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Direct and indirect effects of pollinators and seed predators to selection on plant and floral traits

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Although flowering traits are often assumed to be under strong selection by pollinators, significant variation in such traits remains the norm for most plant species. Thus, it is likely that the interactions among plants, mutualists, and other selective agents, such as antagonists, ultimately shape the evolution of floral and flowering traits. We examined the importance of pollination vs pre-dispersal seed predation to selection on plant and floral characters via female plant-reproductive success in *Castilleja linariaefolia* (Scrophulariaceae). *C. linariaefolia* is pollinated by hummingbirds and experiences high levels of pre-dispersal seed predation by plume moth and fly larvae in the Rocky Mountains of Colorado, USA, where this work was conducted. We first examined whether female reproduction in *C. linariaefolia* was limited by pollination. Supplemental pollination only marginally increased components of female reproduction, likely because seed predation masked, in part, the beneficial effects of pollen addition. In unmanipulated populations, we measured calyx length, flower production, and plant height and used path analysis combined with structural equation modeling to quantify their importance to relative seed set through pathways involving pollination vs seed predation. We found that the strength of selection on calyx length, flower production, and plant height was greater for seed predation pathways than for pollination pathways, and one character, calyx length, experienced opposing selection via pollination vs seed predation. These results suggest that the remarkable intraspecific variation in plant and floral characters exhibited by some flowering plants is likely the result of selection driven, at least in part, by pollinators in concert with antagonists, such as pre-dispersal seed predators. This work highlights the subtle but complex interactions that shape floral and vegetative design in natural ecosystems.

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Plants exhibit remarkable variation in plant and floral traits. Although this variation may be driven, in part, by a diverse pollinator assemblage (Schemske and Horvitz 1989, Herrera 1995, Waser et al. 1996, Thompson 2001), pollinator-mediated selection often does not fully explain variation in floral characters within and among populations of the same plant species (reviewed by Galen 1999a). In natural populations, most flowering plants experience simultaneous interactions with both mutualistic pollinators as well as antagonisitic visitors, such as herbivores, nectar robbers, and seed predators (Inouye 1983, Proctor et al. 1996, Karban and Baldwin 1997). Plants may face trade-offs in attracting pollinators while also attracting antagonists, and such trade-offs may moderate direct selection on floral traits exerted by pollinators (Brody 1992,

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Lohman et al. 1996, Ehrlén 1997, Galen 1999b, Galen and Cuba 2001). The net effect of such interactions will likely shape the evolution of plant and floral design in natural systems. We examined the relative importance of pollinators vs pre-dispersal seed predators to female components of plant-reproductive success and to selection on plant and floral traits.

Trade-offs in attracting pollinators while at the same time incurring visitation by antagonists may be particularly critical for plants experiencing pre-dispersal seed predation (Hainsworth et al. 1984, Molau et al. 1989, Campbell 1991, Brody 1992, Brody and Mitchell 1997, Kudoh and Whigham 1998, Campbell et al. 2002, Ehrlén et al. 2002). Pre-dispersal seed predation is common among flowering plants (reviewed by Crawley 1992) and can have strong effects on plant fitness (Louda and Potvin 1995). Plants may lose as much as 80–90% of their seed crop due to pre-dispersal seed predators (Randall 1986, Crawley and Gillman 1989). Pre-dispersal seed predators rely on flowers to set fruit to provision their developing larvae with food resources. Although in some well-known instances pre-dispersal seed predators also act as pollinators of their host plants (yucca moths: Aker and Udovic 1981, Addicott 1986, Addicott and Tyre 1995; fig wasps: Wiebes 1979, Bronstein et al. 1990) or lay eggs after fruit or seed development (Desouhant 1998), in other instances, pre-dispersal seed predators oviposit on flowers prior to pollination (Zimmerman 1980, Pettersson 1992). In these instances, pre-dispersal seed predators selecting plants that are highly attractive to pollinators may serve to maximize the likelihood that larvae have adequate food resources. Seed predators might use the same or correlated cues as those used by pollinators to select oviposition sites. Whatever the mechanism of seed-predator selection of oviposition sites, the result for plants will be conflicting selection pressures imposed by pollinators vs seed-consuming pre-dispersal seed predators.

