatments or years similarly (Table 4.3), suggesting differences in the rank order and degree of nestedness of taxa among N treatments and years. The only instance in this study where plants and pollinators held similar positions in the nested matrix between N treatments was in 2007, where I found a significant positive relationship of Idiosyncratic Temperatures between control and low-N treatments (Table 4.3).

(4) Do the members of the generalist core vary among N treatments and years?

The identity of plants and pollinators comprising the core group of generalists was similar across N treatments within a year (Fig. 4.4). Contrary to my prediction, I did not find more total plant and pollinator taxa categorized as generalists in low-N addition treatments due to novel visitation by pollinators.

The identity and rank order of the core plants and pollinators differed substantially among years (Fig. 4.4). *Heliomeris*, *Helianthella*, *Potentilla*, and *Erigeron* were all important members of the core in 2005, while *Potentilla* dominated as the primary core plant species in 2006 and 2007. Apidae dominated the core group of pollinator generalists in 2005, Halictidae in 2006, and Anthomyiidae in 2007.

Discussion

Investigating how changes in the resource supply of a community affect higher trophic levels and species interactions is central to understanding the structure and organization of communities and how these communities will respond to environmental

change (Polis 1994; Vitousek et al. 1997; Tilman and Lehman 2001). The consequences of resource manipulations have been studied extensively in the context of food webs (Wallace et al. 1997; Bukovinszky et al. 2008), providing evidence of bottom-up impacts on the abundance of consumers (Peterson et al. 1993; Polis et al. 1998) and the structure of species interactions (Stiling and Rossi 1997; Forkner and Hunter 2000). However, little is known about the effects of nutrient resources on the structure of consumer-resource mutualisms, such as plant-pollinator mutualisms. After three years of nitrogen addition to meadow plots, I found no effects of nutrient enrichment on network structure and nestedness or on the core group of generalist plants and pollinators, even though floral abundance and presentation varied greatly among N treatments (this dissertation, Chapter 3, and also see Campbell and Halama 1993; Sperens 1997; Munoz et al. 2005). However, there were differences between N treatments in how plant and pollinator taxa were packed into the networks, indicating that N addition affected the generalization and nestedness of many taxa in the community but that these effects were not strong enough to alter the network structure. Interestingly, there were large changes in species interactions and network structure among years, presumably due to variation in pollinator composition and abundance among years (e.g., Herrera 1988; Petanidou and Ellis 1993; Williams et al. 2001). These results suggest that the structure of plant-pollinator interactions may be buffered from bottom-up effects of N enrichment at this scale but that other biotic and abiotic factors acting at larger spatial and temporal scales, such as climate and top-down effects of predators, may play pivotal roles in the structure of plant-pollinator networks.

Positive, negative, and neutral bottom-up effects of nutrient enrichment on food web structure have been observed. For example, in a *Spartina* salt marsh, Gratton & Denno (2003) found that nutrient enrichment had positive effects on primary productivity and the abundance of taxa at higher trophic levels. In contrast, in a seagrass system, high levels of nutrients simplified the food web through a loss of species diversity (Tewfik et al. 2007). Similar to the absence of strong effects of nitrogen addition on plant-pollinator network structure, food web structure may not be affected by nutrient enhancement in all systems. For example, in a different seagrass system, nutrient addition did not affect productivity or the biomass and abundance of seagrass epiphytes (Heck et al. 2000). Lack of bottom-up effects on food web structure may be due to top-down effects reducing or moderating bottom-up effects (Hunter and Price 1992; Armitage and Fong 2006). Prey diversity, intraguild predation, omnivory, cannibalism, and strong web connectivity can also influence the strength of bottom-up effects (Schmitz et al. 2000; Snyder and Wise 2001; Hart 2002; Rosenheim and Corbett 2003). Comparable forces may contribute to the structuring of plant-pollinator networks, although the mechanisms remain unexplored.

In most cases, N addition did not affect the diversity (richness and evenness) of plants used by pollinators and of pollinators visiting plants. Thus, the approach of quantifying the effects of N addition on the diversity of plant-pollinator interactions (Albrecht et al. 2007) gave qualitatively similar results to the network analyses. The lack of large bottom-up effects of N addition on the structure of plant-pollinator networks at this spatial and temporal scale was surprising given that previous work in this system, and other systems, has shown that the abundance and quality of floral rewards of some plant species are affected by nutrient addition and that species composition shifts towards

grasses at the expense of forbs in high nitrogen environments (e.g., Bowman et al. 1993; Campbell and Halama 1993; Gardener and Gillman 2001, this dissertation, Chapter 3).

One possible explanation for a lack of effect on plant-pollinator networks is that, due to morphological and phenological constraints, pollinators in this system have limited opportunities to alter their patterns of visitation (Stang et al. 2007). However, this explanation seems unlikely for most bees, flies, and butterflies in this system given that the majority of plant species had an open morphology that is accessible to a wide variety of pollinator types (see Appendix 4.4). An alternative explanation for the lack of effect may be the invariance of nestedness of plant-animal mutualisms (e.g., Bascompte et al. 2003; Nielsen and Bascompte 2007). Irrespective of network size, plant-pollinator interactions typically center around a core of generalist species that maintain a proportionally large number of interactions (Jordano et al. 2006). Thus, changes in floral abundance or the composition of non-core species due to N addition may not affect the generalist core and associated network structure. In addition, previous work has shown that nestedness of the same plant-pollinator community in different years was very similar (Petanidou et al. 2008, *in press*; Alarcón *et al.*, *in review*), indicating that background environmental conditions in another system did not notably affect nestedness. Additional experiments manipulating basal resources in plant-pollinator networks, plant or pollinator morphology, and the abundance of members of the generalist core will provide further insight into the potential importance of bottom-up effects, or lack thereof, on plant-pollinator network structure.

Two caveats are important in the interpretation of my results. The first caveat is that the spatial scale of observation can affect estimates of network nestedness (Wright et

al. 1998). My plots were 16 m² in area and may have been too embedded in the same overarching plant-pollinator community for us to detect changes in nestedness as a result of N addition. Larger shifts in interactions may occur if N availability was altered at the scale of the watershed, which may remove the ability of pollinators to choose among areas of different resource base if they are limited in flight range, or if chronic N addition leads to wide-scale changes in plant species composition. In a similar vein, large spatial-scale changes in plant species composition associated with plant extinctions and invasions have altered pollination webs, resulting in co-extinctions and altered patterns of pollinator visitation and pollination (Memmott et al. 2004; Lopezaraiza-Mikel et al. 2007). Such large-scale studies of nutrient enrichment on plant-pollinator networks remain unexplored. The second caveat is that my results suggest that given more time for observations, additional plant-pollinator links would be recorded (Fig. 4.1). Thus, even with substantial observation effort, many of the rare plant-pollinator links will be missed, and less extensive observations may give a misleading representation of plant-pollinator interactions within a community (Petanidou and Potts 2006). Because the same methods and sampling effort were used throughout this study, my comparisons among N treatments and years were valid, but caution should be used when making quantitative comparisons of the networks reported here with other networks from published studies.

The majority of plant-pollinator interactions in my networks were generalized and the networks exhibited nested structure, in agreement with recent work documenting the organization of many mutualistic interactions (e.g., Waser et al. 1996; Jordano et al. 2003b; Jordano et al. 2006 but see Dicks et al. 2002). The nested structure of mutualistic interactions is different from the compartmentalized structure of antagonistic interactions

(e.g., predator-prey, herbivore-plant), where there are strong interactions within compartments (subgroups of species) but little interaction among compartments (e.g., Prado and Lewinsohn 2004; Bascompte and Jordano 2006; Guimaraes et al. 2006). Thus, despite the consumer-resource nature of some mutualisms, there appear to be fundamental and predictable differences in the structure of mutualistic vs. antagonistic interactions, suggesting that the form of an interaction may mediate its effects on community dynamics. The generalization of plant-pollinator interactions and the nested structure of networks have important implications for the degree to which species and interactions will be affected by perturbations (e.g., Bascompte et al. 2003; Memmott et al. 2004; Bascompte and Jordano 2006). For example, plant species diversity is expected to decline in conjunction with the loss of pollinator species (Biesmeijer et al. 2006), but Memmott et al. (2004) demonstrated the relative tolerance of pollination networks to plant extinction with the removal of pollinator species due to the nested structure of these networks compared to the rapid losses of species from compartmentalized food webs. Similarly, Fortuna & Bascompte (2006) found that real plant-animal mutualist networks begin to lose species more quickly after simulated habitat loss but persist for higher levels of habitat destruction than random communities. Further research comparing mutualistic and antagonistic networks and their response to resource addition and environmental change is necessary to understand the causes and consequences of their structure.

The small effects of N addition that I observed on network structure paled in comparison to interannual variation in structure, independent of nitrogen. Surprisingly few studies have examined interannual variation in the structure of pollination webs (Waser and Ollerton 2006). Of the limited research that has considered such interannual

variation, the amount of interannual variation that I observed is not uncommon (Medan et al. 2006; Petanidou and Potts 2006; Petanidou et al. 2008, *in press*; Alarcón *et al.*, *in review*). The interannual differences in networks appear to be due, in large part, to differences in the pollinator community among years (Herrera 1988, Petanidou and Ellis 1993, Williams et al. 2001). In this study, the dominant pollinator group was different in each year. Bumble bees, solitary bees, and flies were the most common pollinators observed in 2005, 2006, and 2007, respectively. In a similar vein, in a two-year study in the shortgrass prairie in Wyoming, USA, plant-pollinator interactions showed considerable variation among years; bumble bee-flower interactions dropped from 65% to 9% of all bees observed from 1975 to 1976 (Tepedino 1980). These differences in plant-pollinator interactions may be consequences of pollinator abundance and population dynamics or, alternatively, influenced by large-scale climatic or other environmental factors. The presence of large interannual variation in pollinator abundances indicates the importance of temporal scale, especially when drawing conclusions about specialization and generalization in pollination partnerships and their ecological and evolutionary consequences (Petanidou and Potts 2006).

In summary, by combining concepts and techniques from food web ecology and network theory, I tested the bottom-up effects of nitrogen enrichment on plant-pollinator network structure. Despite predictions based on food web, competition, and optimal foraging theories, I found that bottom-up effects of nitrogen addition on plant-pollinator network structure were small relative to interannual variation in interactions, indicating the presence of other factors acting at large temporal, and possibly spatial, scales to influence network structure. Some of these other factors may include other environmental

conditions, competitive interactions, and/or top-down effects of predators. Studies that consider both intrinsic and extrinsic factors at larger spatial and temporal scales may provide additional insight into the factors controlling network structure and variation. Such an approach provides the opportunity to both understand and predict community dynamics in the face of environmental change across a range of species interactions.

Table 4.1. Comparison of plant-pollinator network structure between nitrogen treatments (C = control, L = low nitrogen addition, and H = high nitrogen addition) within and between years. The m^2 statistic is a measure of goodness-of-fit between the Animal by Plant matrices and is bounded between 0 (good fit; similarity in the identity and frequency of plant-pollinator interactions between matrices) and 1 (poor fit; differences in the identity or frequency of interactions between matrices). The significance of m^2 was determined by a permutation test and is reported as P . The size of the matrix is given as A (number of animal pollinator families) by P (number of plant species).

	m^2	P	A x P
2005			
C vs. H	0.01	0.0001	23 x 33
C vs. L	0.02	0.0001	23 x 33
H vs. L	0.02	0.0001	23 x 33
2006			
C vs. H	0.06	0.0001	25 x 31
C vs. L	0.02	0.0001	25 x 31
H vs. L	0.04	0.0001	25 x 31
2007			
C vs. H	0.09	0.0001	32 x 31
C vs. L	0.04	0.0001	32 x 31
H vs. L	0.06	0.0001	32 x 31
2005-2007			
2005 vs. 2006	0.36	0.0002	35 x 38

2006 vs. 2007	0.48	0.0003	35 x 38
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2005 vs. 2007	0.81	0.057	35 x 38
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Table 4.2. Pollinator families that exhibited the largest changes in interactions between nitrogen treatments (C = control, L = low nitrogen addition, and H = high nitrogen addition) within and between years. The five most affected families are shown for each N treatment or year comparison. Large vector residuals indicate large changes in position between the two matrices. The absolute value of changes in the number of visits and links to plant species are reported to illustrate the magnitude of the differences between matrices.

