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The effects of nutrient addition on floral characters and pollination in two subalpine plants, *Ipomopsis aggregata* and *Linum lewisii*

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Abstract 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. We performed resource (water and nutrients) and pollination (open and supplemental) treatments on two species of flowering plants, *Ipomopsis aggregata* and *Linum lewisii*, that differed in life-history, and we measured how resource addition affected floral characters, pollination, and reproduction (both male and female function). We separated the direct effects of resources versus indirect effects on female function via changes in pollination using a factorial experiment and path analysis. Resource addition affected *I. aggregata* and *L. lewisii* differently. *Ipomopsis aggregata*, a monocarp, responded to fertilization in the year of treatment application, 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 effect on growth or reproductive output in the year of treatment on *L. lewisii*, a perennial, except that fertilization lengthened 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, there were no effects of resource addition on male function, and the direct effects of fertilization on female function were relatively stronger than the indirect effects via changes in pollination. Although we studied only two plant species, our results suggest that life-history traits may play an important role in determining the reproductive responses of plants to soil nutrient and pollen additions.

Keywords Floral traits · Life-history · Nutrient limitation · Plant reproduction · Pollen limitation · Water addition

Introduction

Flowering plants vary in their reproductive success, and in many systems, the availability of soil and pollination resources are often the main contributors to this variation (e.g., Haig and Westoby 1988; Dimling 1992; Campbell and Halama 1993; Mattila and Kuitunen 2000; Asikainen and Mutikainen 2005;

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Pina et al. 2007). Yet, we lack a synthetic understanding of how pollination mutualisms are affected by abiotic resources and of the relative strength of the direct effects of abiotic resources on plant reproduction compared to the indirect effects mediated through changes in floral characters and pollination. Plant resource allocation and life-history theories generate testable predictions about how plants should respond to nutrient addition (e.g., Feeny 1976; McKey 1979). For example, in fast-growing annuals or monocarpic perennials, nutrient addition may invoke strong, short-term 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 quality (from the perspective of herbivores) may increase due to elevated nutrient concentrations relative to carbon content (Bryant et al. 1987), or plants may store nutrients for future use (e.g., Bollmark et al. 1999; Novotny et al. 2007), resulting in weak short-term or delayed effects of nutrient addition on productivity (e.g., Chiarucci et al. 1999; Paschke et al. 2000; Monaco et al. 2003).

Plant allocation and life-history theories and their predictions, however, do not explicitly take pollination into account, and thus, we have a less complete understanding of how nutrient availability affects the reproduction of flowering plants via changes in floral characters important for pollination interactions (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 toward reproduction but not necessarily toward 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, but only if pollinators can cue in on changes in floral traits and plants are pollinator-limited for seed set. Although some studies suggest that nutrient addition may have positive effects on floral rewards and pollination, nutrient addition may alternatively disrupt the stoichiometric nutrient balances (e.g., Sterner and Elser 2002) important for flowering plants and floral traits and negatively affect plant reproduction. By integrating the direct and indirect effects of nutrients on female and male plant function and how these effects might be influenced by plant life-history, this study provides insight into how abiotic resources may affect plant reproduction through changes in species interactions (e.g., Galen 2005).

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 underlying basis of this variation, an approach that limits understanding the links between environmental variation, plant growth, and fitness. Knowledge of these links is a first step toward understanding the importance of nutrient availability on the population dynamics of plants and pollinators, natural selection on floral traits, and the 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 plant life-history (semelparous versus iteroparous) and to investigate the relative importance of the direct effects of soil resources on plant reproduction versus the indirect effects via changes in pollination. Based on differences in their life-history characters, and given that many floral characters can respond to environmental conditions, we predicted that both species would respond to nutrient addition in terms of enhanced growth, floral traits, and reproduction but that the effects would be more immediate and stronger in the semelparous *I. aggregata* compared to the iteroparous *L. lewisii*.

Methods

Study system

Fieldwork was conducted at the Rocky Mountain Biological Laboratory (RMBL), Gothic, Gunnison County, Colorado, USA (latitude: 38°57′29″ N, longitude: 106°59′06″ W, and 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 g NH_4^+ $m^{-2}year^{-1}$, 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 baseline for experimentally investigating the effects of changes in resource availability on pollination and plant reproduction.

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 ± 1 SD = 85 ± 66 flowers produced), and dying (Campbell 1989). 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 and Halama 1993; Juenger and Bergelson 1997; Irwin 2006). In a single-year study, nutrient fertilization of *Ipomopsis* (using a 20:20:20 NPK fertilizer) had 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 degree to which the effects of pollen and nutrient supplementation to *I. aggregata* are consistent across years is unknown.