The aim of this study was to examine the importance of pollination vs seed predation for female plant-reproductive success in *Castilleja linariaefolia* (Scrophulariaceae) in the Rocky Mountains of Colorado, USA. In addition, we examined the strength of selection exerted through pollination and seed predation on floral and whole-plant characters. To better understand the relative role of pollination vs seed predation to female plant reproduction, we experimentally manipulated pollination, measured the loss of fruits and seeds to seed predators, and quantified subsequent plant reproduction. We then used path analysis combined with structural equation modeling to test the importance of three floral and plant characters (calyx length, flower production, and plant height) to relative seed set through pathways involving pollination and seed predation.

Methods

Study system

We studied distinct populations of *Castilleja linariaefolia* Bentham (Scrophulariaceae) during the summers of 2000 and 2001 in meadows near the Rocky Mountain Biological Laboratory (RMBL; lat. 38°45′ N, long. 106°59′ W, alt. 2900 m), Gothic, Gunnison County, CO, USA. *Castilleja linariaefolia* is a long-lived perennial, common throughout the western United States and Canada. *Castilleja linariaefolia* is a facultative root hemiparasite. It is photosynthetic but also acquires water, nutrients, and secondary compounds from a variety of hosts via root haustoria connections to the host vascular system (reviewed by Adler 2000, Adler et al. 2001). We selected focal *C. linariaefolia* at random for our study; therefore, it is unlikely that our results are biased by any one host of *C. linariaefolia*.

Around the RMBL, *C. linariaefolia* blooms from mid-June through August (Caruso 1999), producing numerous flowering stalks with obscure flowers surrounded by bright red calyces. In *C. linariaefolia*, the bright red calyces are more conspicuous than the flowers and bracts (Cronquist et al. 1984) and likely are important in pollinator and seed predator attraction. Individual plants produce a mean ± 1 SE of 16.7 ± 2.2 flowers and a range of 2 to 105 flowers in a single flowering season (R. E. Irwin and A. K. Brody, unpubl.). *Castilleja linariaefolia* is self-incompatible (Carpenter 1983) and relies primarily on broad-tailed (*Selasphorus platycercus*) and rufous (*S. rufus*) hummingbirds for pollination (Caruso 1999). In the Sierra Nevada, female-reproductive success of *C. linariaefolia* is not limited by pollinator visitation (Carpenter 1988). But it is unknown whether populations of *C. linariaefolia* experience pollen limitation in other parts of its range. *Castilleja* spp. around the RMBL are attacked by at least two florivores/seed predators. Larvae of the plume moth, *Platyptila pica* (Lepidoptera: Pterophoridae), consume flowers, fruits, and seeds before the seeds disperse from the fruit, and larvae of the fly, *Phytomyza* spp. (Diptera: Agromyzidae), consume developing seeds before the seeds disperse from the fruit (Adler 2002). Hereafter, we refer to *P. pica* as a seed predator because we only assessed consumption of seeds by *P. pica* and not floral consumption, per se.

Castilleja linariaefolia provides an interesting system to address multiple sources of selection. Plant and floral characters vary widely within and among *C. linariaefolia* populations (R. E. Irwin and A. K. Brody, unpubl.), and the importance of such variation to interactions with pollinators and pre-dispersal seed predators and their effects on plant reproduction are largely unknown.

Limits of pollination vs pre-dispersal seed predation to female reproduction in *C. linariaefolia*

We tested whether the female-reproductive success of *C. linariaefolia* was limited by pollen receipt in the summers of 2000 and 2001. In 2000, we haphazardly chose 60 flowering *C. linariaefolia* in one population and randomly assigned 30 plants each to either a pollen-supplementation treatment or an open-pollinated control treatment. In 2001, we haphazardly chose 40 plants in each of three *C. linariaefolia* populations and randomly assigned 20 plants in each population to either the pollen-supplementation or control treatment. For the pollen-supplementation treatment, we collected anthers from at least 10 non-target *C. linariaefolia* growing approximately 5 m away from our study plants and mixed the anthers together. We then saturated the stigma of each female-phase flower with pollen by brushing the pollen mixture against the stigmas. To control for flower handling in the open-pollinated control treatment, plants were physically handled but no pollen was applied to stigmas. In 2000, treatments were performed once on 11 July at peak *C. linariaefolia* flowering to all open female-phase flowers on the focal plants. The calyces of open female-phase flowers in the pollen-supplementation and control treatments were marked with a small dot of indelible ink (Sharpie™), and only these fruits were scored to determine the importance of pollen addition to female plant reproduction. In 2001, treatments were performed twice weekly on all open female-phase flowers throughout the blooming period of the focal plants.