C vs. H				C vs. L				H vs. L			
Pollinator	Residuals	Δ visits	Δ links	Pollinator	Residuals	Δ visits	Δ links	Pollinator	Residuals	Δ visits	Δ links
2005											
Bombyliidae	0.066	31	3	Bombyliidae	0.089	24	0	Halictidae	0.076	27	4
Megachilidae	0.040	16	0	Halictidae	0.071	20	0	Bombyliidae	0.053	7	3
Trochilidae	0.033	8	1	Andrenidae	0.044	12	1	Andrenidae	0.050	12	1
Apidae	0.026	77	5	Syrphidae	0.035	16	4	Syrphidae	0.045	11	4
Syrphidae	0.023	5	0	Muscidae	0.035	12	2	Apidae	0.033	10	2
2006											
Syrphidae	0.190	30	1	Halictidae	0.063	19	2	Syrphidae	0.153	3	4

Halictidae	0.085	49	2	Apidae	0.060	46	3	Apidae	0.078	65	1
Rhagionidae	0.051	21	1	Anthomyiidae	0.050	14	2	Lycaenidae	0.052	8	0
Lycaenidae	0.048	10	2	Syrphidae	0.044	27	3	Rhagionidae	0.040	23	1
Tachinidae	0.042	8	2	Megachilidae	0.027	2	3	Andrenidae	0.039	15	0
2007											
Apidae	0.184	50	0	Apidae	0.131	53	0	Halictidae	0.125	15	0
Anthomyiidae	0.153	43	2	Bombyliidae	0.065	38	2	Anthomyiidae	0.122	77	6
Halictidae	0.105	8	2	Andrenidae	0.055	12	2	Bombyliidae	0.101	47	2
Syrphidae	0.065	31	4	Nymphalidae	0.045	15	2	Andrenidae	0.061	26	3
Tachinidae	0.063	11	1	Lycaenidae	0.038	14	1	Megachilidae	0.060	7	1
2005 vs. 2006				2005 vs. 2007				2006 vs. 2007			
Halictidae	0.345	404	4	Apidae	0.694	1108	12	Anthomyiidae	0.486	518	12
Syrphidae	0.291	501	8	Anthomyiidae	0.654	698	19	Apidae	0.417	506	3
Apidae	0.278	602	9	Halictidae	0.265	249	2	Syrphidae	0.193	332	5
Bombyliidae	0.2632	223	4	Sarcophagidae	0.175	239	15	Sarcophagidae	0.170	214	12

Anthomyiidae	0.168	180	7	Andrenidae	0.116	151	9	Bombyliidae	0.160	163	2
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Table 4.3. Spearman rank correlations of Idiosyncratic Temperatures of plants and pollinators between treatments (C = control, L = low nitrogen addition, and H = high nitrogen addition) within and between years. Idiosyncratic Temperature is a measure of how a species' pattern of links deviates from the pattern expected in a perfectly nested matrix, and the analysis reported here illustrates whether species are occupying similar positions in the nested communities (significant positive correlation). The strength and direction of the correlations are given by ρ , the significance of the test by P , and the sample size by n . Statistically significant P-values are bolded.

	ρ	P	n
2005			
C vs. H	-0.19	0.34	25
C vs. L	0.013	0.94	25
H vs. L	0.028	0.88	25
2006			
C vs. H	0.0043	0.96	39
C vs. L	0.21	0.19	39
H vs. L	0.22	0.17	39
2007			
C vs. H	0.28	0.082	38
C vs. L	0.36	0.027	38
H vs. L	0.28	0.089	38
2005-2007			
2005 vs. 2006	0.054	0.72	45

2006 vs. 2007	0.11	0.45	45
2005 vs. 2007	0.054	0.72	45

Figure 4.1. Rarefaction curves (solid lines) and their 95% confidence intervals (dashed lines) of the number of plant-pollinator links as a function of the number of visits from each of the three years of observations.

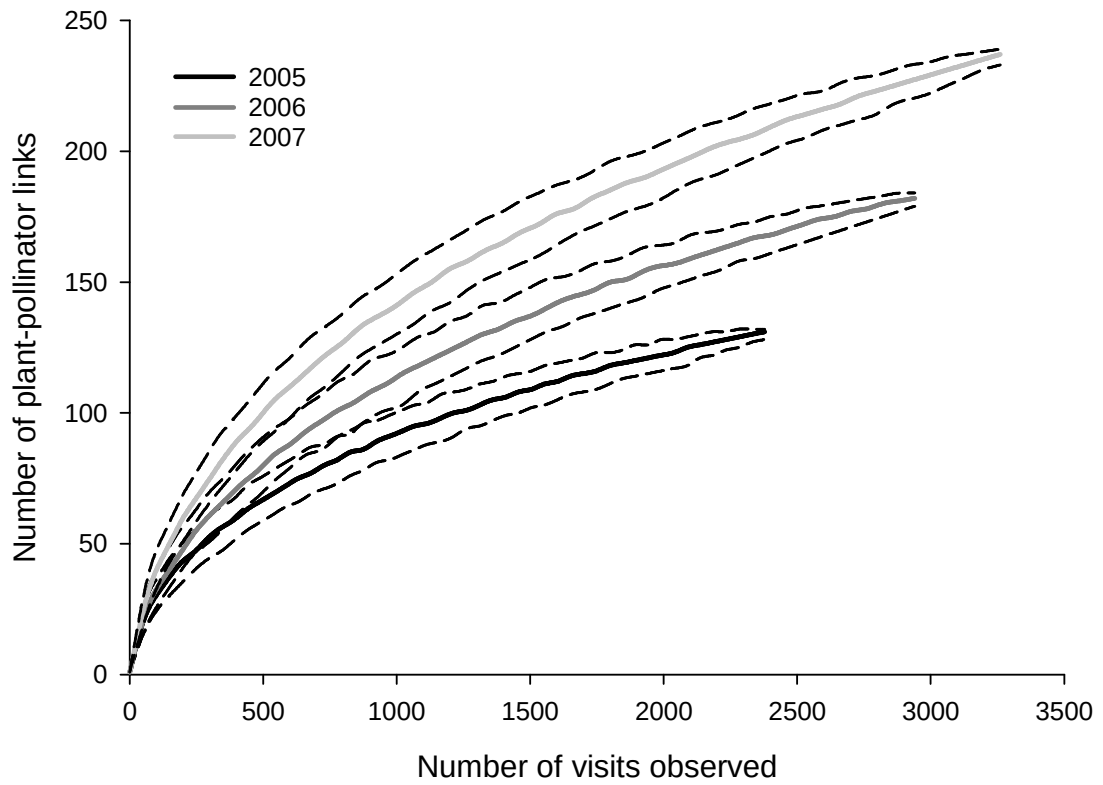


Figure 4.2. The mean rarefied richness (a) and evenness (b) of pollinator families visiting all plants did not differ among nitrogen treatments (C = control, L = low-N addition, H = high-N addition) but varied across the three years of treatment (2005 = black, 2006 = light gray, 2007 = dark gray).

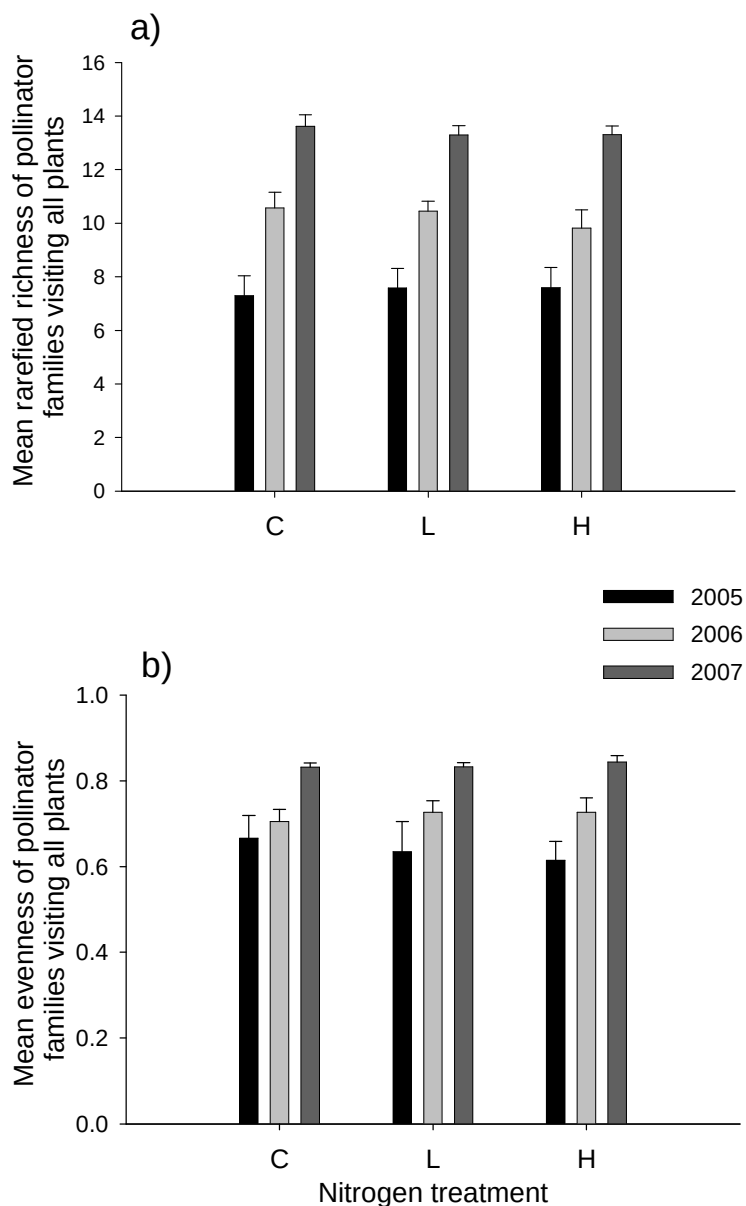
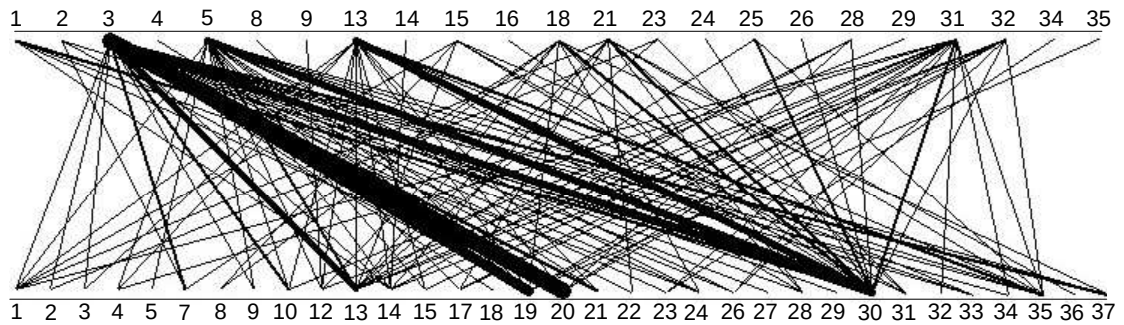
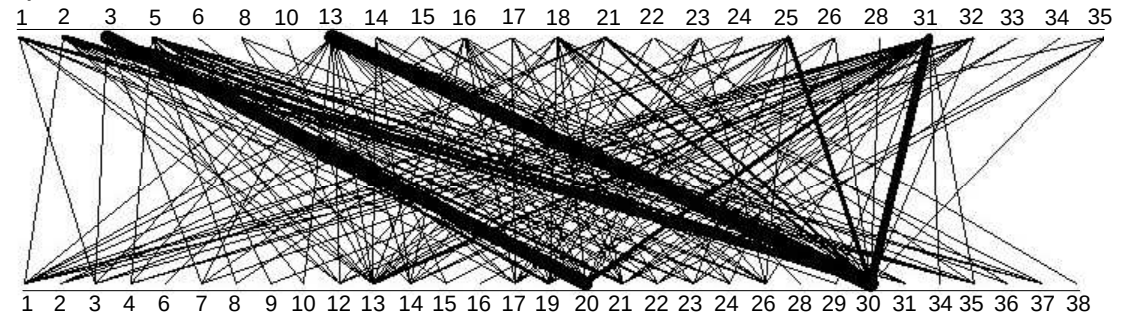