Linum lewisii Pursh is a perennial, pollinated by a variety of small bees and flies (Kearns 1992). The hermaphroditic 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 for seed set, 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 and August, 2004, 72 individuals of each species with flower buds were haphazardly chosen and randomly 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 us to investigate the individual and combined effects of resources and pollination on plant reproduction and the direct and indirect (pollinator-mediated) effects of resource additions. Plants assigned to treatments did not vary significantly in initial plant size based on the number, height, and diameter of stalks ($P > 0.49$ for both species). Drip emitters attached with tubing to plastic cups were used to administer water (400 ml) to the base of individual plants 4 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 gK per plant per season). Plants in the hand-pollination treatment received supplemental pollen to all pistillate-phase flowers 3 days per week for *I. aggregata* and 6 days per week for *L. lewisii* throughout the blooming period, so that most flowers on each plant in 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 natural pollination.

Throughout summer, we measured three classes of floral characters: flower production, flower size, and nectar. For flower production, we counted the total number of flowers produced per plant during the season and measured bloom duration as the number of days that each plant was in bloom. For flower size, we 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, we measured nectar production on bagged, newly opened flowers over 48 h and nectar sugar concentration (using a hand-held refractometer) in sucrose equivalents. We were 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). For example, for both *I. aggregata* and *L. lewisii* in this study, the mean variation (standard deviation) in flower size among plants was over twice as great as the variation within plants. We 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 and Halama 1993; Sperens 1997; Munoz et al. 2005).

To mechanistically investigate the response of pollination to resource treatments, we estimated pollinator visitation using stigma pollen loads. Because neither *I. aggregata* nor *L. lewisii* autogamously self-pollinate due to dichogamy and/or herkogamy, all pollen on stigmas is a result of pollinator activity; therefore, pollen receipt can be used as a proxy for pollinator visitation, as has been shown in *I. aggregata* (Engel and Irwin 2003; Price et al. 2005; Irwin 2006). To estimate pollen receipt, we collected up to three stigmas from each plant once per week throughout 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). We stained stigmas in basic fuchsin dye (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).

As 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 on plant reproduction, we also recorded herbivory and seed predation. Mule deer, *Odocoileus hemionus*, is the primary herbivore of *I. aggregata*, consuming portions of individuals during the reproductive phase of these plants (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. We censused aphid herbivory once per week as the proportion of stalks of each plant present with aphids. We measured predispersal seed predation as the percent of fruits destroyed by fly seed predators (*Hylemya* sp., Diptera: Anthomyiidae) of *I. aggregata* and unknown lepidopteran larvae of *L. lewisii*.

We estimated plant reproductive success through male and female components. We estimated male function using pollen production per flower and pollen quality (percent N). Pollen production is positively correlated with seeds sired in some, but not all, species (reviewed in Stanton et al. 1992). We recognize the limitation of using pollen production as an estimate of male function (Ashman 1998) and interpret our results accordingly. For 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, 1994; Poulton et al. 2001), suggesting that the concentration of pollen N and P may indicate pollen quality in some systems. Here, we focused on measuring the concentration of pollen N only because pollen is composed primarily of N (Roulston et al. 2000), N has widespread effects on many plant functions and traits, including biomass, flower production, and pollen performance (e.g., Tilman and Wedin 1991; Munoz et al. 2005; Lau and Stephenson 1993), and trade-offs in resource allocation might be observed. No study to our knowledge has tested for a relationship between pollen N concentration and pollen performance, although soil N addition can increase pollen competitive ability, presumably through an increase in pollen N concentration (Lau and Stephenson 1993). 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 hemocytometer 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 function, we collected all of the fruits from each plant at the end of the summer, and we counted all of the seeds. We also weighed the seeds of each plant, as seed mass can be an estimate of quality, such as potential germination, growth, and flowering, in some species (Stanton 1984). We calculated female function per plant as percentage fruit set (number of successful fruits divided by total flowers × 100%), mean seeds per fruit (total seeds divided by successful fruits), total seeds per plant, and mean mass per seed.

At the end of the season, we collected and dried plants and measured dry biomass to assess if plant growth was affected by resource addition. Because *I. aggregata* typically die after flowering, we measured both the shoot and root biomass of each plant in 2004. As *L. lewisii* are perennial, we measured only their aboveground dry biomass (all aboveground stalks collected) in 2004. We measured seed set and plant biomass of the surviving *L. lewisii* for three subsequent years (2005–2007) to determine if resource addition had delayed effects, as the storage of resources for future growth and reproduction may be common in perennials (Munoz et al. 2005). We did not measure floral traits or stigma pollen receipt in 2005–2007 for *L. lewisii*.

Statistical analyses

All statistical analyses were performed in SAS 8.2 unless otherwise noted. Multiple comparisons (Tukey HSD) 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. For all tests, data met the necessary model assumptions.

Effects of resource treatments on floral and plant characters

For *I. aggregata*, we assessed 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 belowground) using a MANOVA (PROC GLM). We 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). A significant MANOVA was followed by univariate ANOVAs for each response variable (Scheiner 1993).

