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Beyond biomass: measuring the effects of community-level nitrogen enrichment on floral traits, pollinator visitation and plant reproduction

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Summary

1. Nitrogen (N) limits primary productivity in many systems and can have dramatic effects on plant–herbivore interactions, but its effects on mutualistic interactions at the community level are not well-understood. The reproduction of many plants depends on both soil N and pollination, and N may affect floral traits, such as flower number or size, which are important for pollinator attraction to plant individuals and communities.

2. Thus, N may influence plant biomass and reproduction directly as well as indirectly via changes in pollination. The degree to which the effects of N enrichment scale from plant individuals to assemblages through emerging community-level changes in species interactions, like pollination, is relatively unknown.

3. For 4 years, we tested how N addition to subalpine plant assemblages in Colorado, USA, affected primary productivity and species diversity, floral traits and plant–pollinator interactions, and components of female and male plant reproduction.

4. At the community level, we found that high-N addition favoured the biomass and seed production of grasses, whereas low-N addition promoted forb growth, flower production and pollinator visitation. However, using a pollen supplementation experiment, we found no evidence that N addition altered patterns of pollen limitation of seed production. Pollinators distributed themselves evenly across floral resources such that per-flower visitation rate did not differ among N treatments. Thus, individual plants did not incur any extra benefit or cost from community-level changes in plant–pollinator interactions that resulted from N enrichment, and the effects of N on forb reproduction were direct.

5. *Synthesis*. Understanding how mutualistic and antagonistic species interactions influence individual and community responses to abiotic resources may provide insight to the dominant forces structuring communities and is especially important in the context of predicting the effects of environmental change. In this case, the direct effects of N addition on plants were stronger than the indirect effects mediated through plant–pollinator interactions, thus supporting the concept of bottom-up resource limitation controlling plant response.

Key-words: indirect effects, *Ipomopsis aggregata,* mutualism, nutrient limitation, *Potentilla pulcherrima,* species interactions

Introduction

Nitrogen plays a fundamental role in all biological systems, and its effects can scale up through ecosystems, limiting productivity and affecting plant quality, consumer preference and performance as well as community composition and species interactions (e.g. Tilman 1987; Wallace *et al.* 1997). Although community ecologists have elucidated a host of effects of nitrogen enrichment on plant assemblages and typical consumer–resource interactions (e.g. plant–herbivore interactions), the effects of nitrogen on mutualistic interactions have rarely been addressed at the community level. Nitrogen enrichment has the potential to affect plant–pollinator interactions by altering plant traits, such as floral, nectar and pollen characters, which

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are essential for pollinator attraction. Although pollination ecologists have deeply investigated species-specific direct and indirect effects of nutrient enrichment on plant individuals (e.g. Campbell & Halama 1993; Asikainen & Mutikainen 2005; Munoz *et al.* 2005), patterns of pollinator visitation and plant reproduction are strongly influenced by the community-level presentation of floral traits (Potts *et al.* 2003). The responses of neighbouring plant species to nitrogen availability could indirectly affect a focal plant's reproduction through competition or facilitation for pollinators. These community-level indirect effects are difficult to predict from experiments of nitrogen addition to individual plant species, given that they are emergent properties (Wootton 1993). Integrating community and pollination approaches to study the effects of nutrient enrichment may provide novel insights into the mechanisms that govern the reproductive success of flowering plants.

The reproduction of many terrestrial plants is strongly influenced not only by access to soil nitrogen (e.g. 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 number and size (e.g. Mitchell 1994; Galen 1999). Thus, nitrogen can influence plant biomass and reproduction directly as well as indirectly via changes in floral traits and species interactions. The direct effects of nitrogen on plant reproduction may be insignificant in magnitude compared with indirect effects mediated through community-level plant–pollinator interactions. Trait-mediated indirect interactions are increasingly recognized as important drivers in natural systems (Wootton 1994) and may also be influential drivers of how nutrients affect plant reproduction through changes in floral traits and mutualisms (Poveda *et al.* 2005; Wolfe, Husband & Klironomos 2005).

In this study, using a 4-year nitrogen (N) enrichment regime in subalpine meadows, we tested how N addition affected traditional plant community metrics, including above-ground primary productivity and species diversity, as well as floral traits, plant–pollinator interactions and subsequent reproductive responses. We predicted that productivity, floral traits and plant reproduction would respond positively to N addition, given that communities as well as individual plants are often N-limited (e.g. Bowman *et al.* 1993; Munoz *et al.* 2005). However, we also predicted that the responses 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) and given that perennials often experience a delayed response to N addition relative to annuals (e.g. Monaco *et al.* 2003). We expected plant mating system to play a role in plant responses to N, such that self-incompatible plant species would be more affected by indirect effects of N addition via changes in plant–pollinator interactions compared with self-compatible plants. We expected N addition to result in emergent, community-level facilitative or competitive effects among plants for pollinators. We compared productivity and male and female components of plant reproduction at the whole-plot level, for plant functional groups and for individual dominant forb species. In addition, we used a pollen supplementation experiment to investigate whether pollination success was a mechanism by which female reproduction responded to N addition. By combining concepts and methods from studies of pollination and community ecology, this work explores how N enrichment scales up from individual flower and plant presentation of floral traits to pollinator preference at the community level, and the importance of direct effects of nitrogen addition vs. the indirect effects on plant reproduction through changes in pollination over multiple years.

Materials and methods

STUDY SYSTEM

We explored the effects of N enrichment at the flower, plant and plot levels (Table 1) in subalpine meadows near the Rocky Mountain Biological Laboratory (RMBL), in western Colorado, USA (38°57′29″N, 106°59′06″W, 2900 m a.s.l.). Mountain ecosystems often have low nutrient supply (Bowman & Fisk 2001), and above-ground net primary productivity (ANPP) in these systems can be limited by soil N (e.g. Bowman *et al.* 1993; Brancaleoni *et al.* 2007; but see Cross & Harte 2007). Nitrogen deposition rates are low around the RMBL [mean = 0.4 g nitrate (NO_3^-) and 0.06 g ammonium (NH_4^+) m^{-2} $year^{-1}$; NADP (2006)] compared with other areas in the Rocky Mountains (Baron *et al.* 2000; Fenn *et al.* 2003; NADP 2006), so the RMBL serves as a baseline for investigating the potential effects of changes in N availability.