Figure 4.3. Graphic representation of the plant-pollinator community in (a) 2005, (b) 2006, and (c) 2007. Plants are along the bottom and pollinators are along the top of each web. The identities of plants and pollinators are indicated by numbers and are the same across years (see Appendix 4 for names). The width of the links is proportional to the frequency of the interaction within a year.

a) 2005



b) 2006



c) 2007

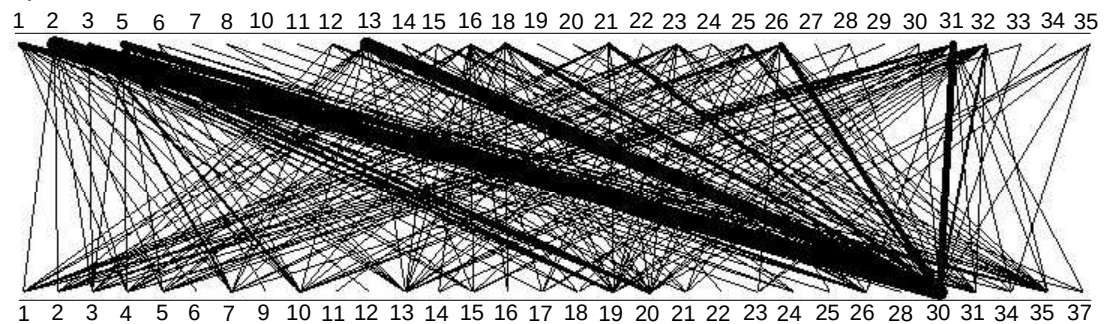
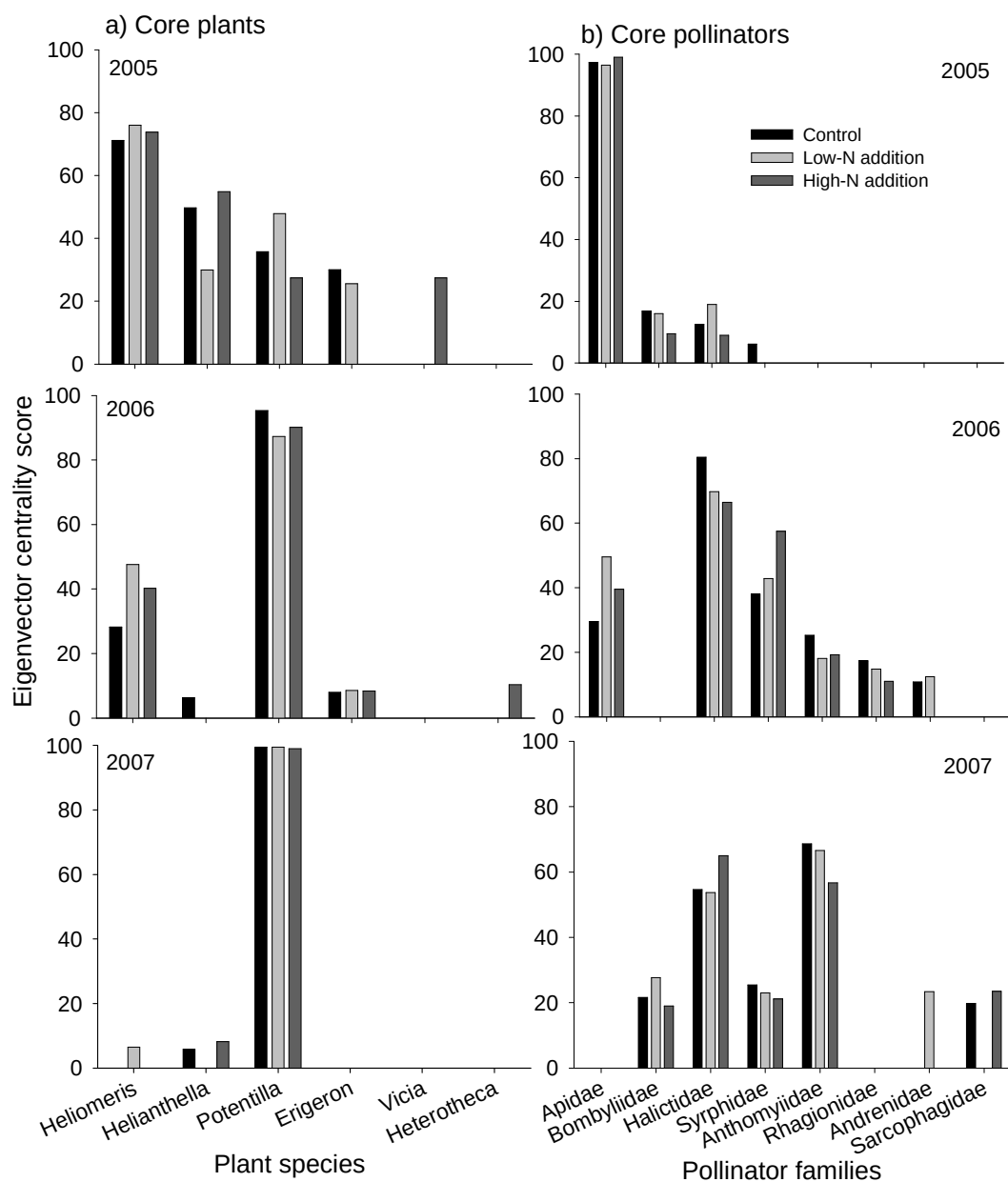


Figure 4.4. The identity and rank order (ranked by eigenvector centrality score) of plants and pollinators comprising the core group of generalists was similar across N treatments (control, low nitrogen addition, or high nitrogen addition) but different among years. Larger eigenvector centrality scores indicate that the family (pollinator) or species (plant) was more generalized. Plants are referred to by their genus (full species names are given in Appendix 1).



CHAPTER 5: NECTAR SUGAR LIMITS SOLITARY BEE LARVAL PERFORMANCE

Summary

The bottom-up effects of plant food quality and quantity can affect the growth, survival, and reproduction of herbivores. The larvae of solitary bee pollinators, consumers of nectar and pollen, can also be considered herbivores. Although pollen quantity and quality are known to be important for larval growth, little is known about how nectar quality limits solitary bee performance. By adding different levels of nectar sugar directly to solitary bee provisions in the subalpine of Colorado, I tested the degree to which larval performance (development time, mass, and survival) was limited by nectar sugar. I found that larval growth increased with nectar sugar addition, with the highest larval mass in the high nectar-sugar addition treatment (50% honey solution). The shortest larval development time was observed in the low nectar-sugar addition treatment (25% honey solution). Neither low nor high nectar-sugar addition affected larval survival. This study suggests that, in addition to pollen, nectar-sugar concentration can limit solitary bee larval growth and development, and thus, the availability and sugar content of nectar may scale up to affect bee fitness, population dynamics, and plant-pollinator mutualisms. Nectar resources should be considered in predictions of solitary bee foraging preferences and reproduction and in conservation management decisions.

Introduction

The quality and quantity of food resources are known to have dramatic effects on higher trophic levels, affecting individual performance, population size, and species interactions (Hunter and Price 1992; Bukovinszky et al. 2008). A wealth of studies have tested the bottom-up effects of food quality and quantity on foliar herbivores and secondary consumers (Stiling and Rossi 1997; Stiling and Moon 2005; Forkner and Hunter 2000), with foliage quality typically enhancing herbivore performance (reviewed in Awmack and Leather 2002). In particular, host plant quality has been shown to increase the growth, survival, and reproduction of insect herbivores (e.g., Mattson 1980; Scribner and Slansky 1981; Hemmi and Jormalainen 2002; Chen et al. 2004). As consumers of plant nectar and pollen, pollinators can also be considered herbivores (e.g., Williams 2003). Some pollinators can perceive differences in pollen quality and prefer to forage on and provision their offspring with high quality (high protein) pollen (Cook et al. 2003; Fewell and Winston 1992; Rasheed and Harder 1997; Waddington et al. 1998), which is important for bee colony growth and individual larval growth and development (McCaughey et al. 1980; Schmidt et al. 1987; Genissel et al. 2002; Roulston and Cane 2002). The effects of nectar quality on pollinator consumers, however, are less well understood. A growing body of evidence indicates that some insect pollinators, such as honey bees and butterflies, prefer high-quality nectar (high amino acid content), which may enhance their fecundity as adults (Alm et al. 1990; Mevi-Schutz and Erhardt 2005). Here, I used solitary bee pollinators that use nectar and pollen to provision their offspring to test how nectar sugar quality affected larval performance.

Solitary bees vary in growth and reproduction based on resource availability. For example, adult solitary bees vary in body size, which is influenced by their larval pollen provision size and can vary seasonally with floral nectar and pollen resources (Kim and Thorp 2001). Subsequently, large adult female solitary bees produce more and larger eggs than small females (Tengo and Baur 1993; Kim 1997). In addition, when available floral resources are enhanced (potted flowering plants in flight cages), brood cell production rate can increase (Goodell 2003). Moreover, more and heavier *Megachile apicalis* offspring are produced when floral resources are increased (flower bouquets in flight cages) (Kim 1999). Large offspring often have higher survival and fecundity through territory defense, nest usurpation and better mating opportunities (e.g., Tepedino and Torchio 1994; Alcock 1995; Kim 1997; Roulston and Cane 2002; but see Bosch and Vicens 2006).

Variation in nectar resources across the landscape is common (e.g., Zimmerman 1981; Marden 1984; Rathcke 1992), and solitary bees can detect nectar-rewarding flowers based on visual and olfactory cues (Nuttman et al. 2006; Howell and Alarcon 2007). Furthermore, abiotic environmental conditions, such as soil nutrient enrichment, can affect the quality and quantity of floral rewards and patterns of pollinator visitation (Campbell and Halama 1993; Munoz et al. 2005; this dissertation, Chapter 3). In particular, nutrient enrichment can increase the amino acid and sugar concentrations of nectar (Petanidou et al. 1999; Gardener and Gillman 2001), suggesting pathways by which soil nutrients could scale up to affect solitary bee larval performance.