Pollination and herbivore responses to resource and pollen treatments

We analyzed the effects of resource treatments on mean stigma pollen receipt per flower per plant of *I. aggregata* (conspecific only) using ANOVA and *L. lewisii* (conspecific and heterospecific) using MANOVA. We did not analyze the quantity of heterospecific pollen deposited on *I. aggregata* stigmas because it was low across all treatments (<6% of total pollen received). *Ipomopsis aggregata* susceptibility to mule deer (presence or absence of herbivory) and *L. lewisii* susceptibility to aphids (proportion of stalks infested) in different resource and pollen treatments were assessed using a chi-square test (JMP 4.0) and two-way ANOVA, respectively. The effect of nutrient and pollen treatments on *I. aggregata* and *L. lewisii* susceptibility to seed predators (percent of fruits destroyed) were evaluated using two-way ANOVAs.

Effects of resource treatments on male function

For both *I. aggregata* and *L. lewisii*, we analyzed the effects of resource treatments on mean pollen production per flower per plant and pollen quality (percent N) using MANOVAs.

Effects of resource and pollen treatments on female function

We analyzed the effects of resource and pollen treatments on female function per plant (percent fruit set, mean seeds per fruit, total seeds per plant, and mean mass per seed) in *I. aggregata* and *L. lewisii* using MANOVAs 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 function. A significant interaction would indicate that resource additions affected the level of pollen limitation, suggesting indirect effects of resources on reproduction via changes in pollination.

Delayed effects of resource treatments on L. lewisii

The effects of resource treatments on *L. lewisii* female function (percent fruit set, mean seeds per fruit, total seeds per plant, and mean weight per seed) in 2005 and 2006 were assessed using a MANOVA for each year. There were no effects of pollen treatment in the previous year on female function in any subsequent year ($P > 0.65$ in all cases), so we report only effects of resource treatments on subsequent female function and biomass. 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. We could not use MANOVAs to investigate the overall effect of resource treatments on female function and biomass because individual plants often had biomass values but did not reproduce (which would bias our results toward 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 (in all years, $\chi^2 > 0.49$). Furthermore, correlations of aboveground vegetative biomass and of 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 or growth. Although female function of *L. lewisii* was also measured in 2007, only 3 out of the 29 remaining individuals produced fruit in that year and were not included in the analyses.

Path analysis: direct versus indirect effects of fertilization on I. aggregata

To further tease apart and understand the relative strength of the direct effects of fertilization on reproduction in *I. aggregata* via resource allocation to flower and seed production versus the indirect effects via changes in floral characters and pollination, we performed a path analysis. We did not perform a parallel analysis for *L. lewisii* because of the lack of effects of resource and pollen treatments in the main year of study. We summarized the effects of fertilization on floral traits (nectar and flower production), pollination (estimated using stigma pollen receipt), and female function (seed production) of *I. aggregata* using one mechanistic path model. Only open-pollinated plants were included in the path analysis. We hypothesized that fertilization affects nectar and flower production (Campbell and Halama 1993), that nectar and flower production affect pollen receipt because these are traits important for pollinator attraction (e.g., Mitchell 1994; Galen 1999), and that pollen receipt and flower production affect seeds per fruit because *I. aggregata* are often pollen limited (e.g., Campbell and Halama 1993) and there might be a trade-off between flower and seed production if nutrients are limiting (e.g., Lawrence 1993; Greenway and Harder 2007). We also hypothesized that fertilization might directly affect seeds per fruit and that seeds per fruit together with flower production determine total seeds. We included a correlation (double arrowed path) between flower and nectar production because there could be a correlation between these response variables regardless of nutrient addition. We were most interested in exploring if this model fit the observed data and if so, the relative strength of different pathways by which nutrients affected seed production. Due to the lack of effects of the watering treatment in this study (see “Results”), the water and control treatments were combined and, along with the fertilizer treatment, coded as a categorical variable in SAS 8.2. To incorporate a categorical variable in the path analysis (Schemske and Horvitz 1988; Campbell and Halama 1993), we used the POLYCHOR macro to create a tetrachoric correlation matrix. We then used PROC CALIS on this correlation matrix to calculate the goodness-of-fit of the model, standardized path coefficients, and significance levels for each path.