Based on flower abundance, the dominant forbs in this system include *Delphinium nuttallianum*, *Erigeron speciosus*, *Helianthella quinquenervis*, *Heliomeris multiflora*, *Ipomopsis aggregata* and *Potentilla pulcherrima* (Table S1). 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. Water and/or N addition to individual plants can increase biomass, flowering, nectar production per flower, pollinator visitation rates and seed set of some of these species (Zimmerman 1983; de Valpine & Harte 2001).

To understand the effects of N addition on ANPP and plant reproduction (Table 1), we focused our measurements on the community level, on plant functional groups (forbs, grasses, N-fixers) and on dominant plants, with particular focus on *Ipomopsis aggregata* and *Potentilla pulcherrima* (hereafter referred to by genus). *Ipomopsis* and *Potentilla* differ in their life history. By studying these two species in depth, we could gain insight into how community-level N addition affected the biomass and reproduction of these different species.

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 during one season and then dying; thus, we could estimate lifetime reproduction in one season. Increased seed set generally translates into increased seedling and juvenile recruitment (Price *et al.* 2008). The red, trumpet-shaped flowers are hermaphroditic, protandrous and bloom for 3–5 days (Pleasants

Table 1. Summary of the response variables to nitrogen (N) addition measured at the flower, plant and plot levels

Response variable	Level	Metrics	Target plant species
ANPP or biomass	Plant level	Roots and shoots	*Ipomopsis*
		Shoots	*Potentilla*
	Plot level	Total	All species combined
		Functional group	Grasses, forbs, N-fixers
Species diversity	Plot level	Species richness	All species combined
		Species evenness	All species combined
Floral traits	Flower level	Flower size	*Ipomopsis*, *Potentilla*
		Nectar production rate	*Ipomopsis*
		Nectar sugar concentration	*Ipomopsis*
	Plant level	Per-plant flower production	*Ipomopsis*, *Potentilla*
	Plot level	Per-species flower production	Each forb species separately[1]
		Total flower production	All forb species combined[2]
Pollinator visitation[3]	Flower level	Stigma pollen receipt	*Ipomopsis*
		Time spent per flower	*Potentilla*, all forb species combined[2]
		Per-flower visitation rate	*Potentilla*, all forb species combined[2]
	Plant level	Plant visitation rate	*Potentilla*, all forb species combined[2]
	Plot level	No. flowers visited per foraging bout	*Potentilla*, all forb species combined[2]
Female reproduction	Flower level	Forb seeds per fruit	Each forb species separately[4]
		Forb mass per seed	Each forb species separately[4]
	Plant level	Percent fruit set	*Ipomopsis*, *Potentilla*[5]
		Total seeds per plant	*Ipomopsis*, *Potentilla*[5]
	Plot level	Forb seeds per plot	Each forb species separately[4,5]
		Grass seed mass per plot	All grasses combined[6]
Male reproduction	Flower level	Pollen production per flower	Each forb species separately[7,8,9]
	Plant level	Pollen production per plant	*Ipomopsis*,[8] *Potentilla*[9]
	Plot level	Pollen production per plot	Each forb species separately[7,8,9]

Variables were measured from 2005 to 2008 unless otherwise noted.
[1]Dominant forbs: *Agoseris aurantiaca*, *Arabis hirsuta*, *Campanula rotundifolia*, *Delphinium nuttallianum*, *Erigeron speciosus*, *Helianthella quinquenervis*, *Heliomeris multiflora, Ipomopsis aggregata, Lathyrus leucanthus* and *Potentilla pulcherrima*.
[2]See Table S1 for the full list of species.
[3]Variables measured from 2005 to 2007.
[4]2007: *Agoseris aurantiaca*, *Arabis hirsuta*, *Campanula rotundifolia*, *Delphinium nuttallianum*, *Erigeron speciosus*, *Helianthella quinquenervis* and *Heliomeris multiflora*.
[5]2005–2007: *Ipomopsis aggregata* and *Potentilla pulcherrima*.
[6]2007–2008: grasses.
[7]2005 and 2007: *Achillea lanulosa*, *Campanula rotundifolia*, *Erigeron speciosus* and *Helianthella quinquenervis*; 2007 only: *Heliomeris multiflora* and *Vicia americana*.
[8]2005–2007: *Ipomopsis aggregata*.
[9]2005 and 2007: *Potentilla pulcherrima*.

1983). *Ipomopsis* is self-incompatible and is pollinated primarily by broad-tailed (*Selasphorus platycercus*) and rufous (*Selasphorus rufus*) hummingbirds around the RMBL (Price *et al.* 2005). *Ipomopsis* is pollen-limited for seed set in some years (e.g. Hainsworth, Wolf & Mercier 1985; Campbell & Halama 1993; Irwin 2006). Nutrient fertilization of individual *Ipomopsis* in a single year (using a 20:20:20 NPK fertilizer) had direct, positive effects on floral rewards and seed production but minimal indirect effects on seed production mediated through changes in pollinator behaviour (Campbell & Halama 1993). The direct and indirect effects of N addition alone and over multiple years 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 and can autogamously self-pollinate, but requires pollinators and outcrossing for maximal seed set (R. Irwin, C. Danaher and J. Reithel, unpubl. data). Plants can reproduce vegetatively through the production of additional stalks emerging near the base of the parent plant (L. Burkle, pers. obs.). The direct and indirect effects of N addition on *Potentilla* are unexplored.

NITROGEN TREATMENTS

In 2005, we identified 24 plots (4 × 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 four consecutive summers, 2005–2008): control, 'low' N addition (1 g N m^{-2} $year^{-1}$) and 'high' N addition (20 g N m^{-2} $year^{-1}$). Treatment plots within blocks were at least 6 m apart from each other, and blocks were up to 2.7 km apart. We 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 control plots received the same amount of water. The low-N treatment represented a level similar to atmospheric N deposition in the Front Range of the Colorado Rocky Mountains, USA (Sievering, Rusch & Marquez 1996). In the high-N treatment, N should have been abundant to plants even after chemical and microbial immobilization (Eviner, Chapin & Vaughn 2000). Our N treatments translated into expected increases in soil N availability, measured using ion-exchange resin bags (Binkley 1984; MANOVA, $\lambda = 0.48$, $F_{4,40} = 4.41$, $P = 0.005$).