Here, by adding nectar sugar to solitary bee larval provisions, I tested the degree to which individual larval performance was nectar limited. I focused on testing nectar-

sugar limitation of larval performance in solitary bees in the Megachilidae because they are common floral visitors in the subalpine and other systems (Price and Waser 1998; Blionis and Vokou 2001; Alston et al. 2007; C. A. Kearns, *unpub data*). Moreover, solitary bees are commonly used in feeding trials, and some species are affected by pollen quantity and quality at the larval stage (Williams 2003; Praz et al. 2008). This study provides insight to the potential for food limitation of solitary bee survival, growth, and reproduction, an important topic given the significance of solitary bees as pollinators and of the threatened status of some bee pollinators worldwide (Williams and Kremen 2007, NRC 2007).

Methods

Study System

This study was conducted in the summer of 2007 near the Rocky Mountain Biological Laboratory (RMBL) in Gothic, Gunnison County, Colorado, USA (elev. 2900 m). This area is characterized by large, open subalpine meadows dominated by wildflowers and bordered by aspen-fir forests. Solitary bees are frequent visitors to flowers in subalpine meadows (e.g., Campbell 1987; Eickwort et al. 1996; Price and Waser 1998); around the RMBL, solitary bees make up 25% of pollinator visitors to wildflowers (this dissertation, Chapter 3.). I focused on bees in the genera *Megachile*, *Hoplitis*, and *Osmia* (family Megachilidae). These mason and leafcutter bees lay a single egg on each provision of pollen and nectar and use a variety of materials, including mud, leaf material, and pebbles, to partition their offspring (Michener 2000). At the genus level, the solitary bees in this study are generalists, and the mass of their provisions

ranges from 26.8 to 420.4 mg (mean $\pm$ SE = 171.9 ± 2.7 mg; N = 462 provisions; *this study*), usually 50% nectar and pollen each by mass (Williams 2003). Larvae in these genera typically have five larval instars and reach pupation in three weeks (Torchio 1989). In my study area, adults provisioned a mean $\pm$ SE of 4.81 ± 0.33 offspring per nesting tube (N = 96 artificial nesting tubes). Female eggs are typically provisioned on larger pollen provisions at the back of the tube, while males are provisioned on smaller pollen provisions near the tube opening (Torchio 1989).

Field and laboratory methods

Nesting blocks. I used 30 solitary bee pine nesting blocks (20 x 18 x 4 cm), each with 20 evenly spaced 1.25 cm diameter holes, 15 cm deep. I lined each hole with cardboard guard tubes and paper liners (Knox Cellars, Bellingham, WA, USA) and placed all nesting blocks in the field on or near existing dead wood (e.g., fallen trees, old wooden structures). I allowed female solitary bees to naturally provision their offspring in the nesting blocks throughout the summer. Filled tubes were removed from the nesting blocks up to bi-weekly and brought into the laboratory.

I differentiated two categories of bees: those that partitioned cells with walls of mud and/or leaves (in the genera *Megachile*, *Hoplitis*, and *Osmia*) and those that enclosed each provision and egg inside a cylinder constructed of leaf pieces (in the genus *Megachile*; Michener 2000; L. A. Burkle, *unpub. data*). Thus, for all tubes, I recorded nesting material (i.e., leaves, mud and/or pebbles) and style (i.e., single divisions of mud or leaves between cells, entire provision and egg wrapped in leaves) to help differentiate among bee genera.

Each egg or larva, along with its provision, was transferred to a plastic well (cryogenic tube 10 mm wide cut to a height of 12 mm; Perfector Scientific Cryo-Store®, Atascadero, CA, USA) and weighed to the nearest 0.01 mg. Larvae that were already feeding on their provision were excluded from the study. Whether the larvae were females or males was assessed by their location within the tube and their relative provision size by weight. To ensure that there were no systematic biases in pollen species among nectar treatments (described below), two small pollen samples from each provision were stained in basic fuchsin (Kearns and Inouye 1993) and identified to species under a compound microscope using a pollen reference collection. The entire slide was scanned, one representative field of view chosen randomly, and proportion of each pollen species determined. I assigned nectar treatments across pollen species without systematic biases (data not shown).

Nectar treatments. The eggs or larvae were randomly assigned to one of four treatments: control (no addition), water addition (10 μ L), low nectar-sugar addition (10 μ L of 25% honey water), or high nectar-sugar addition (10 μ L of 50% honey water). The water addition treatment controlled for any effects associated with additional moisture addition. I added 10 μ L of liquid because it is the volume roughly equivalent to the amount of nectar that could be collected from 100 flowers in this system (Waser 1978; Zimmerman 1986) or 20% of the amount required for one provision (assuming 50% of the average provision is composed of nectar by mass; Wall 1990; Williams 2003). Honey water concentrations of 25% and 50% were chosen to reflect low and high nectar-sugar concentrations of flowers commonly found in the field (Waser 1978; Pleasants 1983). I used local high altitude Colorado honey (Ambrosia Honey Co., Parachute, CO, USA) for

the honey water solutions. I added honey water, rather than sugar water, to bee provisions to provide a food containing other natural nectar components (Baker 1977). I assumed that each nesting tube was provisioned by one adult female solitary bee (Tepedino and Torchio 1982); thus, when possible I divided nectar treatments within sex within each tube (i.e., likely the offspring of one female) to control for gender and maternal effects. However, there were not enough offspring per tube (i.e., four females and four males per tube) to block nectar treatment by tube in statistical analyses (see below). All nectar treatments were added to the provisions with an Eppendorf pipetter (Hamburg, Germany) before larvae began feeding. For the larvae wrapped in leaf cylinders, I temporarily removed the leaf cap at the top of the cylinder, delivered the nectar treatment onto the provision, and replaced the leaf cap. I treated a total of 450 larvae, with 113 controls, 117 water addition, 110 low nectar-sugar addition, and 110 high nectar-sugar addition larvae.

Larval performance. I estimated larval performance as larval development time, larval mass just prior to pupation (wet and dry mass), and survival. All offspring were allowed to develop in a dark growth room at 21-25°C. I monitored the larvae daily and measured their larval development time as the duration between the date of first feeding to the date of initial cocoon spinning. For bees that encased their provisions in leaves, I was not able to observe the spinning date and did not measure their development time. After five months of simulated winter (4°C) diapause, I measured larval survival and the wet mass (to the nearest 0.0001 mg) of larvae that survived. Larvae were then dried at 20°C and reweighed for dry mass.

Predictions and data analyses. I predicted that nectar-sugar addition would reduce larval development time by providing access to energy-rich food, as has been

shown in Ichneumonid wasps (Winkler et al. 2006), and would increase larval mass and the probability of survival relative to the control treatment (Haggstrom and Larsson 1995). I predicted that high nectar-sugar addition would have the greatest benefits on larval performance and low nectar-sugar addition would have intermediate benefits relative to the control. In addition, I expected that there would be no difference in bee performance between the control and the water addition treatments, assuming that liquid addition has no effect on larval performance.

I tested the effects of nectar treatment and nesting style on larval development time and larval mass with initial provision mass included as a covariate using separate linear contrasts. I included provision mass as a covariate in these analyses, instead of my estimates of larval sex, because provision mass was measured with certainty and should be correlated with sex (female provisions being larger than male provisions). To test the effects of nectar treatment on larval survival, I used multiple logistic regression. I used separate tests for each component of bee performance because development time could not be recorded for all larvae (see *Larval performance*) and mortality during development removed some larvae from the analyses. I tested for an interaction between nectar treatment and nest style in each analysis; I found no significant interactions ($P > 0.05$ in all cases), suggesting that nectar treatment had similar effects on bees across nesting styles. Therefore, I reported the results without interactions in the models. In addition, because I collected offspring throughout the summer, there may have been interactions between date and larval development time or between date and nectar treatment; I tested for these interactions and again found no significant interactions ($P > 0.05$ in all cases). Thus, I did not include interactions with time (date) in the models. The linear contrasts

and logistic regressions were conducted using JMP 4.0. I report least squared means due to unbalanced sample sizes among nectar treatments.

Results

Nectar treatment affected components of larval performance. Larval wet mass increased with nectar-sugar addition (Fig. 5.1a; $F_{1,125} = 4.37$, $P = 0.039$), with 18% larger larvae in the high nectar-sugar addition treatment compared to the control. Larval dry mass showed the same positive pattern with nectar-sugar addition ($F_{1,125} = 5.50$, $P = 0.021$). Larval development time did not decrease with nectar-sugar addition, as predicted (Fig 5.1b; $F_{1,150} = 0.42$, $P = 0.52$), but larvae developed 24% faster in the low nectar-sugar addition treatment compared to the control. Larval survival was not affected by nectar-sugar treatments ($\chi^2 = 1.32$; $P = 0.72$). Overall, survival to the final (i.e., fifth) larval instar was 35% in the control treatment ($N = 40$), 34% in the low nectar addition treatment ($N = 37$), 31% in the water treatment ($N = 36$), and 29% in the high nectar addition treatment ($N = 32$).

In general, across nectar treatments, provision mass positively affected larval mass (Fig. 5.2.; $r^2 = 0.23$; $N = 131$; $P < 0.0001$), but not larval development time ($r^2 = 0.015$; $N = 156$; $P = 0.13$). In addition, larval wet mass ($t_{129} = 3.2$; $P = 0.0016$), dry mass ($t_{129} = 11.08$; $P = 0.0011$), development time ($t_{154} = 4.8$; $P < 0.0001$), and survival ($\chi^2 = 51.7$; $P < 0.0001$) differed between the two nesting styles (see Table 5.1 for means). Both provision mass and nesting style explained a significant amount of the variance in larval mass and development time in all contrasts ($P < 0.0003$ in all cases). Nesting style

explained a significant amount of the variance in survival ($\chi^2 = 44.93$, $n = 450$, $P < 0.0001$), while provision mass did not ($\chi^2 = 0.91$, $n = 450$, $P = 0.34$).

Discussion

The performance of herbivores can be strongly affected by the quality and quantity of plant food resources (Awmack and Leather 2002). Such bottom-up effects can scale up through ecological communities, influencing the abundance of individuals and species interactions (e.g., Hunter and Price 1992; Bukovinszky et al. 2008). Pollinators, such as solitary bees, are herbivores, feeding on nectar and pollen, and their preference and performance as adults may be affected by the quality and quantity of floral resources they received as larvae. Pollen is known to be important for solitary bee larval growth (Roulston and Cane 2002; Williams 2003), and although solitary bee adults and larvae consume nectar, little is known about the degree to which nectar quality limits larval growth. By adding nectar with different sugar concentrations to provisions, I tested nectar limitation of larval performance. Larval mass increased with nectar-sugar concentration, a signal evident after controlling for provision size and nesting style (proxy for bee genus). In addition, larvae receiving intermediate levels of nectar sugar displayed the fastest development times. Thus, in addition to pollen, nectar can also limit solitary bee reproduction, and its quality and availability may have important implications for plant-pollinator mutualisms and conservation.

Nectar-sugar addition had a positive effect on larval mass, an effect that may subsequently increase adult mass and fecundity (Roulston and Cane 2002). Assuming that female bees do not alter the sugar concentration of nectar and provide similar

nectar quantities to each provision, plant species that produce nectar with higher sugar concentration may disproportionately contribute to the growth of solitary bee offspring. In particular, if bees can cue in on plants with higher nectar-sugar concentration, then larvae could benefit from selective foraging by their mothers. Interestingly, although nectar-sugar addition did not linearly decrease larval development time as I predicted, larvae in the low nectar-sugar addition treatment had the shortest development times (as measured by the amount of time until cocoon spinning). It was surprising that low nectar-sugar addition reduced larval development time but that high nectar sugar addition did not. One potential explanation for this result is that the effects of the high nectar-sugar addition treatment were dominated by an extended feeding, growing, and development period. Thus, it is possible that at high nectar-sugar levels, the energy provided by the additional sugar was allocated to growth, while at low nectar-sugar addition levels, the extra energy may not have been enough to substantially increase body mass and was instead allocated to a rapid development time (Chown and Nicolson 2004). More detailed observations of larval development and additional experiments manipulating nectar sugar across more levels are needed to assess the validity of this explanation.