As fruits reached maturity, we collected the flowering stalks of each focal plant and counted the number of expanded fruits, the number of aborted fruits, and the number of seeds in each fruit. We calculated three measures of female reproduction per plant: (1) proportion fruit set (number of successful fruits divided by the total number of flowers produced; arcsine square-root transformed), (2) mean seed set per fruit (mean number of seeds produced per seed-bearing fruit in fruits not attacked by seed predators; square-root transformed), and (3) total seed production per plant (natural-log transformed). In 2000, these estimates of female plant reproduction were only determined for the treatment flowers marked with indelible ink. We assumed that flowers that had an expanded fruit capsule but were destroyed by seed predators were successfully fertilized, and these flowers were classified as having set fruit. We measured seed predation per plant by counting the number of fruits with exit holes (indication of damage by fly larvae), frass (indication of damage by plume moth larvae), or the presence of larvae inside the fruits. We calculated the proportion of fruits attacked by larvae (plume moth and fly larvae combined) as: number of fruits attacked by the larvae divided by the total number of non-aborted fruits produced (arcsine square-root transformed).

To test the importance of pollen supplementation to percent fruit set, mean seed set per fruit, and total seed production per plant simultaneously, we used a MANOVA with pollination treatment as a fixed effect. We tested the two years of study separately because methods differed slightly between the two years. In all 2001 analyses (here and below), we included site as a random factor. Because percent fruit set, seed set per fruit, and total seed production per plant may be intercorrelated, a MANOVA was used initially to control for Type I error (Rencher 1995). A significant MANOVA was followed by univariate ANOVAs for each response variable. To examine whether plants in the pollen-supplementation treatment were more susceptible to seed predation than plants in the open-pollinated control treatment in 2000 and 2001, we used an ANOVA with pollination treatment as a fixed effect, site as a random factor (2001 only), and proportion of fruits attacked by larvae as the response variable. In addition, we also wanted to examine whether plants that lost more fruits to seed predators might also have lost more fruits overall. To do so, we examined the correlation between the proportion of flowers that set fruit and the proportion damaged by seed predators across treatments and sites in 2000 and 2001. All statistical analyses described here and below were performed with SAS statistical software (SAS Institute Inc., version 8.2).

Selection by pollinators vs seed predators on floral and plant characters in *C. linariaefolia*

We examined the importance of pollinators vs seed predators to selection on floral and plant characters via female-reproductive success in one natural population of *C. linariaefolia* in the 2000 flowering season. The population was greater than 1 km from the populations in which we tested for pollen limitation above. As plants initiated flowering, we haphazardly chose 60 *C. linariaefolia*. At peak flowering, we measured the height of the tallest flowering stalk (measured to the nearest 0.1 cm). We also measured calyx length (measured from the base to the tip of the red calyx) on three haphazardly chosen female-phase flowers on each plant. Calyx length was measured to the nearest 0.01 mm using digital calipers and was averaged across flowers on the same plant. In *C. linariaefolia*, the calyces (not the flowers) are brightly colored and are likely important in pollinator and seed predator attraction. As plants senesced, we collected all plants and counted the total number of reproductive structures produced (hereafter referred to as 'total flower production'). We measured stalk height, calyx length, and number of flowers produced because these traits are variable in this and other

systems and influence interactions with mutualistic and/or antagonistic visitors and subsequent plant reproduction (Hainsworth et al. 1984, Campbell 1989, Brody and Mitchell 1997, Adler et al. 2001, Caruso 2001).

To estimate pollinator visitation to *C. linariaefolia*, we used stigma pollen loads as indices of pollinator visitation. To do so, we collected three stigmas from three female-phase flowers on each plant at approx. peak flowering in this site (18 July, 2000). Because stigmas protrude beyond the corolla opening before becoming receptive, it is likely that all pollen deposited on the stigma was transported by floral visitors. We assumed that pollen grain receipt was correlated with pollinator visitation – e.g. increased pollinator visitation resulted in increased pollen grain receipt. Stigmas were squashed in a basic fuchsin dye (Kearns and Inouye 1993) and the number of *C. linariaefolia* pollen grains on each stigma were counted under a compound microscope at $40\times$. Pollen counts were averaged across stigmas on the same plant.

To estimate female-reproductive success, we collected all fruits of each plant and counted the total number of seeds produced. Total seed production per plant was converted to a relative fitness estimate by dividing by the mean value across all study plants in the population.

To estimate levels of seed-predator attack per plant, we counted the total number of fruits attacked by seed predators (plume moth and fly larvae combined) and expressed these as a percentage of the total fruits produced (arcsine square-root transformed). Because we did not measure oviposition events by the two species, we could not assess how floral characters influenced oviposition selectivity. We could, however, use the counts of damaged fruits as estimates of net selection by the seed predators on plant reproduction as a function of the floral and plant characters we measured.