Results

Effects of resource treatments on floral and plant characters

Resource addition affected floral and plant 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

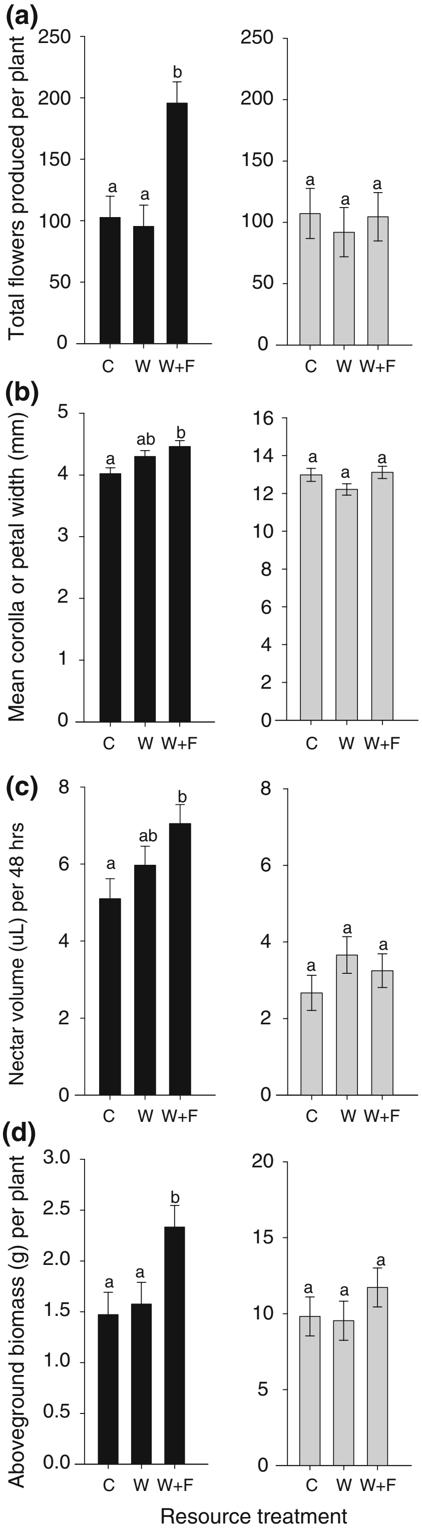

Fig. 1 Effects of resource treatments (C = control, W = water, W + F = fertilizer) on mean **a** flower production, **b** flower size, **c** nectar production, and **d** aboveground plant biomass in *Ipomopsis aggregata* (black bars) and *Linum lewisii* (gray bars) in the treatment year, 2004. Error bars are ±1 SE. Letters above bars indicate significant treatment differences within species at $P < 0.05$ ▶

the responses were species specific. For *I. aggregata*, fertilized plants produced 91% and 105% more total flowers than control and watered plants, respectively (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 (Fig. 1b, $F_{2,68} = 5.2$, $P = 0.0078$). Resource addition also affected *I. aggregata* nectar, with fertilized plants producing 38% more nectar per flower over 48 h (Fig. 1c, $F_{2,67} = 3.74$, $P = 0.03$), but of moderately lower concentration ($F_{2,67} = 2.84$, $P = 0.065$), than control plants. Both fertilized and watered *I. aggregata* bloomed on 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. 1d, $F_{2,68} = 5.74$, $P = 0.005$), 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 (Fig. 1b, MANOVA: Wilks' $\lambda = 0.88$, $F_{4,102} = 1.61$, $P = 0.18$), and nectar production (Fig. 1c, $F_{2,49} = 1.13$, $P = 0.33$) were not affected by resource treatments. Fertilized *L. lewisii* bloomed 9 days longer on an 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. 1d, $F_{2,69} = 0.86$, $P = 0.43$). However, resource addition had a delayed effect on *L. lewisii*, with fertilized plants producing 50% and 83% more aboveground biomass than control and watered plants (respectively) in the year following treatments (2005, Fig. 2a, $F_{2,56} = 3.54$, $P = 0.036$). However, this effect was temporary and was not evident 2 (2006: $F_{2,48} = 1.28$, $P = 0.29$) or 3 years (2007: $F_{2,26} = 1.38$, $P = 0.27$) after treatments.

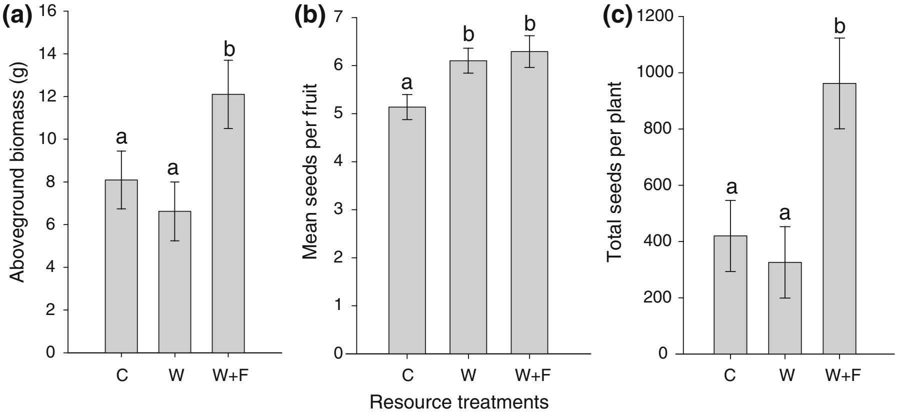

Fig. 2 Delayed effects of resource treatments (C = control ($N = 21$), W = water ($N = 21$), and W + F = fertilizer ($N = 16$)) in 2004 on **a** aboveground biomass, **b** seeds set per fruit, and **c** seeds per plant of *Linum lewisii* in 2005. Error bars are ±1 SE. Letters above bars indicate significant treatment differences at $P < 0.05$