Two caveats are important in the interpretation of the results. First, larvae were reared in a controlled laboratory setting in the absence of parasitism and predation, such as parasitic bees that kill the resident larva and eat its provision, bird predators, or other parasites (e.g., Munster-Swendsen and Calabuig 2000; Goodell 2003; Wcislo et al. 2004). In the field, faster development may decrease the amount of time larvae are vulnerable to parasitism and predation, possibly enhancing their survival. For example, fast-growing leaf beetles have been shown to survive better in the presence of predation (Haggstrom

and Larsson 1995). Although survival was not affected by nectar treatment in this experiment, rearing larvae in the field may reveal indirect positive effects of nectar addition on survival via changes in parasitism and predation. Alternately, parasites and predators may show preference for larger or more nutritious prey (e.g., Osenberg and Mittelbach 1989), and larvae feeding on high-sugar diets may be preferred, assuming that bee predators can perceive the food quality of their larval prey. Raising larvae in the field and tracing the effects of nectar-sugar addition through adulthood will provide a more complete understanding of nectar on solitary bee performance. The second caveat is that the identity of solitary bee species was not determined in this study. Thus, the exact nature of species-specific differences in performance remains unknown. Independent of nectar treatments, provision mass and nesting style often influenced larval performance measures, reinforcing the importance of pollen availability for solitary bee reproduction and the possibility of species specific differences in performance and life-history characters. Mud and leaf nesters were larger, took longer to develop and had higher survival than those that constructed a leaf cylinder to envelop each offspring.

This study is in agreement with work showing that nectar sugar is important to solitary bees and other pollinators. Honey bees select inflorescences that provide them with more nectar sugar (Higginson et al. 2006) and can change their foraging behavior to maximize their rate of nectar sugar (energy) intake (Dedaj and Delaplane 2005). The overwintering survival of honey bee colonies can depend on the size of their honey reserves (Seeley and Visscher 1985). Furthermore, honey bee colonies foraging in the field in an area of sparse floral resources that were also provided with a daily concentrated sugar solution gained weight compared to unfed colonies, indicating that

energy is a limiting resource for colony reproduction (Seeley and Mikheyev 2003). The growth, survival and reproduction of bumble bee colonies are also likely limited by the rate of energy gain (Bowers 1985). One recent study has shown that nectar addition can increase solitary bee performance in *Osmia lignaria* (Elliott et al. 2008). Often the only readily available energy source in the environment used by pollinators, nectar resources and their quality may be more important for the growth and reproduction of individuals and populations than previously appreciated.

Understanding what factors limit the growth and reproduction of species is fundamental to investigation of their distribution and species interactions, and becomes more important when species, or the ecosystem services they provide, are threatened (Kremen et al. 2007). Solitary bees and other pollinators may be declining worldwide (NRC 2007); yet many basic aspects of solitary bee natural history are unknown. In particular, the main factors limiting the reproductive success of solitary bees and their relative importance are not fully understood (Morato and Martins 2006), but likely include nest sites and materials and food resources (Gathmann and Tschardt 2002). Only recently have ecologists begun to investigate the influence of floral rewards on solitary bee reproduction and not just foraging patterns in the field (Kim 1999; Kim and Thorp 2001; Goodell 2003). Here, I conclude that nectar-sugar resources, in addition to pollen, are important for larval performance of solitary bees. Further work is necessary to determine how nectar quality and availability in the field influence larval performance and how these factors may scale up to affect adult bee fitness, bee abundance, and plant-pollinator mutualisms. With the inclusion of the effects of floral rewards from the perspective of pollinator reproduction, a more comprehensive understanding of how

environmental change may affect species, their interactions, and the ecosystem services they provide can be achieved.

Table 5.1. Overall means $\pm$ 1 SE (and sample sizes) of larval wet and dry mass, development time, and survival of solitary bee larvae between the two nesting styles observed (mud and leaf partitions or provisions and larvae wrapped in leaves).

	Nesting style	
	mud and leaves	wrapped in leaves
Larval wet mass (mg)	44.7 $\pm$ 2.1 (81)	33.9 $\pm$ 2.6 (50)
Larval dry mass (mg)	21.7 $\pm$ 1.1 (81)	16.2 $\pm$ 1.0 (50)
Development time (days)	16.8 $\pm$ 0.8 (96)	10.6 $\pm$ 1.0 (60)
Survival (%)	51.4% (94)	19.1% (51)

Figure 5.1. (a) Larval wet mass increased with nectar-sugar addition, but (b) low nectar-sugar addition resulted in a decrease in development time. Error bars are ± 1 SE for least-squared means. Sample sizes for each treatment are indicated above error bars.

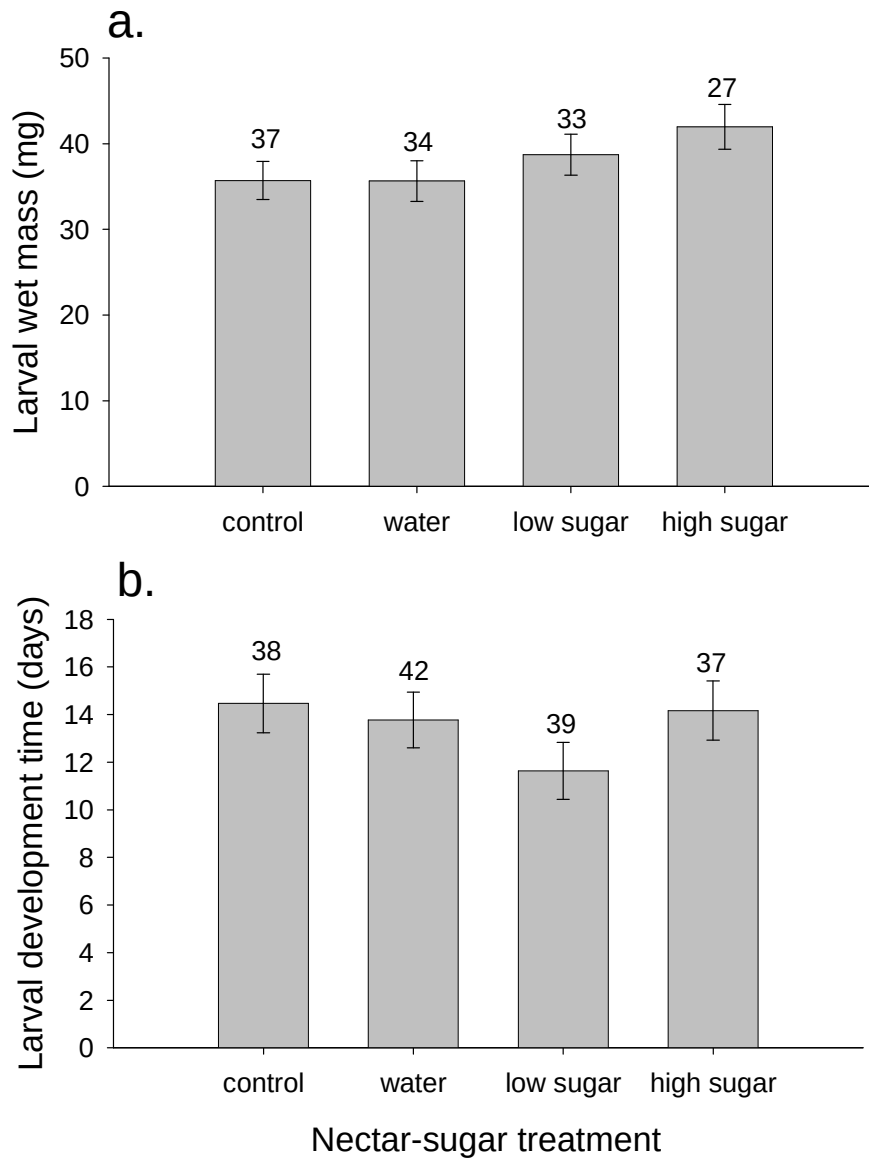
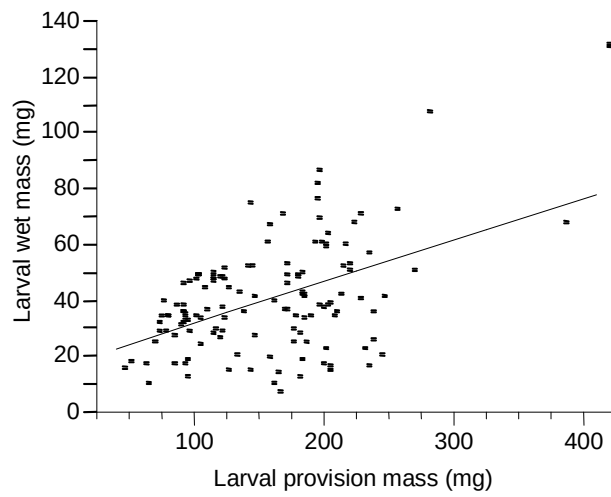


Figure 5.2. The effects of provision mass on larval wet mass across all nectar treatments.

Larval mass = 17.3 + 0.15*provision mass.



APPENDICES

Appendix 3.1. Forb species occurring in the study plots near the Rocky Mountain Biological Laboratory in Colorado, USA, their floral morphology, primary pollinators, and root morphology. For plant species, I followed the nomenclature of Weber and Wittmann (1996) (exceptions marked with a footnote). Abbreviations for primary pollinators are as follows: bumble bees (BB), flies (F), hummingbirds (HB), Lepidoptera (L; butterflies and moths), and solitary bees (SB).

Plant species	Plant family	Floral morphology	Primary pollinators	Root morphology
<i>Achillea lanulosa</i>	Asteraceae	umbel	F, L	shallow-rooted
<i>Agoseris aurantiaca</i>	Asteraceae	open	F, L	shallow-rooted
<i>Agoseris glauca</i>	Asteraceae	open	BB, F, SB	shallow-rooted
<i>Androsace septentrionalis</i>	Primulaceae	open	F	shallow-rooted
<i>Arabis hirsuta</i>	Brassicaceae	open	F, L, SB	shallow-rooted
<i>Arenaria congesta</i>	Caryophyllaceae	open	SB	shallow-rooted
<i>Campanula rotundifolia</i>	Campanulaceae	bell	BB, SB	shallow-rooted
<i>Collinsia parviflora</i>	Scrophulariaceae	zygomorphic	F	shallow-rooted
<i>Collomia linearis</i>	Polemoniaceae	open	F	shallow-rooted

<i>Delphinium nuttallianum</i> ¹	Ranunculaceae	zygomorphic	BB, HB	shallow-rooted
<i>Dugaldia hoopesii</i>	Asteraceae	open	L	Unknown
<i>Erigeron flagellaris</i>	Asteraceae	open	F	shallow-rooted
<i>Erigeron speciosus</i>	Asteraceae	open	BB, F, L, SB	shallow-rooted
<i>Eriogonum umbellatum</i>	Polygonaceae	umbel	F, L	shallow-rooted
<i>Erysimum inconspicuum</i>	Brassicaceae	open	F, L, SB	shallow-rooted
<i>Fragaria virginiana</i>	Rosaceae	open	L, SB	shallow-rooted
<i>Galium boreale</i>	Rubiaceae	umbel	F, L	shallow-rooted
<i>Hackelia floribunda</i>	Boraginaceae	open	F, SB	Unknown
<i>Helianthella quinquenervis</i>	Asteraceae	open	BB, F, L, SB	tap-rooted
<i>Heliomeris multiflora</i>	Asteraceae	open	BB, F, SB	shallow-rooted
<i>Heterotheca villosa</i>	Asteraceae	open	BB, F, SB	Unknown
<i>Hydrophyllum fendleri</i>	Hydrophyllaceae	umbel	SB	Unknown
<i>Ipomopsis aggregata</i>	Polemoniaceae	tube	HB	shallow-rooted
<i>Lathyrus leucanthus</i>	Fabaceae	zygomorphic	BB, SB	shallow-rooted