Plot size was chosen for two reasons: first, to reflect the scale at which pollinators make foraging decisions once inside a meadow (Klinkhamer, Jong & Linnebank 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 2005–2008 and in each plot, we marked flowering individuals of our focal species, *Ipomopsis* and *Potentilla* (*c*. 8 plants per species per plot), to investigate individual plant biomass and reproduction (see below, Table 1). Because *Ipomopsis* is monocarpic, new focal plants were marked in each plot at the beginning of each season. Because *Potentilla* is perennial, the same plants were followed across years.

EFFECTS OF N TREATMENTS ON ANPP AND SPECIES DIVERSITY

Plant level

We collected the above- and below-ground biomass of focal individuals of *Ipomopsis* and the above-ground biomass of *Potentilla* at the end of each growing season.

Plot level

To assess if N addition affected total ANPP, functional group (grasses, forbs and N-fixers) ANPP and species diversity (richness and evenness), we collected, separated by species, dried and weighed above-ground plant biomass of three randomly located quadrats (0.0625 m^2) per plot at the end of each growing season.

EFFECTS OF N TREATMENTS ON FLORAL TRAITS

In each year, we measured floral traits to provide a mechanistic understanding of the direct effects of N treatments on characters important to pollination at the flower, individual plant and plot levels (e.g. Pleasants 1981; Galen 1999; Biernaskie & Cartar 2004).

Flower level

We measured flower size in *Ipomopsis* as corolla length and width (Campbell 1996). For *Potentilla*, we measured the length and width of one haphazardly chosen petal. Measurements were made on up to three flowers per focal plant in all plots. We estimated per-flower nectar rewards in *Ipomopsis* by measuring nectar production rate (over 48 h on bagged flowers) and sugar concentration on a maximum of four flowers per focal plant. We were unable to measure nectar traits in *Potentilla* because measurement precision was not high enough for the small quantities of nectar produced.

Plant level

We estimated total per-plant flower production of *Ipomopsis* and *Potentilla* as the number of initiated and aborted fruits of each focal plant in all plots at the end of the season.

Plot level

We measured flower production as the number of open flowers approximately every 3 days throughout the blooming season (2005–2007; once per week in 2008) for each forb species in all plots and calculated total and per-species flower production per plot for each sampling day.

EFFECTS OF N TREATMENTS ON POLLINATOR VISITATION

Throughout each flowering season, we observed plant–pollinator interactions in each plot for *c*. 1 h per week during peak insect activity (09:00–16:00). We observed plots for a total of 126 h in 2005, 178 h in 2006 and 168 h in 2007. All N treatments were observed equally within a given summer ($F_{2,21} < 0.99$, $P > 0.39$). We 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. We only recorded visitors that contacted the sexual organs of flowers; thus, it is likely that our estimates of visitation are for effective pollinators. Because we wanted to observe pollinator behaviour in the plots, including the number of flowers and plants probed and time spent per flower, we did not collect visitors for identification to species. Instead, we 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). We found similar effects of N addition on estimates of pollinator visitation (described below) when testing by pollinator functional group and across all functional groups; thus, we report the results across all pollinators.

Flower level

Using these pollinator observations, we calculated the mean time pollinators spent per flower and per-flower visitation rate for *Potentilla* and for all forb species together in each plot. *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, we estimated pollinator visitation using stigma pollen loads (Table 1). Because *Ipomopsis* does not autogamously self-pollinate, pollen receipt is a reliable proxy for pollinator visitation rate (Engel & Irwin 2003). We collected up to three stigmas from each focal plant once per week throughout each summer, stained them in basic fuchsin dye, and counted conspecific and heterospecific pollen deposition. We calculated mean pollen receipt per flower for each plant.

Plant level

We used the pollinator observations to calculate mean plant visitation rate for *Potentilla* and for all forb species together in each plot.

Plot level

We calculated the mean number of flowers visited per pollinator foraging bout for *Potentilla* and for all forb species together in each plot.

EFFECTS OF N TREATMENTS ON FEMALE PLANT REPRODUCTION

Flower level

We randomly collected up to 10 fruits per plot from nine common forb species (Table 1) and counted seeds per fruit and measured mass per seed to understand the effects of N on individual species that spanned a diversity of flower forms (Table S1).

Plant level: direct and indirect effects of N treatments

We used pollen supplementation treatments to investigate whether pollination success was a mechanism by which female reproduction of *Ipomopsis* and *Potentilla* responded to N addition. Half of the focal *Ipomopsis* and *Potentilla* plants 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 onto receptive stigmas. Anthers were collected from outside each plot, *c.* 5–10 m away. At the end of each season, we collected all of the fruits from each *Ipomopsis* and *Potentilla* focal plant and counted all of the seeds.

Plot level

To understand the effects of N on reproduction of the forb community, we used flower production and seeds per fruit of each species per plot to estimate the total number of seeds produced per plot for nine forb species (Table 1). To estimate female grass reproduction at the plot level, we divided grass biomass (see ANPP above) into vegetative (grass blades) and reproductive (seeds) components and weighed these separately.

EFFECTS OF N TREATMENTS ON MALE PLANT REPRODUCTION

Flower level

We measured pollen production per flower in eight forb species, including *Ipomopsis* and *Potentilla* (Table 1). Up to 30 flowers on separate plants of each species in each plot were sampled during peak flowering. Per-flower pollen production has been shown to increase with N addition (Lau & Stephenson 1993), and pollen production is often correlated with male siring success (reviewed in Snow & Lewis 1993; but see Ashman 1998).

Plant level

We used measures of flower production per plant and pollen production per flower to estimate mean total pollen production per plant for *Ipomopsis* and *Potentilla* in each plot.

Plot level

We used measures of flower production per plot and pollen production per flower to estimate total pollen production per plot (all eight forbs).

STATISTICAL ANALYSES

For each response variable, means for each plot were calculated, and repeated-measures ANOVAS (rm-ANOVAS) were used to test for the effects of N addition (see exceptions below). Significant year effects in the rm-ANOVAS were followed by individual tests to determine which year(s) was/were driving the response. We did not include block in these analyses because differences among blocks were not statistically significant ($P > 0.22$ in all cases). There was no difference in the results when we rarefied species richness and evenness (ECOSIM 7.72; Gotelli & Entsminger 2009), so we report the unrarified data. To determine whether N addition *per se* or whether the effects of N addition via community-level changes in flower production were associated with differences in pollinator visitation to *Ipomopsis* or *Potentilla* or to all forbs combined, the mean *Ipomopsis* or *Potentilla* flowers per plot and the mean number of total flowers per plot respectively were included as covariates in the analyses.