We used path analysis (Wright 1921, 1934, Li 1975) combined with structural equation modeling (SEM; reviewed by Mitchell 1992, 1993) to analyze the importance of pollination vs seed predation to selection on plant and floral characters via female reproduction. Path analysis is increasingly used in studies of plant–herbivore and plant–pollinator interactions (Schemske and Horvitz 1988, Campbell and Halama 1993, Mothershead and Marquis 2000, Gómez and Zamora 2000, Adler et al. 2001) because it allows for the dissection of complex direct and indirect relationships among variables. An additional benefit of path analysis is that path coefficients are equivalent to Lande and Arnold's (1983) selection coefficients (Kingsolver and Schemske 1991). Using SEM, we determined which of three a priori path diagrams (described below) best fit the observed data. Then, using path analysis, we compared the relative direction and strength of selection on plant and floral characters via pathways involving pollination vs seed predation. Despite its advantages, path analysis should not be used to infer causation among variables (Mitchell 1993, Shipley 1999). Rather, path analysis identifies correlations among variables and possible targets of selection that can be further tested using an experimental approach (Kingsolver and Schemske 1991, Mitchell 1992, Petraitis et al. 1996).

We developed three competing a priori hypotheses concerning the relationships among plant and floral traits, visitation by pollinators vs seed predator attack, and subsequent effects on relative seed production. We developed these hypotheses, represented as path diagrams in Fig. 1, based on previous studies examining the relationships among plant and floral characters, pollination, seed predation, and/or plant reproduction in other systems (Schemske and Horvitiz 1988, Mothershead and Marquis 2000, Adler et al. 2001). In Model A (Fig. 1), calyx length, plant height, and number of flowers produced directly influence pollinator selectivity of plants and levels of seed predation and indirectly influence relative seed set through changes in pollination and seed predation. The number of flowers produced also has a direct effect on relative seed set, and pollinator visitation influences levels of seed predation, assuming seed predators select oviposition sites that are more likely to set fruit. Finally, calyx length, plant height, and number of flowers produced are intercorrelated due to genetic and/or environmental conditions (double-headed arrows indicate correlations in Fig. 1). Model B is nested within Model A (Fig. 1), with the effect of pollinator visitation on seed predation constrained to zero but all other paths remaining identical. We include this second model because for some types of seed predators that also act as florivores (plume moth larvae in this study), there may be no direct reliance of larval success on the production of seeds. Finally, in Model C (Fig. 1), we hypothesize that plant and floral characters have no effect on pollinator visitation, and changes in pollinator visitation have no effect on relative seed production if plants are not pollen limited in the year of study at this site. All other paths remain identical to Model A. We do not propose a model parallel to Model C that includes pollinators but leaves out seed predators because previous research on a related *Castilleja* spp. around the RMBL suggested that pollination did not limit seed production (L. S. Adler, unpubl.) while seed predators consumed a significant proportion of the yearly seed crop (Adler 2002).

Using SEM, we statistically tested which competing path hypothesis, Model A, B, or C (Fig. 1), provided the most appropriate fit to the observed data (Hayduk 1987, Loehlin 1987, Mitchell 1992, 1993) using a goodness-of-fit statistic which has an approximate χ^2 distribution with df = the difference between the number of observed correlations minus the number of coefficients. A non-significant χ^2 value indicates that the expected correlations in the path diagram do not differ significantly from the observed correlations in the data,