<i>Ligusticum porteri</i>	Apiaceae	umbel	F, L	Unknown
<i>Linum lewisii</i> ²	Linaceae	open	F, SB	shallow-rooted
<i>Lithospermum ruderale</i>	Boraginaceae	open	BB	Unknown
<i>Mertensia fusiformis</i>	Boraginaceae	bell	SB	shallow-rooted
<i>Pneumonanthe parryi</i>	Gentianaceae	tube	BB	tap-rooted
<i>Potentilla pulcherrima</i>	Rosaceae	open	BB, F, SB	shallow-rooted
<i>Pseudocymopterus montanus</i>	Apiaceae	umbel	F, L, SB	shallow-rooted
<i>Ranunculus inamoenus</i>	Ranunculaceae	open	F	shallow-rooted
<i>Senecio canus</i>	Asteraceae	open	BB, F, SB	Unknown
<i>Solidago multiradiata</i>	Asteraceae	open	BB, F	shallow-rooted
<i>Taraxacum officinale</i>	Asteraceae	open	F, L, SB	tap-rooted
<i>Valeriana occidentalis</i>	Valerianaceae	umbel	F, SB	shallow-rooted
<i>Vicia americana</i>	Fabaceae	zygomorphic	BB, SB	shallow-rooted
<i>Viola praemorsa</i>	Violaceae	zygomorphic	L	shallow-rooted

¹*Delphinium* is in the Helleboraceae in Weber and Wittman (1996).

²*Adenolinum lewisii* in Weber and Wittman (1996)

Appendix 3.2. *Herbivory*. Because plant nutrient quality can affect plant susceptibility to herbivores, which could obscure any beneficial effects of nitrogen or pollen additions, I also recorded herbivory to focal plants. *Ipomopsis* experience damage by three main types of herbivores: foliar herbivores, root borers, and seed predators. Mule deer, *Odocoileus hemionus*, were the primary foliar herbivores of *Ipomopsis*, consuming above-ground portions of individuals during their reproductive phase (Paige and Whitham 1987). In each year, the number of flowering stalks with herbivory and the presence/absence of root borers (species unknown) were recorded for all *Ipomopsis* focal plants. In addition, pre-dispersal seed predation by larvae of *Hylemya* sp. (Diptera: Anthomyiidae) was assessed in *Ipomopsis* as percent of fruits destroyed. Seed predators may cue in on fertilized plants and/or plants that have higher seed set for oviposition (Brody and Waser 1995, Brody 1992). The effects of N addition on the average proportion of *Ipomopsis* stalks browsed and the proportion of plants containing root borers per plot were tested using separate rm-ANOVAs with year as the repeated factor. The effects of N addition and pollination on the percent of *Ipomopsis* fruits destroyed by seed predators were assessed using rm-ANOVAs. *Potentilla* flowering stalks were occasionally browsed by unknown herbivores (likely ground squirrels and/or mule deer), and I recorded the number of damaged stalks on all focal plants. The effects of N addition on the average proportion of browsed *Potentilla* stalks per plot were tested using a rm-ANOVA with year as the repeated factor.

Herbivory and seed predation did not vary among N treatments for *Ipomopsis* or *Potentilla*. Nitrogen addition had no effect on the proportion of browsed *Ipomopsis* (rm-ANOVA, $F_{2,8} = 0.10$, $P = 0.90$) or *Potentilla* stalks (rm-ANOVA, $F_{2,18} = 1.55$, $P = 0.24$).

There was a marginal effect of N addition on the susceptibility of *Ipomopsis* to root borers (rm-ANOVA, $F_{2,8} = 3.65$, $P = 0.07$), with high-N plots having a greater proportion of plants with root borers compared to low-N and control plots during all three years of study. Neither N addition nor pollination affected percent fruit damage by pre-dispersal seed predators in *Ipomopsis* (rm-ANOVA, $F_{5,17} = 2.19$, $P = 0.20$).

Appendix 3.3. ANOVA table for ANPP and biomass responses of plant functional groups and focal species to nitrogen addition across three years of treatment. These tests were used to understand the effects of nitrogen on different plant species at different scales and possible delayed effects. Statistically significant P-values are bolded.

Response variable	Test	df	F	P
Plot-level:				
Total ANPP	rm-ANOVA	2,21	14.94	< 0.0001
2005	ANOVA	2,21	1.79	0.19
2006	ANOVA	2,21	23.35	< 0.0001
2007	ANOVA	2,21	8.07	0.0025
Forb ANPP	rm-ANOVA	2,21	16.34	< 0.0001
2005	ANOVA	2,21	1.33	0.29
2006	ANOVA	2,21	11.61	0.0004
2007	ANOVA	2,21	9.44	0.0012
Grass ANPP	rm-ANOVA	2,21	23.5	< 0.0001
2005	ANOVA	2,21	0.91	0.42
2006	ANOVA	2,21	22.17	< 0.0001
2007	ANOVA	2,21	27.32	< 0.0001
N-fixer ANPP	rm-ANOVA	2,14	0.76	0.48
Focal plant biomass:				
<i>Ipomopsis</i>				
Aboveground	rm-ANOVA	2,8	0.68	0.54
Belowground	rm-ANOVA	2,8	0.58	0.58

Potentilla

Aboveground	rm-ANOVA	2,18	2.2	0.13
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Appendix 3.4. ANOVA table for species richness and evenness responses to nitrogen addition across three years of treatment. These tests were used to determine if nitrogen affected measures of species diversity of plant assemblages. Statistically significant P-values are bolded.

Response variable	Test	df	F	P
Species richness	rm-ANOVA	2,21	0.71	0.5
	time (year)	2,20	5.55	0.012
Evenness	rm-ANOVA	2,21	5.93	0.0091
	time (year)	2,20	1.69	0.21
2005	ANOVA	2,21	1.34	0.28
2006	ANOVA	2,21	5.22	0.014
2007	ANOVA	2,21	3.21	0.061

Appendix 3.5. ANOVA table for flower production (total and for dominant forb species) to nitrogen addition across three years of treatment. Results are from whole-model rm-ANOVAs. Statistically significant P-values are bolded.

Response variable	df	F	P
Total flower production			
2005	2,21	2.6	0.098
2006	2,21	5.59	0.011
2007	2,21	9.3	0.0013
<i>Agoseris</i>			
2005	2,9	1.02	0.40
2006	2,7	4.38	0.058
2007	2,6	5.38	0.046
<i>Arabis</i>			
2005	2,13	0.07	0.93
2006	2,9	1.23	0.34
2007	2,19	1.92	0.17
<i>Campanula</i>			
2005	2,7	0.24	0.79
2006	2,8	0.20	0.82
2007	2,8	0.083	0.92
<i>Erigeron</i>			
2005	2,20	0.01	0.99
2006	2,20	3.65	0.045

2007	2,18	3.50	0.050
<i>Helianthella</i>			
2005	2,14	0.6	0.56
2006	2,14	1.13	0.35
2007	2,14	9.86	0.0021
<i>Heliomeris</i>			
2005	2,9	0.92	0.43
2006	2,9	1.37	0.30
2007	2,10	1.81	0.21
<i>Ipomopsis</i>			
2005	2,9	0.58	0.58
2006	2,12	4.20	0.041
2007	2,11	3.93	0.049
<i>Lathyrus</i>			
2005	2,13	3.41	0.065
2006	2,16	4.93	0.022
2007	2,11	4.18	0.045
<i>Potentilla</i>			
2005	2,18	0.80	0.47
2006	2,18	3.60	0.048
2007	2,18	4.78	0.022

Appendix 3.6. ANOVA table for the effects of nitrogen addition on the number of flowering stalks per plot of dominant forb species across three years of nitrogen treatment. Results are from whole-model rm-ANOVAs. Statistically significant P-values are bolded.

Plant species	df	F	P
<i>Agoseris</i>	2,4	0.007	0.99
<i>Arabis</i>	2,15	1.92	0.18
<i>Campanula</i>	2,8	1.03	0.40
<i>Delphinium</i>	2,13	0.12	0.89
<i>Erigeron</i>	2,17	0.29	0.75
<i>Helianthella</i>	2,14	0.88	0.45
<i>Heliomeris</i>	2,6	0.60	0.58
<i>Ipomopsis</i>	2,9	2.12	0.18
<i>Lathyrus</i>	2,17	0.26	0.78
<i>Potentilla</i>	2,18	0.86	0.44

Appendix 3.7. ANOVA table for flowering phenology (bloom duration) responses of dominant forb species to nitrogen addition across the three years of treatment.

Statistically significant P-values are bolded.

Plant species	Test	df	F	P
<i>Agoseris</i>	ANCOVA, N trt	2,15	10.89	0.0012
2005	ANOVA	2,9	1.35	0.31
2006	ANOVA	2,7	27.85	0.0005
2007	ANOVA	2,6	7.15	0.026
<i>Arabis</i>	ANCOVA, N trt	2,25	0.29	0.75
<i>Campanula</i>	ANCOVA, N trt	2,19	1.74	0.20
<i>Erigeron</i>	ANCOVA, N trt	2,37	7.12	0.0024
2005	ANOVA	2,20	5.66	0.31
2006	ANOVA	2,15	1.47	0.26
2007	ANOVA	2,18	2.19	0.14
<i>Helianthella</i>	ANCOVA, N trt	2,32	6.22	0.0052
2005	ANOVA	2,14	0.58	0.57
2006	ANOVA	2,14	0.48	0.63
2007	ANOVA	2,14	5.95	0.014
<i>Heliomeris</i>	ANCOVA, N trt	2,22	5.74	0.0099
2005	ANOVA	2,9	1.48	0.28
2006	ANOVA	2,9	0.76	0.49
2007	ANOVA	2,10	1.35	0.3
<i>Ipomopsis</i>	ANCOVA, N trt	2,24	2.15	0.14

<i>Lathyrus</i>	ANCOVA, N trt	2,30	4.22	0.024
2005	ANOVA	2,13	0.87	0.44
2006	ANOVA	2,17	0.23	0.80
2007	ANOVA	2,12	5.66	0.018
<i>Potentilla</i>	ANCOVA, N trt	2,34	7.04	0.0028
2005	ANOVA	2,12	2.99	0.088
2006	ANOVA	2,18	2.68	0.095
2007	ANOVA	2,18	0.29	0.75

Appendix 3.8. ANOVA table for flower size responses of *Ipomopsis* and *Potentilla* focal plants to nitrogen addition across the three years of treatment. Statistically significant P-values are bolded.

Response variable	Test	df	F	P
<i>Ipomopsis</i>				
Corolla length	rm-ANOVA	2,8	3.48	0.08
2005	ANOVA	2,8	0.70	0.52
2006	ANOVA	2,11	7.1	0.01
2007	ANOVA	2,11	4.03	0.049
Petal width	rm-ANOVA	2,8	8.88	0.0093
2005	ANOVA	2,8	0.44	0.66
2006	ANOVA	2,11	4.2	0.044
2007	ANOVA	2,11	5.47	0.023
<i>Potentilla</i>				
Petal length	rm-ANOVA	2,18	4.47	0.027
2005	ANOVA	2,18	0.83	0.45
2006	ANOVA	2,18	2.25	0.13
2007	ANOVA	2,18	16.63	< 0.0001
Petal width	rm-ANOVA	2,18	3.07	0.071
2005	ANOVA	2,18	0.75	0.49
2006	ANOVA	2,18	1.95	0.17
2007	ANOVA	2,18	10.95	0.0008

Appendix 3.9. ANOVA table for nectar responses of *Ipomopsis* focal plants to nitrogen addition across three years of treatment. Statistically significant P-values are bolded.