At the flower level, mean female reproduction of forbs was analysed using MANOVAS for each species. For *Ipomopsis* and *Potentilla* focal plants, we tested for the effects of N treatment and pollen supplementation on all four measured components of female reproduction (Table 1) using MANOVAS with N treatment (control, low, high), pollen-supplementation treatment (supplemented or control), year, plot (nested within N treatment to account for repeated sampling of the plots; Quinn & Keough 2002) 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 degree of pollen limitation. Significant MANOVA results were further analysed by appropriate univariate tests. There were no significant N treatment × year interactions for any of the response variables for *Ipomopsis* or *Potentilla* ($P > 0.11$ in all cases), so we removed the interaction from the model.

COMPARISON OF THE RELATIVE RESPONSES OF ANPP VS. REPRODUCTION

To directly compare the strengths of the relative responses of ANPP vs. seed and pollen production to N addition, we calculated effect sizes. For each block (low- and high-N additions compared with controls), we calculated mean log-response ratios (Hedges, Gurevitch & Curtis 1999) for ANPP and female and male reproductive success of all plants, functional groups (grasses and forbs) and focal species (*Ipomopsis* and *Potentilla*) over the first 3 years (2005–2007) 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 the reproduction of individual functional groups (and thus calculation of reproductive success of all plants per plot) were only available for 2007. We used a random-effects model, including both sampling error and random variation between blocks, for calculating effect sizes (Rosenberg, Adams & Gurevitch 2000). We calculated 95% confidence intervals with bias-corrected bootstrapping using MetaWin (Rosenberg, Adams & Gurevitch 2000). If the confidence intervals did not overlap zero, effect sizes were considered statistically significant (Gurevitch & Hedges 2001). We compared these effect sizes to determine whether the magnitude and direction of plant responses to N treatments differed between productivity and reproduction.

Results

EFFECTS OF N TREATMENTS ON ANPP AND SPECIES DIVERSITY

Plant level

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 across 4 years of study. Individual *Potentilla* focal plants were affected by N addition ($F_{2,18} = 4.29$, $P = 0.030$), but this effect was delayed, with 45% higher shoot biomass in low-N compared with high-N plots only after 4 years of treatments (2008: $F_{2,18} = 3.81$, $P = 0.042$).

Plot level

Nitrogen addition increased total ANPP ($F_{2,21}$ = 14.96, 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 (Table S2). In 2007 and 2008, both the low- and high-N treatments supported double the ANPP compared with the control (Table S2).

Nitrogen addition affected plant functional groups differently (Fig. 1). The ANPP of both forbs ($F_{2,21}$ = 13.78, P = 0.0002) and grasses ($F_{2,21}$ = 29.46, P < 0.0001) were affected by N addition, but the ANPP of nitrogen-fixing legumes was not ($F_{2,13}$ = 0.021, P = 0.98). The 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 up to eight times higher grass ANPP in the high-N plots in 2006, 2007 and 2008 compared with controls (Table S2). Species richness was not affected by N addition ($F_{2,21}$ = 0.84, P = 0.44), but species evenness reflected the differential effects of N addition on plant functional groups, with 10–40% greater evenness in controls compared with low- and high-N treatments in 2006, 2007 and 2008 ($F_{2,21}$ = 5.26, P = 0.014). Both forbs (in the low-N plots) and grasses (in the high-N plots) were driving these differences in species evenness.

EFFECTS OF N TREATMENTS ON FLORAL TRAITS

Flower level

There were effects of N addition on flower size in both *Ipomopsis* and *Potentilla*. We also found a significant effect of year (P < 0.05 in both cases), such that the effects of N addition on flower size were delayed. For *Ipomopsis*, although we saw no effects of N addition on components of flower size in 2005, we 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 2006–2008 (F > 4.03, P < 0.049 in all years). 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 and 2008 (F > 10.95, P < 0.015 in both years). Nitrogen addition affected nectar production ($F_{2,6}$ = 7.19, P = 0.026) but not nectar sugar concentration ($F_{2,6}$ = 0.64, P = 0.56) in *Ipomopsis*. In each year, plants in the low-N plots produced 64% more nectar on average than plants receiving the other treatments.

Plant level

Flower production by *Ipomopsis* was not affected by N treatments ($F_{2,9}$ = 0.90, P = 0.44). There was a delayed effect of N addition on *Potentilla* flower production ($F_{2,18}$ = 3.67, P = 0.046), with no effects in 2005 or 2006, but 79% and 55% greater flower production per plant in control and low-N plots respectively, than in high-N plots in 2007.

Plot level

Nitrogen addition affected total flower production, with greater flower production in the low-N plots compared with control or high-N plots (Fig. 2). These effects were, however, delayed, i.e. present only in 2006–2008 ($F_{2,21}$ > 5.29, P < 0.014). This increase in flower production not only held for individual forb species at the plot level, including *Ipomopsis* (driven by increased numbers of *Ipomopsis* flowering stalks in low-N plots) and *Potentilla*, but we also observed some species-specific variation (Table S3). This species-specific variation in the response of flower production to N addition could not be attributed to plant family.