Pollination and herbivore responses to resource and pollen treatments

Resource treatments affected pollination in *I. aggregata* and *L. lewisii* differently. In *I. aggregata*, resource addition affected stigma pollen receipt, an estimate of pollinator visitation, with fertilized plants receiving 26% more conspecific pollen grains per stigma ($F_{2,67} = 4.17$, $P = 0.02$) than control plants (means ± SE = 131.0 ± 8.75 (control), 148.0 ± 8.56 (water), and 165.6 ± 8.21 (fertilizer)). In contrast, resource treatments did not affect stigma pollen receipt in *L. lewisii* (MANOVA: Wilks' $\lambda = 0.93$; $F_{4,70} = 0.65$, $P = 0.63$). Heterospecific pollen represented 34% of the average total pollen receipt in *L. lewisii.*

We found no statistically significant effects of resource or pollination treatments on herbivory or seed predation in *I. aggregata* or *L. lewisii*. In *I. aggregata*, we found no effect of nutrient or pollen treatment on herbivory by mule deer ($\chi^2 = 3.91$, $P = 0.27$). Watered *L. lewisii* harbored 62% and 31% more aphid-infected stalks than control and fertilized plants, respectively, although this difference 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 function

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 ($F_{2,63} = 2.02$, $P = 0.14$) showed no statistically significant effects of resource addition, 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 ± SE = 8.16 ± 0.28% (control), 7.52 ± 0.25% (water), and 7.08 ± 0.22% (fertilized)).

Effects of resource and pollen treatments on estimates of female function

Resource and pollen treatments affected female function of *I. aggregata* and *L. lewisii* differently. Female function in *I. aggregata* was affected by the addition of both nutrients and pollen (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. 3c, $F_{2,64} = 5.56$, $P = 0.006$), with fertilized plants producing 94% more seeds than control plants across pollen treatments. However, there was no effect of pollen treatment on seed production ($F_{1,64} = 1.38$, $P = 0.25$). The effects of resource and pollination treatments on seeds per fruit were marginally significant (Fig. 3b, $F_{5,64} = 2.19$, $P = 0.066$), with a trend for hand-pollinated plants to have 27% more seeds per fruit than open-pollinated controls across resource treatments. There was no effect of either treatment on percent fruit set (Fig. 3a, resource treatment: $F_{2,64} = 0.56$, $P = 0.58$; pollination treatment: $F_{1,64} = 0.38$, $P = 0.54$) or on mean mass per seed (Fig. 3d, 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 effects of resource and pollen treatments or their interaction on any measures of female function in *L. lewisii* (Fig. 4, 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 function in *L. lewisii* (MANOVA: Wilks' $\lambda = 0.65$, $F_{8,98} = 2.92$, $P = 0.006$),

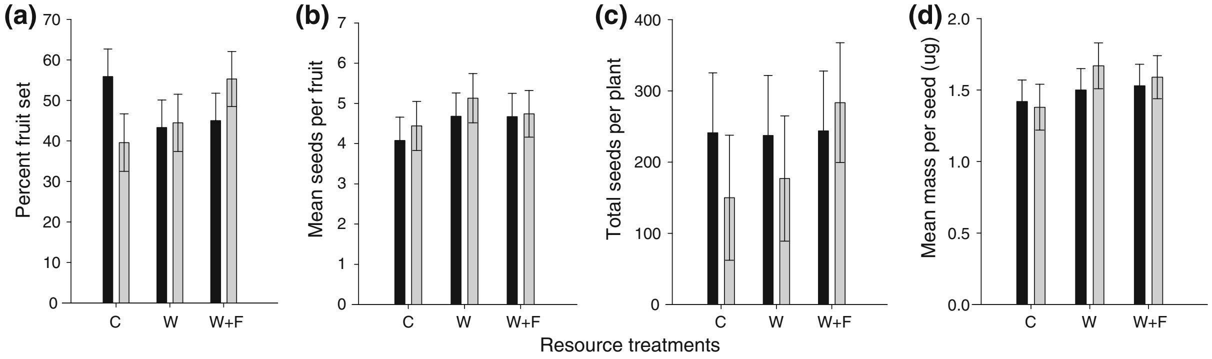

Fig. 3 Effects of nutrient (C = control, W = water, and W + F = fertilizer) and pollen treatments (natural pollination = black, supplemental pollination = gray) on **a** percent fruit set, **b** seeds per fruit, **c** seeds per plant, and **d** mass per seed of *Ipomopsis aggregata* in the treatment year, 2004. Error bars are ±1 SE