Response variable	Test	df	F	P
<i>Ipomopsis</i>				
Nectar production	rm-ANOVA	2,7	13.89	0.0037
2005	ANOVA	2,8	20.37	0.0007
2006	ANOVA	2,10	10.91	0.0031
2007	ANOVA	2,9	7.62	0.01
Nectar sugar concentration	rm-ANOVA	2,7	0.94	0.44

Appendix 3.10. ANOVA table for pollinator visitation responses to nitrogen addition across the three years of treatment. For plant visitation rate in 2006 (all flowers only) and 2007, this test was repeated including flower production as a covariate to determine whether N addition *per se* or its effects on community-level floral traits was driving the response. Statistically significant P-values are bolded.

Response variable	Test	df	F	P
All flowers combined:				
Plant visitation rate	rm-ANOVA	2,21	3.7	0.042
2005		2,21	0.22	0.84
2006		2,21	4.59	0.022
	ANCOVA	3,20	6.46	0.0031
	N treatment	2,20	0.72	0.50
	flower production	1,20	7.41	0.013
2007		2,21	4.28	0.028
	ANCOVA	3,20	9.16	0.0005
	N treatment	2,20	0.59	0.56
	flower production	1,20	13.73	0.0014
Flowers per bout	rm-ANOVA	2,21	0.94	0.41
Seconds per flower	rm-ANOVA	2,21	1.71	0.21
Per-flower visitation rate	rm-ANOVA	2,21	0.21	0.81
<i>Potentilla</i> :				
Plant visitation rate	rm-ANOVA	2,18	7.64	0.0040
2005		2,18	1.26	0.31

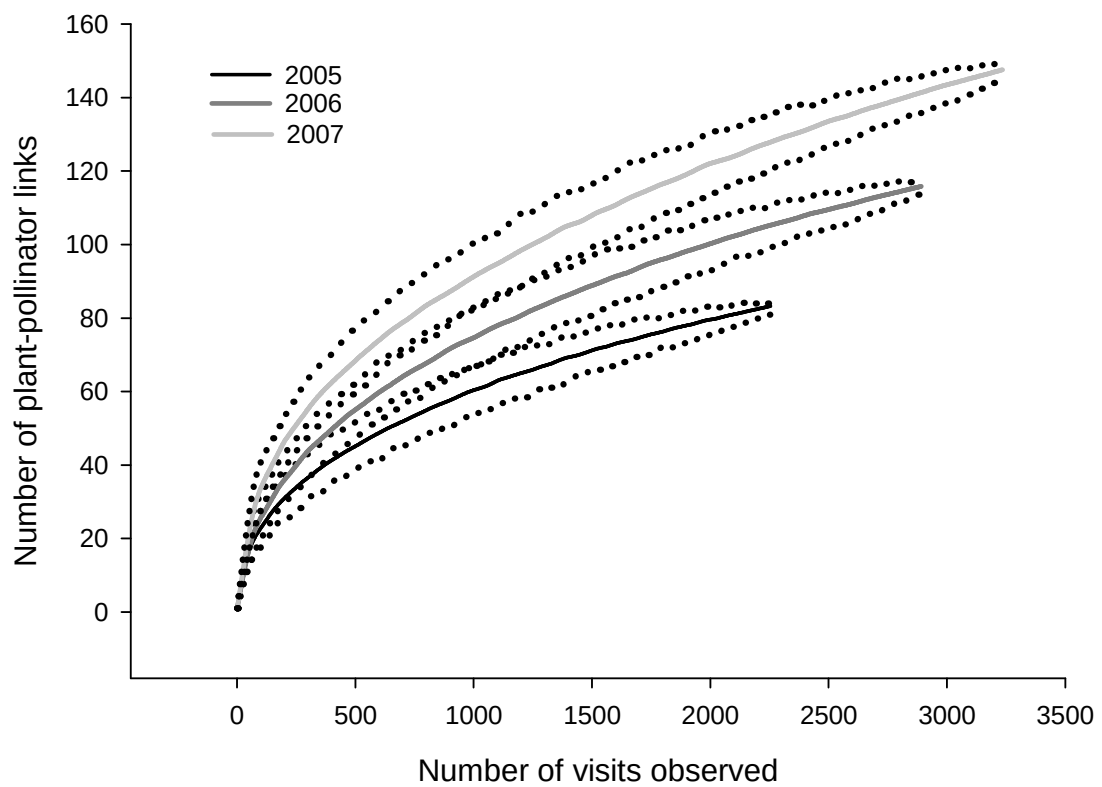
2006		2,18	2.92	0.081
2007		2,18	6.41	0.0079
	ANCOVA	4,16	10.97	0.0002
	N treatment	2,16	4.84	0.023
	Total flower production	1,16	0.018	0.90
	<i>Potentilla</i> flwr. prod'n	1,16	11.79	0.0034
Flowers per bout	rm-ANOVA	2,18	2.15	0.15
Seconds per flower	rm-ANOVA	2,18	2.11	0.15
Per-flower visitation rate	rm-ANOVA	2,18	0.78	0.47

Appendix 4.1. Flowering plants and pollinators observed from 2005-2007. Black dots represent the presence of a plant-pollinator interaction in at least one year.

Plant family	Plant genus and species	Andrenidae	Anthomyiidae	Apidae	Arctidae	Bombyliidae	Brachonidae	Buprestidae	Calliphoridae	Chrysidae	Cleridae	Coccinellidae	Colletidae	Halictidae	Hesperiidae	Ichneumonidae	Lycanidae	Masariidae	Megachilidae	Meloidae	Miridae	Muscidae	Noctuidae	Nymphalidae	Pieridae	Rhagionidae	Sarcophagidae	Siricidae	Sphecidae	Sphingidae	Stratiomyidae	Syrphidae	Tachinidae	Tenthredinidae	Trochilidae	Vespidae
Apiaceae	<i>Ligusticum porteri</i>		●																				●													
Apiaceae	<i>Pseudocymopterus montanus</i>	●	●	●			●						●			●	●		●			●		●		●	●					●	●			
Asteraceae	<i>Achillea lanulosa</i>		●	●		●							●			●	●	●				●		●	●	●	●					●	●			●
Asteraceae	<i>Agoseris aurantiaca</i>		●										●	●			●					●		●	●							●	●			
Asteraceae	<i>Agoseris glauca</i>	●	●	●		●						●	●			●						●		●		●	●					●	●			
Asteraceae	<i>Dugaldia hoopesii</i>																							●												
Asteraceae	<i>Erigeron flagellaris</i>	●	●			●				●				●					●								●					●				
Asteraceae	<i>Erigeron speciosus</i>	●	●	●		●								●	●		●					●	●	●			●			●		●	●			
Asteraceae	<i>Helianthella quinquenervis</i>		●	●		●			●					●	●		●			●		●		●	●	●	●					●	●			●
Asteraceae	<i>Heliomeris multiflora</i>	●	●	●	●	●			●			●	●	●	●	●	●		●			●		●	●	●	●		●		●	●	●	●		●
Asteraceae	<i>Heterotheca villosa</i>	●		●		●							●				●	●								●			●			●	●	●		●
Asteraceae	<i>Senecio canus</i>		●			●							●																							
Asteraceae	<i>Solidago multiradiata</i>		●	●												●											●					●				●
Asteraceae	<i>Taraxacum officinale</i>	●	●	●		●					●		●						●	●	●	●		●	●	●	●					●	●			
Boraginaceae	<i>Hackelia floribunda</i>		●	●		●							●														●						●			
Boraginaceae	<i>Lithospermum ruderales</i>		●																																	
Boraginaceae	<i>Mertensia fusiformis</i>		●	●										●					●			●														
Brassicaceae	<i>Arabis hirsuta</i>		●			●							●				●							●			●					●				
Brassicaceae	<i>Erysimum inconspicuum</i>	●	●			●							●				●									●		●				●	●			
Campanulaceae	<i>Campanula rotundifolia</i>	●	●	●		●						●	●					●								●	●					●				
Caryophyllaceae	<i>Arenaria congesta</i>															●																				
Fabaceae	<i>Lathyrus leucanthus</i>	●		●		●							●				●			●							●									
Fabaceae	<i>Vicia americana</i>			●		●								●					●													●				
Gentianaceae	<i>Pneumonanthe parryi</i>			●																												●				
Hydrophyllaceae	<i>Hydrophyllum fendleri</i>			●		●							●					●				●										●	●			●
Linaceae	<i>Linum lewisii</i>	●	●	●		●							●	●	●	●	●		●					●		●						●	●			
Polemoniaceae	<i>Collomia linearis</i>			●		●																										●				
Polemoniaceae	<i>Ipomopsis aggregata</i>		●	●									●				●									●	●					●	●			●
Polygonaceae	<i>Eriogonum umbellatum</i>	●		●		●								●	●							●		●		●						●	●			
Primulaceae	<i>Androsace septentrionalis</i>	●	●			●							●				●			●		●		●								●	●			
Ranunculaceae	<i>Delphinium nuttallianum</i>	●	●	●		●	●						●						●			●					●					●	●			
Ranunculaceae	<i>Ranunculus inamoenus</i>																															●	●			
Rosaceae	<i>Fragaria virginiana</i>																●		●													●	●			
Rosaceae	<i>Potentilla pulcherrima</i>	●	●	●		●	●	●	●		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Rubiaceae	<i>Galium boreale</i>		●			●	●		●								●	●				●	●	●			●	●				●	●			
Scrophulariaceae	<i>Collinsia parviflora</i>					●										●			●			●														
Valerianaceae	<i>Valeriana occidentalis</i>												●									●														
Violaceae	<i>Viola praemorsa</i>																					●														

Appendix 4.2. All analyses repeated with plants and pollinators classified to family.

Appendix 4.2.1. Rarefaction curves (solid lines) and their 95% confidence intervals (dashed lines) of the number of plant-pollinator links as a function of the number of visits from each of the three years of observations. Here, both plants and pollinators were classified to family.



Appendix 4.2.2. Comparison of plant-pollinator network structure between nitrogen treatments (C = control, L = low nitrogen addition, and H = high nitrogen addition) within years and between years. The m^2 statistic is a measure of goodness-of-fit between the Animal by Plant matrices and is bounded between 0 (good fit; similarity in the identity and frequency of plant-pollinator interactions between matrices) and 1 (poor fit; differences in the identity or frequency of interactions between matrices). The significance of m^2 was determined by a permutation test and is reported as P . The size of the matrix is given as A (number of animal pollinator families) by P (number of plant families).

	m^2	P	$A \times P$
2005			
C vs. H	0.0081	0.0001	23 x 17
C vs. L	0.010	0.0001	23 x 17
H vs. L	0.011	0.0001	23 x 17
2006			
C vs. H	0.043	0.0001	25 x 20
C vs. L	0.0094	0.0001	25 x 20
H vs. L	0.028	0.0001	25 x 20
2007			
C vs. H	0.11	0.0001	32 x 16
C vs. L	0.042	0.0001	32 x 16
H vs. L	0.062	0.0001	32 x 16
2005-2007			

2005 vs. 2006	0.37	0.0001	35 x 21
2006 vs. 2007	0.50	0.0003	35 x 21
2005 vs. 2007	0.83	0.059	35 x 21

Appendix 4.2.3. Pollinator families that exhibited the largest changes in interactions between nitrogen treatments (C = control, L = low nitrogen addition, and H = high nitrogen addition) within and between years. The five most affected families are shown for each N treatment or year comparison. Large vector residuals indicate large changes in position between the two matrices. The absolute value of changes in the number of visits and links to plant families are reported to illustrate the magnitude of the differences between matrices.