EFFECTS OF N TREATMENTS ON POLLINATOR VISITATION

Flower level

There were no effects of N addition on the number of seconds pollinators spent per flower or the per-flower visitation rate to *Potentilla* or to all forbs combined in any year (F < 2.11, P > 0.15). For focal *Ipomopsis*, mean per-flower stigma receipt of conspecific ($F_{2,8}$ = 1.38, P = 0.31) and heterospecific pollen ($F_{2,8}$ = 0.17, P = 0.85) was unaffected by N addition. Neither *Ipomopsis* flower production per plot nor total

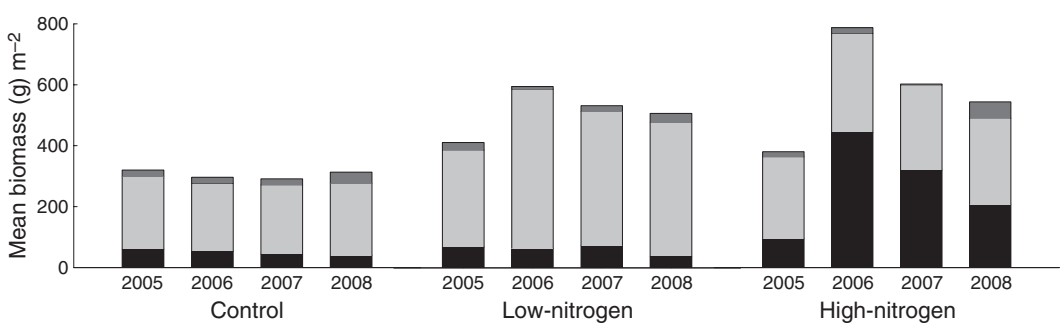

Fig. 1. Annual net primary productivity of grasses (black), forbs (light grey) and nitrogen (N) fixers (dark grey) in control, low-N addition and high-N addition treatments over 4 years of N enrichment (2005–2008). Error bars not shown.

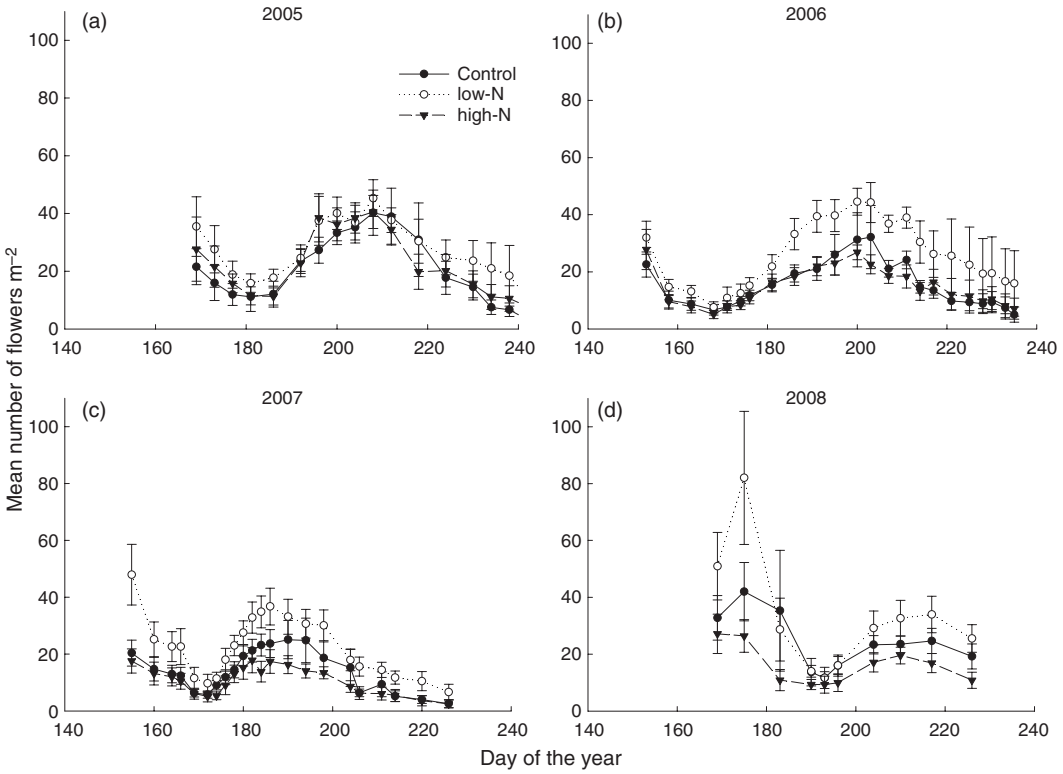

Fig. 2. Mean number of total flowers per m^2 in control (black circles), low-nitrogen (N) (white circles) and high-N (black triangles) plots over the flowering season in (a) 2005, (b) 2006, (c) 2007 and (d) 2008. Day of the year indicates how many days have passed since January 1 in a given year. Error bars are ±1 SE.

flower production per plot influenced *Ipomopsis* stigma pollen receipt in any year ($F < 1.28$, $P > 0.37$).

Plant level

Nitrogen addition affected plant visitation rate by pollinators to *Potentilla* ($F_{2,18} = 7.64$, $P = 0.004$) and to all forbs ($F_{2,21} = 3.7$, $P = 0.042$), with plants in low-N plots having a higher rate of visitation than plants in control or high-N plots. The effect on *Potentilla* was driven by both N addition ($F_{2,16} = 4.84$, $P = 0.004$) and *Potentilla* flower production per plot ($F_{1,16} = 11.79$, $P = 0.0034$), but not by total flower production per plot ($F_{1,16} = 0.018$, $P = 0.90$), whereas the effect on all forb plants combined was driven by flower production (2006: $F_{1,20} = 7.41$, $P = 0.013$; 2007: $F_{1,20} = 13.73$, $P = 0.0014$) and not N addition *per se* (2006: $F_{2,20} = 0.72$, $P = 0.50$; 2007: $F_{2,20} = 0.59$, $P = 0.56$). We also found a significant effect of year, such that the effects of N addition on plant visitation rate were delayed ($P < 0.042$).

Plot level

Even though plant visitation rate varied among N-treatments, at the plot level, there were no differences among N treatments for *Potentilla* or for all forbs combined in the number of flowers visited per foraging bout in any year ($F < 2.15$, $P > 0.15$).

EFFECTS OF N TREATMENTS ON FEMALE PLANT REPRODUCTION

Nitrogen addition benefited some components of female reproduction of *Ipomopsis* and *Potentilla*, with high-N addition supporting lower female fecundity than control or low-N treatments (Fig. 3, Table S4).

Flower level

For *Ipomopsis*, seed set per fruit was 43% higher in control and low-N plots compared to high-N plots (Table S4). Mass per seed was 32% and 37% greater in control and low-N plots compared with high-N plots. For *Potentilla*, there was no effect of N treatment on seeds per fruit, but mass per seed was 16% higher in control and low-N than in high-N plots (Table S4). For the other forb species (*Agoseris*, *Arabis*, *Campanula*, *Delphinium*, *Erigeron*, *Helianthella and Heliomeris*), we found no strong effects of N addition on per-flower female reproduction (Table S4).