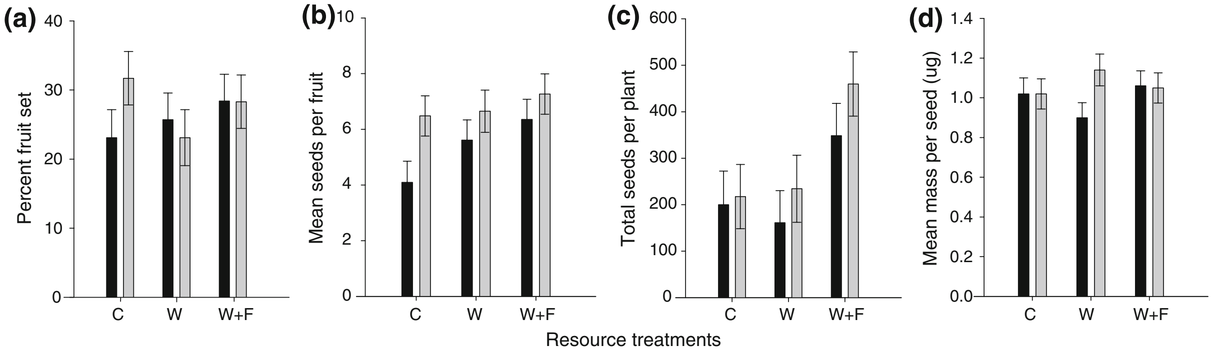

Fig. 4 Effects of resource treatments (C = control, W = water, and W + F = fertilizer) and pollen treatments (natural pollination = black, supplemental pollination = gray) on **a** percent fruit set, **b** seeds per fruit, **c** seeds per plant, and **d** mass per seed of *Linum lewisii* in the treatment year, 2004. Error bars are ±1 SE

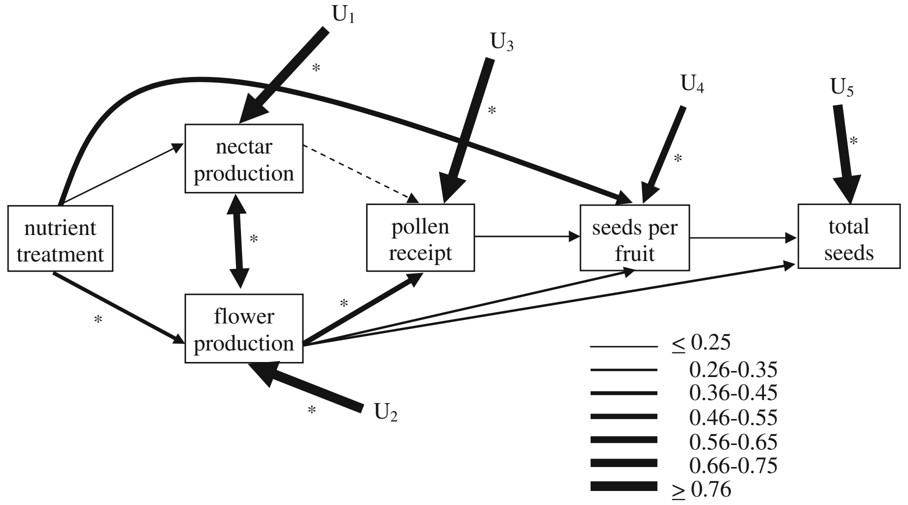

Fig. 5 Path model relating nutrient treatment (NPK treatment coded as "1" and water/control treatment coded as "0") to floral traits (nectar and flower production), pollination (pollen receipt), and female reproduction (seeds per fruit and total seeds) in *Ipomopsis aggregata*. Unidirectional arrows indicate a causal path, with solid lines representing positive effects and dashed lines negative effects. Bi-directional arrows indicate correlations. The thickness of the arrow represents the magnitude of the standardized path coefficient. Unexplained variation is represented by U's. Asterisks denote significance of these coefficients at $P < 0.05$

with fertilized plants producing 129% and 195% more seeds than control and watered plants, respectively, in 2005 (Fig. 2c, $F_{2,52} = 5.24$, $P = 0.0085$). Fertilized and watered *L. lewisii* also had 22% and 19% more seeds per fruit than controls in 2005 (Fig. 2b, $F_{2,52} = 5.08$, $P = 0.0096$). 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. These effects of resource treatments on female function were not sustained; no effects were observed in 2006, 2 years after treatment application (MANOVA: Wilks' $\lambda = 0.96$, $F_{8,62} = 0.17$, $P = 0.3$).