C vs. H				C vs. L				H vs. L			
Pollinator	Residuals	Δ visits	Δ links	Pollinator	Residuals	Δ visits	Δ links	Pollinator	Residuals	Δ visits	Δ links
2005											
Bombyliidae	0.059	32	2	Halictidae	0.0639	19	0	Halictidae	0.0761	26	2
Halictidae	0.034	7	2	Bombyliidae	0.0461	27	1	Andrenidae	0.0379	12	1
Megachilidae	0.0265	17	0	Andrenidae	0.0347	12	1	Syrphidae	0.0304	9	2
Syrphidae	0.0242	7	0	Syrphidae	0.0284	16	2	Bombyliidae	0.0278	5	1
Trochilidae	0.0232	8	1	Megachilidae	0.0249	6	1	Megachilidae	0.0254	11	1
2006											
Syrphidae	0.1491	32	1	Halictidae	0.0459	20	1	Syrphidae	0.1137	3	2

Halictidae	0.1105	51	1	Syrphidae	0.0425	29	3	Halictidae	0.0777	71	2
Rhagionidae	0.0482	21	1	Anthomyiidae	0.0418	14	1	Lycaenidae	0.0501	8	1
Lycaenidae	0.0439	10	0	Megachilidae	0.0279	2	0	Rhagionidae	0.0419	23	3
Anthomyiidae	0.0437	27	1	Andrenidae	0.0245	9	1	Andrenidae	0.0384	7	0
2007											
Apidae	0.2337	48	2	Apidae	0.1467	51	1	Anthomyiidae	0.1255	77	3
Anthomyiidae	0.1616	41	1	Bombyliidae	0.068	37	1	Halictidae	0.1071	15	0
Halictidae	0.089	8	1	Andrenidae	0.0593	12	1	Bombyliidae	0.1002	46	0
Syrphidae	0.0643	31	3	Nymphalidae	0.0437	15	1	Apidae	0.0874	3	1
Tachinidae	0.0634	11	0	Lycaenidae	0.0431	14	1	Andrenidae	0.0626	26	4
2005 vs. 2006				2005 vs. 2007				2006 vs. 2007			
Halictidae	0.3809	401	5	Apidae	0.6969	998	5	Anthomyiidae	0.4983	516	5
Syrphidae	0.3322	489	4	Anthomyiidae	0.6443	696	7	Apidae	0.4131	494	3
Apidae	0.2866	504	2	Halictidae	0.3439	250	2	Syrphidae	0.223	320	3
Bombyliidae	0.1818	219	1	Sarcophagidae	0.1799	237	7	Sarcophagidae	0.1822	212	4

Anthomyiidae	0.1493	180	3	Syrphidae	0.1347	169	1	Bombyliidae	0.1577	162	0
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Appendix 4.2.4. The Temperature (T) of networks in N treatments (C = control, L = low nitrogen addition, and H = high nitrogen addition) within and between years.

<i>T</i>	
2005	10.53
C	9.32
L	10.75
H	12.14
2006	12.66
C	11.52
L	6.52
H	9.04
2007	7.37
C	12.74
L	8.57
H	10.06

Appendix 4.2.5. Spearman rank correlations of Idiosyncratic Temperatures of plant and pollinator families between nitrogen treatments (C = control, L = low nitrogen addition, and H = high nitrogen addition) within a year and between years. Idiosyncratic Temperature is a measure of how a families' pattern of links deviates from the pattern expected in a perfectly nested matrix, and the analysis reported here illustrates whether families are occupying similar positions in the nested communities (significant correlation). The strength and direction of the correlations are given by *rho*, the significance of the test by *P*, and the sample size by *n*. Significant P-values are bolded.

	<i>rho</i>	P	<i>n</i>
2005			
C vs. H	0.33	0.11	23
C vs. L	0.15	0.48	23
H vs. L	0.15	0.48	23
2006			
C vs. H	-0.12	0.52	30
C vs. L	0.07	0.70	30
H vs. L	-0.32	0.09	30
2007			
C vs. H	-0.14	0.45	34
C vs. L	-0.15	0.38	34
H vs. L	0.42	0.015	34
2005-2007			
2005 vs. 2006	0.15	0.37	36

2006 vs. 2007	0.05	0.74	36
2005 vs. 2007	-0.20	0.25	36

Appendix 4.2.6. The identity of plant and pollinator families in the core group of generalists in each year and nitrogen treatment (C = control, L = low nitrogen addition, H = high nitrogen addition). Larger eigenvalues indicate that the family was more generalized. Core generalists are ranked according to their eigenvalues.

	2005		2005C		2005H		2005L	
Plant	Rank	Eigenvalue	Rank	Eigenvalue	Rank	Eigenvalue	Rank	Eigenvalue
Asteraceae	1	96.485	1	97.093	1	97.521	1	93.686
Rosaceae	2	23.878	2	22.138	2	17.149	3	32.852
Fabaceae	3	7.879	3	7.651	3	12.417	2	7.46
Pollinator								
Apidae	1	98.438	1	97.837	1	99.254	1	97.825
Bombyliidae	2	11.592	2	14.542	2	8.08	3	15.39
Halictidae	3	11.375	3	11.923	3	7.491	2	12.126
Syrphidae	4	5.575	4	7.179				
Plant	2006		2006C		2006H		2006L	
Rosaceae	1	71.974	1	76.341	2	66.132	1	74.371
Asteraceae	2	69.323	2	64.533	1	74.817	2	66.696
Pollinator								
Apidae	1	61.227	2	56.579	1	64.361	1	60.466
Halictidae	2	57.624	1	64.038	3	48.289	3	60.429
Syrphidae	3	48.198	3	43.017	2	55.831	2	45.493
Anthomyiidae	4	15.949	4	20.205	4	12.975	4	14.811
Rhagionidae	5	11.833	5	14.17	5	8.799	5	12.566
Andrenidae			6	8.732			6	10.17

Plant	2007		2007C		2007H		2007L	
Rosaceae	1	97.834	1	98.143	1	96.478	1	98.315
Asteraceae	2	20.104	2	18.867	2	25.26	2	17.343
Pollinator								
Anthomyiidae	1	64.868	1	68.675	2	57.101	1	66.732
Halictidae	2	56.641	2	53.845	1	63.573	2	53.068
Syrphidae	3	23.803	3	25.795	4	21.808	4	23.296
Bombyliidae	4	23.12	4	21.14	5	19.237	3	27.517
Sarcophagidae	5	21.129	5	20.449	3	23.981	6	19.287
Andrenidae	6	20.253					5	22.994

Appendix 4.3. Summary statistics for plant-pollinator interaction matrices in each of the three years of observation. Control (C), high nitrogen addition (H), and low nitrogen addition (L) are the three treatments included for each year.

Year	2005				2006				2007			
Nitrogen treatment	C	H	L	total	C	H	L	total	C	H	L	total
Sampling hours	40.6	42.4	42.2	125.2	59.5	57.9	58.7	176.1	56.3	55.2	56.7	168.2
Total # taxa (plants & pollinators)	40	42	40	56	46	46	47	56	51	46	51	63
# Pollinator families observed	16	17	15	23	22	23	22	25	29	24	25	32
# Foraging bouts observed	785	803	850	2438	1014	884	1116	3014	1072	1017	1250	3339
# Flower visits per foraging bout	4678	6677	7512	18867	6461	5933	8258	20652	3615	3655	5222	12492
# Links	77	78	73	133	116	98	111	183	133	136	153	239
# Plant species visited	24	25	25	33	24	23	25	31	22	22	26	31
Median links per plant species	2	2	2	3	3	3	3	4	4.5	4.5	5	6
Maximum links per plant species	14	11	13	15	16	15	18	20	24	20	23	28
Median visits by all poll to indiv plant	8	8	8	12	6.5	7	5	14	13.5	8.5	8.5	13
Max visits by all poll to indiv plant	259	250	311	820	530	435	595	1560	735	633	845	2213

Median links per pollinator species	2.5	3	3	3	4.5	3	3.5	5	3	6	5	4
Max links per pollinator species	15	20	18	24	16	15	19	22	12	13	16	22
Connectance	0.20	0.18	0.19	0.175	0.22	0.19	0.20	0.24	0.21	0.26	0.24	0.24
Temperature (T)	8.19	8.77	10.05	6.21	15.64	9.37	8.25	11.3	10.88	11.53	10.87	7.73
Nestedness (100-T/100)	0.92	0.91	0.90	0.94	0.84	0.91	0.92	0.89	0.89	0.88	0.89	0.92

Appendix 4.4. The numerical code for identifying plants and pollinators in Figure 4.3.

Members of insect and hummingbird families were verified as functioning as pollinators in this or other systems (one exception is footnoted) by conducting a search on Web of Science for each family.

Plants	Plant ID#	Pollinators	Pollinator ID#
<i>Achillea lanulosa</i>	1	Andrenidae	1
<i>Agoseris aurantiaca</i>	2	Anthomyiidae	2
<i>Agoseris glauca</i>	3	Apidae	3
<i>Androsace septentrionalis</i>	4	Arctiidae	4
<i>Arabis hirsuta</i>	5	Bombyliidae	5
<i>Arenaria congesta</i>	6	Brachonidae	6
<i>Campanula rotundifolia</i>	7	Buprestidae	7
<i>Collinsia parviflora</i>	8	Calliphoridae	8
<i>Collomia linearis</i>	9	Chrysididae	9
<i>Delphinium nuttallianum</i>	10	Cleridae	10
<i>Dugaldia hoopesii</i>	11	Coccinellidae	11
<i>Erigeron flagellaris</i>	12	Colletidae	12
<i>Erigeron speciosus</i>	13	Halictidae	13
<i>Eriogonum umbellatum</i>	14	Hesperidae	14
<i>Erysimum inconspicuum</i>	15	Ichneumonidae	15
<i>Fragaria virginiana</i>	16	Lycaenidae	16
<i>Galium boreale</i>	17	Masaridae	17
<i>Hackelia floribunda</i>	18	Megachilidae	18

<i>Helianthella quinquenervis</i>	19	Meloidae	19
<i>Heliomeris multiflora</i>	20	Miridae	20
<i>Heterotheca villosa</i>	21	Muscidae	21
<i>Hydrophyllum fendleri</i>	22	Noctuidae	22
<i>Ipomopsis aggregata</i>	23	Nymphalidae	23
<i>Lathyrus leucanthus</i>	24	Pieridae	24
<i>Ligusticum porteri</i>	25	Rhagionidae	25
<i>Linum lewisii</i>	26	Sarcophagidae	26
<i>Lithospermum ruderale</i>	27	Siricidae ¹	27
<i>Mertensia fusiformis</i>	28	Sphecidae	28
<i>Pneumonanthe parryi</i>	29	Sphingidae	29
<i>Potentilla pulcherrima</i>	30	Stratiomyidae	30
<i>Pseudocymopterus montanus</i>	31	Syrphidae	31
<i>Ranunculus inamoenus</i>	32	Tachinidae	32
<i>Senecio canus</i>	33	Tenthredinidae	33
<i>Solidago multiradiata</i>	34	Trochilidae	34
<i>Taraxacum officinale</i>	35	Vespidae	35
<i>Valeriana occidentalis</i>	36		
<i>Vicia americana</i>	37		
<i>Viola praemorsa</i>	38		

¹To my knowledge, the role of Siricid wasps as pollinators has not been previously demonstrated. As adults, they have reduced mouthparts, feeding little (or not at all; Krenn et al. 2005), and this may limit their effectiveness as pollinators

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