Plant level: direct and indirect effects of N treatments

For *Ipomopsis* focal plants, there was no effect of N addition on percent fruit set, and there was a trend for total seeds per plant to be highest in low-N plots, but the trend was not statistically significant (Table S4). For *Potentilla* focal plants,

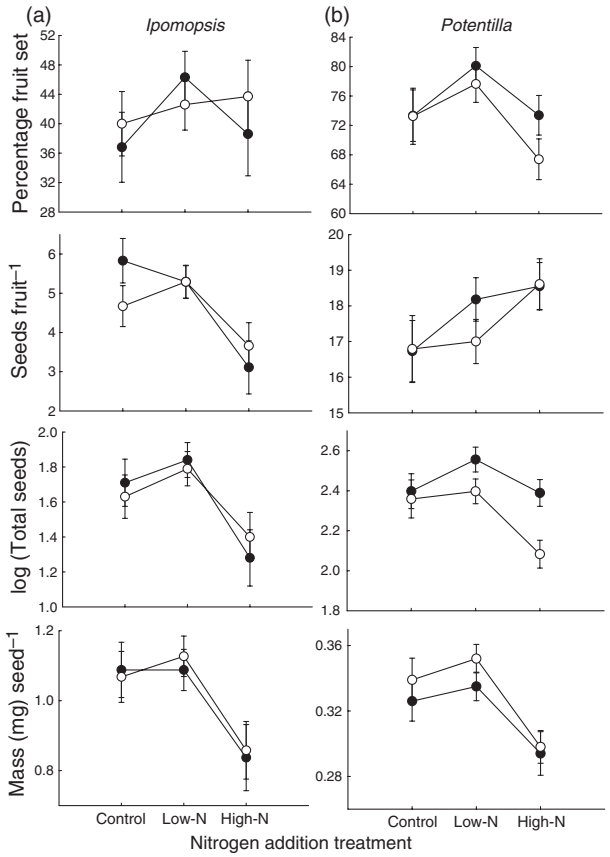

Fig. 3. The effects of nitrogen (N) enrichment on components of female reproduction in (a) *Ipomopsis aggregata* and (b) *Potentilla pulcherrima* with supplemental (filled circles) and natural (open circles) pollination. Values are means across years (2005–2007). Error bars are ±1 SE.

percent fruit set was 13% higher in low-N compared with high-N plots. Total *Potentilla* seeds per plant were 16% higher in control and low-N than in high-N plots. Most components of both *Ipomopsis* and *Potentilla* focal plant reproduction varied among years (Table S4), but there was no year × N treatment interaction (see Materials and methods), suggesting that this yearly variability did not affect the outcome of the N treatments.

Contrary to our expectations, we found no evidence that N addition indirectly influenced the degree of pollen limitation (Fig. 3). For both *Ipomopsis* and *Potentilla*, there were no significant interactions between the N and pollen treatments for any measures of female reproduction ($P > 0.12$ in all cases). In *Ipomopsis*, the supplemental-pollen treatment had no effect on any measurement of female reproduction in any year ($F_{4,225} = 0.24$, $P = 0.92$), suggesting no pollen limitation of seed set. In *Potentilla*, there was a positive 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 with open-pollinated controls in 2007 ($F_{1,112} = 8.00$, $P = 0.006$), suggesting pollen limitation of seed set in some years.

Plot level

Total *Ipomopsis* seeds per m^2 was almost three times higher in low-N plots than in control and high-N plots ($F_{2,24} = 12.68$, $P = 0.0002$); this result was driven by enhanced per-flower seed production and a threefold increase in *Ipomopsis* flower production per m^2 in low-N plots ($F_{2,24} = 17.27$, $P < 0.0001$). Total *Potentilla* seeds per m^2 was 48% higher in low-N plots compared to control and high-N plots ($F_{2,40} = 7.91$, $P = 0.0013$), driven by 55% greater *Potentilla* flower production per m^2 in low-N plots ($F_{2,40} = 18.11$, $P < 0.0001$). For the other forb species, we found no strong per-plot effects of N addition on female reproduction (Table S4). For grasses, we found that N addition increased grass seed biomass per m^2 (rm-ANOVA; $F_{2,20} = 8.83$, $P = 0.0018$), with up to 14 and 4 times greater grass seed mass in high-N than control or low-N plots respectively (2007: $F_{2,21} = 7.1$, $P = 0.0044$; 2008: $F_{2,20} = 5.88$, $P = 0.0098$).

EFFECTS OF N TREATMENTS ON MALE PLANT REPRODUCTION

Flower level

Nitrogen addition did not strongly affect per-flower pollen production of any of the species measured in any year ($F < 1.74$, $P > 0.25$ in all cases).

Plant level

Per-plant pollen production was not affected by N addition in *Ipomopsis* (rm-ANOVA; $F_{2,6} = 0.16$, $P = 0.86$). However, there was a marginal effect of N addition on per-plant *Potentilla* pollen production (rm-ANOVA; $F_{2,18} = 2.76$, $P = 0.090$) with up to 56% and 62% greater pollen production per plant in control and low-N plots respectively, compared with high-N plots. This trend was due to the increase in per-plant *Potentilla* flower production in low-N plots.

Plot level

Per-plot pollen production was not affected by N addition in any species ($F < 1.75$, $P > 0.20$ in all cases, but see comparison of effect sizes below).

Comparison of the relative responses of ANPP vs. reproduction

Measures of ANPP and female and male plant reproduction responded differently in magnitude and direction to N addition in some cases (Fig. 4). 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). For example, grasses and forbs responded differently to N addition. High-N addition strongly increased ANPP and reproduction of grasses, whereas low-N addition increased total forb ANPP and female, but not male, reproduction. N