Path analysis: direct versus indirect effects of fertilization on *I. aggregata*

Our path model did not differ significantly from the observed data ($\chi^2 = 6.6$, $P > 0.24$), suggesting that the model provided an appropriate fit to the data. The model indicated positive effects of fertilization on flower production (Fig. 5, $t_{32} = 2.54$, $P = 0.016$). Although flower production positively affected pollen receipt ($t_{32} = 2.16$, $P = 0.038$), pollen receipt did not significantly affect seeds per fruit ($t_{32} = 0.91$, $P = 0.37$), although the relationship was positive. This result indicates that seed production in *I. aggregata* experienced only low levels of pollen limitation in 2004. There was a strong effect of fertilization on seeds per fruit ($t_{32} = 3.06$, $P = 0.0045$), but a weak effect of seeds per fruit on total seeds per plant ($t_{32} = 0.58$, $P = 0.57$), rendering the pathway from nutrients to total seeds weakly positive (effect magnitude calculated as $p_{\text{seeds per fruit, nutrients}} \times p_{\text{total seeds, seeds per fruit}} = 0.048$). Overall, the direct effect of fertilization on reproduction via allocation to increased flower production ($p_{\text{flowers, nutrients}} \times p_{\text{seeds per fruit, flowers}} \times p_{\text{total seeds, seeds per fruit}} + p_{\text{flowers, nutrients}} \times p_{\text{total seeds, flowers}} = 0.13$) was relatively stronger than indirect effects via changes in pollinator visitation ($p_{\text{nectar, nutrients}} \times p_{\text{pollen, nectar}} \times p_{\text{seeds per fruit, pollen}} \times p_{\text{total seeds, seeds per fruit}} + p_{\text{flowers, nutrients}} \times p_{\text{pollen, flowers}} \times p_{\text{seeds per fruit, pollen}} \times p_{\text{total seeds, seeds per fruit}} = 0.00$).

Discussion

To maximize reproduction in many flowering plants, both nutrients and pollination may be required (e.g., Haig and Westoby 1988; Campbell and Halama 1993). However, these resources are not independent, as belowground resources can indirectly contribute to plant reproductive success through changes in floral traits that may influence pollination (e.g., interaction-modification indirect effects; Wootton 1993; Abrams et al. 1996). Here, we used resource and pollen treatments on two flowering plant species and showed that the direct effects of nutrients, largely through increased flower production, dominated in their effects on reproduction compared to the indirect effects mediated via changes in pollination. For *I. aggregata*, plant and floral traits responded to nutrient addition. However, the timing and magnitude of these effects on plant reproductive success may have been influenced by the life-history of the plant. The monocarp, *I. aggregata*, responded to fertilization in the year of treatment, while the perennial, *L. lewisii*, showed delayed effects. Given that we investigated only two plant species in this study, further demonstrations of the importance of life-history in influencing plant response to nutrient and pollen addition in other systems are necessary to assess the generality of these findings.

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 and pollinator behavior and female and male function. In particular, corolla width in *I. aggregata* is a floral trait important to male function, 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). We must note, however, that the increase in pollen receipt that we observed in fertilized plants may represent both an increase in pollinator per-flower visitation (Engel and 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. Besides 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 mountain habitat. Further investigation of water effects is warranted, however, given the few years of examination relative to the frequency of droughts in this region (e.g., ENSO cycles; Philander 1990).

Nutrient and pollen availability affected female function in *I. aggregata*. Experimentally, we showed that one component of female function, total seeds per plant, was nutrient limited, and we used path analysis to investigate three pathways by which nutrients might affect total seeds. We determined that the direct effect of nutrient addition on female function via flower production was stronger than either the direct effect via seeds per fruit or the indirect effect via pollen receipt. The path model illustrated that nutrient addition had a significant positive effect on flower production, which then positively affected pollen receipt. Hummingbird pollinators respond to increased floral display in *I. aggregata* (Brody and Mitchell 1997; Mitchell 1994) and in other plant species (Rodriguez-Robles et al. 1992; Podolsky 1992). However, although our experimental hand pollination increased seed production per fruit, indicating some degree of pollen limitation, the path model showed no significant relationship between pollen receipt and seeds per fruit among open-pollinated plants. This suggests that the effects of nutrient addition on total seed production per plant via changes in pollinator behavior were relatively weak in comparison to the direct effects through increased flower production. A positive link between flower and seed production is common in flowering plants (e.g., Herrera 1993; Ehrlen 1997), while weak links between pollination and seed set attenuate any effects of traits on reproduction (Herrera 1993; Irwin 2006). In addition, although nutrient addition had a direct, positive effect on seeds per fruit, this pathway to total seeds was also inconsequential due to the weak link between seeds per fruit and total seeds. Our comparison of the results of this study to those obtained by Campbell and Halama (1993) revealed that nutrients may commonly limit growth and reproduction of *I. aggregata* directly, but the degree of limitation likely varies among years (Appendix 1), as does the relative importance of nutrient and pollen limitation.