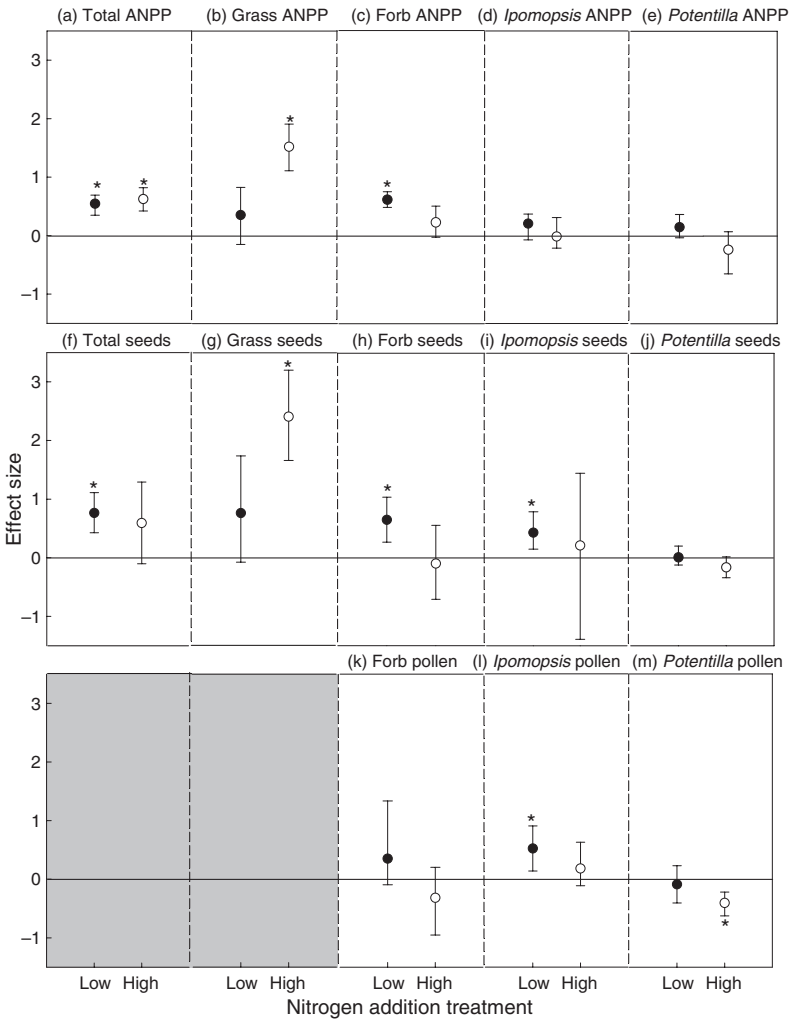

Fig. 4. Mean effect size (log response ratio comparing treatment to the control) of plant responses to low- (black circles) and high-nitrogen (N) addition (open circles) for both above-ground net primary productivity (ANPP; a–e), female reproduction (f–j) and male reproduction (k–m). ANPP was measured as biomass per m^2 for total plants, grasses and forbs, and as biomass per plant for *Ipomopsis* and *Potentilla*. Reproduction was measured as seeds or pollen per m^2 for total plants and forbs, biomass per m^2 for grasses, and total seeds or pollen per plant for *Ipomopsis* and *Potentilla*. Effect sizes are means over the first 3 years of N treatment (2005–2007), except for total seeds, grass seeds and forb seeds (measured in 2007), and forb and *Potentilla* pollen (measured in 2005 and 2007). No estimates of grass pollen per m^2 were measured, so this panel, along with total pollen per m^2, are greyed out. Error bars are bias-corrected 95% confidence intervals. Asterisks (*) denote significant effect sizes at $\alpha = 0.05$.

treatments did not affect all forb species similarly and differentially affected their productivity vs. reproduction (Fig. 4). *Ipomopsis* exhibited increased female and male reproduction, but not biomass, in the low-N treatment. High-N addition had especially negative effects on *Potentilla*, with significantly decreased male reproduction relative to controls and a tendency for decreased biomass and female reproduction. Compared with the repeated-measures analyses reported above, the effect sizes of N addition on male reproduction of *Ipomopsis* and *Potentilla* appear to be more sensitive to differences among treatments, given the large variation in pollen production among individuals over time. At the plot level, both N treatments had moderate, positive effects on total ANPP and total reproductive success. Taken together, these results suggest that there are some species-specific effects of N addition but that plant functional groups with similar life-history characters explained a lot of the variation in response to N addition.

Discussion

The availability of nutrients can limit primary productivity and affect community composition and typical consumer–resource interactions in many systems (Tilman 1987; Siemann 1998; Elser *et al.* 2007). However, our understanding of how nutrient limitation affects mutualistic interactions has not been thoroughly explored at the community level. Here, we built upon previous community studies by adding N to field plots and measuring biomass effects over multiple years, and in addition, we considered how N affected functional traits, species interactions and subsequent indirect effects on plant reproduction. We found that high levels of N addition favoured grasses via increased ANPP and reproductive success. Low levels of N addition positively affected forb ANPP and floral traits important to pollinator attraction, such as flower production, flower size and nectar production. Subsequently, pollinator visitation rate to plants was increased in low-N addition plots. However, from the plants' perspective, there were no effects of N addition on per-flower visitation, and pollen-supplementation of two forbs showed that most components of plant reproduction were not pollen limited. Thus, N addition did not have indirect effects on forb reproduction via changes in pollination. Positive effects of low-N addition on female, and occasionally male, reproduction per plant and per plot were direct, often through the production of additional flowers. This work emphasizes the importance of considering

productivity, reproduction and the mechanisms by which they are affected to more fully understand the bottom-up effects of resource addition to plant communities. Despite large changes in floral traits that affected pollinator behaviour at the plant level, the direct effects of N addition on plant reproduction were stronger than emerging community-level 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. One other study has tested for N limitation in subalpine meadows of the western slope of Colorado. Cross & Harte (2007) found no effect of a 3-year addition of 6 g N m^{-2} $year^{-1}$, a level intermediate between our low- and high-N treatments. Our result is in agreement with a host of other studies documenting N as a major limiting nutrient in terrestrial systems (Elser *et al.* 2007), including subalpine meadows (Brancaleoni *et al.* 2007). The effects we observed 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 (Monaco *et al.* 2003). In high-N plots, increased ANPP was driven by grasses, whereas the 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 (Shaver & Chapin 1986). 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). Additionally, the floral density and bloom duration of *Lathyrus*, an N-fixer, declined in N-addition plots relative to controls (Table S3; L.A. Burkle, unpubl. data), likely because legumes loose their competitive advantage over other species when N is no longer limiting (Suding *et al.* 2005). Although we did not observe any loss of species richness in the N-addition plots, the large changes in biomass of plant functional groups with N addition resulted in decreased evenness. 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.