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. We found effects of nutrient and pollen treatments on the monocarp *I. aggregata* in the year of nutrient treatments, but 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 function 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 and corroborate the important role of plant life-history on plant response to environmental resources (e.g., Chapin et al. 1986; Tilman and Wedin 1991), with short-term 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 or because they used the stored nutrients only in the year following treatment. 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, we did not find evidence of pollen limitation of female function in *L. lewisii* in 2004. 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* (Morgan and Heinrich 1987; Gass and Sutherland 1985). Male plant fitness may be relatively more pollinator-limited than female fitness if this diversity of pollinators results in pollen removal that is rarely deposited on conspecifics (Aizen and Harder 2007). Indeed, we observed high deposition of heterospecific pollen on *L. lewisii* stigmas, but no evidence of pollen limitation of female function.

In this study, we assessed plant reproduction through both male and female components. We estimated male function on a per-flower basis. Although we found no effect of nutrient addition on pollen production per flower and a decrease in pollen N concentration (in *L. lewisii*), possibly representing an allocation trade-off between male and female functions, 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 an average than controls, assuming that per-flower estimates of pollen production can be scaled up to the plant level. For enhanced pollen production to translate to increased male function, pollen production must also be positively correlated with pollen removal, deposition on stigmas of conspecifics, and seed siring (Stanton et al. 1992). To determine the potential male fitness benefits of increased flower production and whole plant pollen production, it would be necessary to measure realized seeds sired.

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 for 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). 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, including the potential importance of 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.

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Appendix 1. Comparison of effect sizes for *Ipomopsis aggregata* between 1990 and 2004

In 1990, Campbell and Halama (1993) manipulated soil resources and pollination to *I. aggregata* at the RMBL using the same nutrient addition amounts and schedules and measured similar response variables as were measured in this study. Thus, we were provided with an opportunity to test whether the effects of resources and pollen were consistent across years at the exact same study site at the RMBL. We calculated and compared effect sizes of *I. aggregata* in the two studies using the standardized mean difference, d (Hedges et al. 1999), the difference between the means of two treatments divided by their pooled standard deviation. A positive effect size indicated positive effects of the treatments and vice versa. An effect size of 0.2 was considered small, 0.5 medium, and greater than 0.8 large (Cohen 1969). Because the water treatment had minimal effects on floral characters and female plant reproduction in Campbell and Halama (1993) and in this study (see "Results"), we limited our comparison to fertilized and control treatments. For corolla width and nectar production, we compared the fertilized and control treatments of each of the two studies. For flower production, seeds per fruit, and total seeds per plant, we compared all factorial combinations of nutrient and pollination treatments.

Overall, we found similar directional responses to nutrient and pollination treatments between the 1990 (Campbell and Halama 1993) and 2004 (here) studies, although some effect sizes varied in magnitude (Table A). Large effect sizes ($d > 0.8$) of nutrient and/or pollen treatments were calculated for most comparisons. Fertilization in this study resulted in larger effect sizes on some floral traits than in Campbell and Halama (1993). In particular, the effect size for corolla width was 5 times larger and for nectar production 4.4 times larger in 2004 than 1990. There were only two comparisons, between total seeds of fertilized open- versus hand-pollinated plants and seeds per fruit of open-pollinated fertilized versus control plants, in which we saw an effect in this study where none was observed in 1990. In addition, there

Table A Effect sizes of nutrient and pollination treatments in 1990 (Campbell and Halama 1993) and 2004 (this study)

Character	Comparison	1990		2004	
		Effect size	% change	Effect size	% change
Nectar production	Fertilized–control	0.73	30 ↑	3.94	38 ↑
Corolla width	Fertilized–control	0.78	5 ↑	4.68	11 ↑
Total flowers	Fertilized(open)–control(open)	9.89	134 ↑	3.19	86 ↑
	Fertilized(hand)–control(hand)	3.91	44 ↑	4.99	94 ↑
	Control(hand)–control(open)	0.49	4 ↑	1.57	42 ↑
	Fertilized(hand)–fertilized(open)	5.83	35 ↓	3.36	49 ↑
Total seeds	Fertilized(open)–control(open)	8.78	134 ↑	2.20	74 ↑
	Fertilized(hand)–control(hand)	3.28	42 ↑	3.66	111 ↑
	Control(hand)–control(open)	3.42	66 ↑	0.26	9 ↑
	Fertilized(hand)–fertilized(open)	0.09	No change	1.68	32 ↑
Seeds per fruit	Fertilized(open)–control(open)	0.25	No change	3.19	55 ↑
	Fertilized(hand)–control(hand)	5.27	23 ↑	1.14	12 ↑
	Control(hand)–control(open)	8.01	44 ↑	3.37	58 ↑
	Fertilized(hand)–fertilized(open)	12.11	75 ↑	1.32	14 ↑

Comparisons are between nutrient (fertilized versus control) and pollen (open- versus hand-pollination) treatments. Pollen treatments are in parentheses was only one comparison, between total flowers of fertilized open- versus hand-pollinated plants, in which the direction of effect changed between studies (negative in 1990 to positive in 2004). These two studies indicate that nutrients and pollen are limiting resources for *I. aggregata* in two different years, but the magnitude of limitation was generally greater in 2004 than in 1990.

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