Positive, community-level effects of N addition on floral traits, such as flower production, were important in influencing pollinator visitation to plants and provided a *potential* pathway for N to affect plant reproductive success. However, we found no evidence of pollen limitation of seed production, nor did we observe differences in per-flower pollinator foraging behaviour among N treatments, both results contributing to the lack of indirect effects of N addition on plant reproduction. 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 (Dreisig 1995; Robertson & Macnair 1995; Ishihama & 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 behaviour, direct manipulations of flower abundance of different species are needed. It is likely that *Potentilla* flower production alone contributes strongly to pollinator attraction at the scale of a meadow given the dominance of *Potentilla* in this flowering assemblage. Indeed, only *Potentilla* flower number per plot, and not total flowers per plot, contributed to the enhanced plant visitation rate that we observed in this species. In addition, we focused the majority of our measures on common or dominant plants, but to make more universal conclusions across species, quantifying the effects of N addition on rare plants may provide additional insights (Feinsinger 1987). In this study, emergent properties of N addition on pollinator behaviour were evident, but they were not important for plant reproduction due to the lack of per-flower visitation effects and the lack of pollen limitation.

At the flower and plant levels, N addition affected female reproduction of *Ipomopsis* and *Potentilla*. For each species, at least two of four components of female reproduction measured, including seeds per fruit, mass per seed, percent fruit set and total seeds per plant, were influenced by N addition, with low-N addition generally increasing female reproductive success and high-N addition decreasing success relative to controls. These results generally match patterns found with ANPP. The low success of forbs in the high-N treatments may have been due to the strong competitive ability of grasses in high-N environments. 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. The lack of effects of pollen supplementation on seed set in *Ipomopsis* was especially surprising given that seed set has been shown to be pollen-limited in some years (e.g. Hainsworth, Wolf & Mercier 1985; Campbell & Halama 1993; Irwin 2006). Moreover, contrary to our expectation, we 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 of N on reproduction associated with changes in pollination. Herbivory and seed predation did not vary among N treatments, suggesting that they did not confound direct or indirect effects of N addition on plant reproduction (unpubl. data). It was surprising that we did not find any conditionality of plant–pollinator mutualisms depending on soil N availability because the outcomes of mutualistic interactions can be context-dependent, hinging in part on the availability of resources in the environment (Bronstein 1994). 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 (Mattila & Kuitunen 2000; Asikainen & Mutikainen 2005), we found that, for 3 years, N limitation was more important for reproduction than pollination, a pattern that may be a common trend (Ne'eman, Ne'eman & Ellison 2006).

At the plot level, N addition greatly affected female forb reproduction directly through changes in flower production. Although the number of seeds produced per fruit was not affected by N enrichment in most forb species, increased flower production by many forbs in low-N plots resulted in enhanced female reproduction at the community level. Whether N-induced increases in community-level flower production occurred through enhanced per-plant flower production or more flowering stalks per plot were species-specific. For example, enhanced per-plot flower production in *Ipomopsis* was due to increased flowering stalks in low-N addition plots, suggesting that low-N addition may increase the survival and transition rates of early life stages, like the bolting of rosettes (Brewer 1995). For *Potentilla*, however, low-N addition increased per-plot flower production through enhanced per-plant flowering, influencing adult fecundity. Further study is needed to link the effects of N addition on individual forbs with plant population and community dynamics, mediated through changes in population growth rates, differential effects on certain life stages and species interactions (Brys *et al*. 2005; Dalgleish *et al*. 2008).

Comparing ANPP and reproduction at different scales (plot level, functional groups and individual forb species) was useful in understanding the variable effect sizes of different levels of N addition. For functional groups (i.e. grasses and forbs), biomass and reproduction generally responded in the same direction to N addition, although not necessarily with the same magnitude. However, for individual forb species, biomass, female reproduction and male reproduction often did not respond similarly in magnitude and direction to N addition. For perennials, such discrepancies between biomass and reproductive responses to environmental resources are likely due to species-specific resource allocation patterns, including resource storage, acquisition of threshold biomass before sexual reproduction, costs of reproduction and flexibility in allocation to biomass vs. reproduction (Reekie & Bazzaz 2005; Jongejans, de Kroon & Berendse 2006). Thus, biomass measures and their response to resource manipulations may not be indicative of reproductive responses and future population parameters. This 4-year study did not always show a positive relationship between biomass and reproduction in perennials, contributing support for this finding across plant species with different life histories (for annuals, see Neytcheva & Aarssen 2008). Furthermore, the effects of N addition on estimates of reproductive success in *Potentilla* illustrate that male and female measures may not respond similarly, suggesting that a complete understanding of reproduction can only be achieved when both components are measured (Strauss, Conner & Rush 1996; Agrawal, Strauss & Stout 1999).

Three 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 we were able to measure seed set for most species. We 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 & 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 reproduction will allow stronger conclusions to be drawn about the effects of N across forb species (Zimmerman & Pyke 1988). Secondly, we were only able to examine estimates of male reproductive success and not the realized number of seeds sired; the latter response variable would more fully document the effects of N addition on male reproduction (Ashman 1998). Lastly, the spatial scale of our 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 N 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 there would be little opportunity for pollinator choice to play a role in plant reproduction at this larger scale.

In summary, 4 years of N enrichment in a perennial plant system affected productivity, floral traits, pollinator behaviour and some components of female and male plant reproduction. Biomass, however, was not always positively related to reproduction of individual forb species and may not necessarily be linked to future population sizes or population dynamics. In addition, consideration of plant functional group was important; grasses did not respond similar to forbs, likely due to the competitive dominance of grasses over forbs in high-N environments. Surprisingly, the direct effects of N on reproduction were stronger than the indirect effects associated with pollination at the community level. Thus, pollinators did not drive differences in plant reproduction as is often predicted, and bottom-up effects of N availability were more important to plant reproduction. This work is novel in its consideration of the effects of resource manipulations on productivity, plant reproduction and the role of pollination at the community level. The generality of these results in other systems remains to be tested.

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Supporting Information

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

Table S1. Forb species occurring in the study plots and their floral morphology, primary pollinators and root morphology

Table S2. ANOVA table for ANPP and biomass responses of plant functional groups to nitrogen addition

Table S3. ANOVA table for flower production responses to nitrogen addition

Table S4. ANOVA table for reproductive responses of forbs and grass to nitrogen addition

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