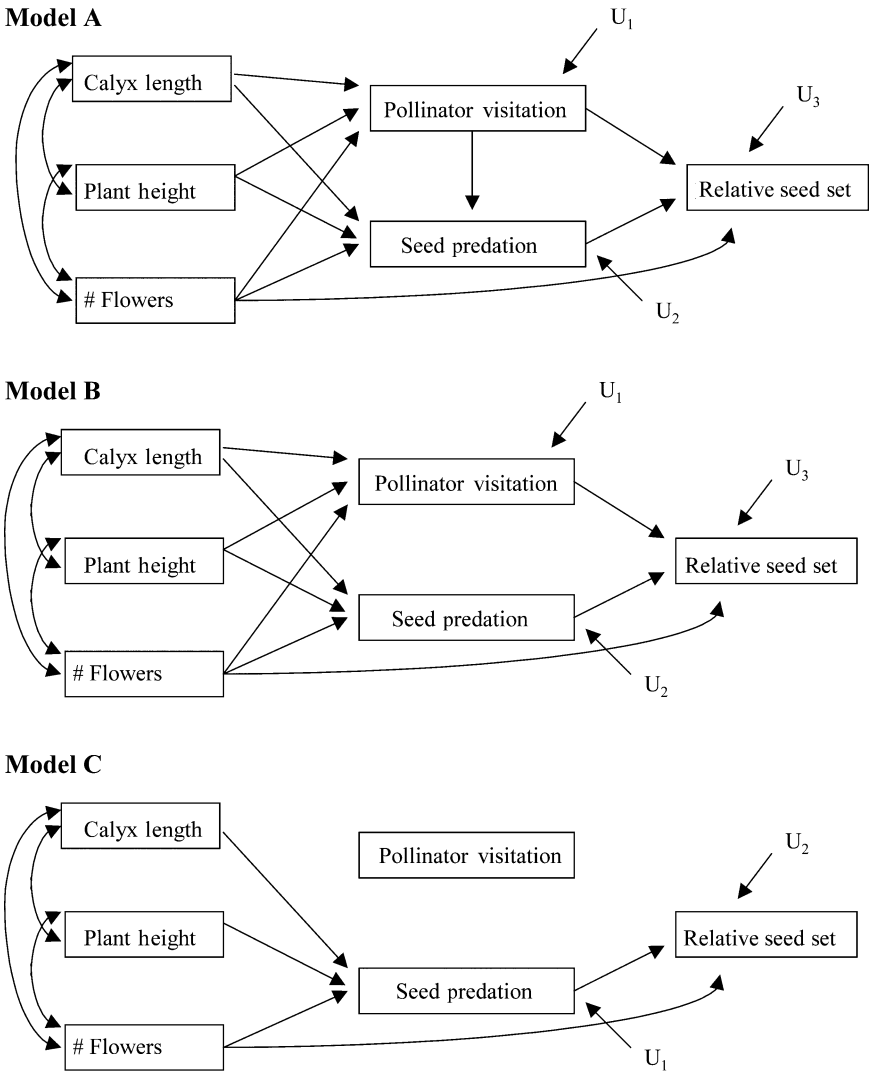


Fig. 1. Three competing path diagrams testing the effects of plant and floral characters (calyx length, plant height, and number of flowers produced) on pollination (estimated as pollen receipt to stigmas), seed predation, and relative seed set in *Castilleja linariaefolia*. In all models, plant and floral characters may be intercorrelated, depicted by the double-headed arrows, and there is unexplained variation $[(1 - R^2)^{0.5}]$ associated with the measurement of pollinator visitation, seed predation, and relative seed set (U_1, U_2, and U_3). In Model A, plant and floral characters influence pollinator visitation and seed predation, pollinator visitation influences plant susceptibility to seed predation, and both seed predation and pollination influence relative seed set. In Model B, which is nested within Model A, all pathways remain identical to Model A, accept the pathway between pollinator visitation and seed predation is constrained to zero. In Model C, plant and floral characters do not influence pollinator visitation, and pollinator visitation does not influence relative seed set, suggesting that pollinator visitation is not a limiting factor to female plant reproduction. All other pathways remain identical to those in Model B.

suggesting the model provides a reasonable fit to the data. We also report Akaike's Information Criterion (AIC); the model that minimizes AIC provides the most reliable fit to the data. For the three competing path models, we used PROC CALIS in SAS (METHOD = ML) to calculate AICs, goodness-of-fit statistics, and significance values. We calculated significance values using both exact variables and variables in standard deviates and found similar results. We report results from the standardized variables only. In addition, to screen for multi-collinearity among the predictor variables, we calculated variance inflation factors (hereafter referred to as VIFs; VIF option in PROC REG). Correlations among variables will inflate VIFs and cause a loss of precision in the models. In all cases, the VIFs for the predictor variables in this study were less than 2; therefore, it is unlikely that multi-collinearity had a strong impact on the results (Myers 1990). For the path diagram that provided the most appropriate fit to the observed data, we calculated direct effects (standardized partial regression coefficients), indirect effects, and significance levels using PROC CALIS of SAS.

Results

Limits of pollination vs pre-dispersal seed predation to female reproduction in *C. linariaefolia*

In 2000, we found that pollen supplementation had a significant effect on female plant reproduction (MANOVA: $F_{3,25} = 3.76$, $P = 0.02$). Plants that received supplemental pollen had significantly higher percent fruit set than plants in the open-pollinated control treatment (ANOVA: $F_{3,27} = 11.98$, $P = 0.002$), experi-

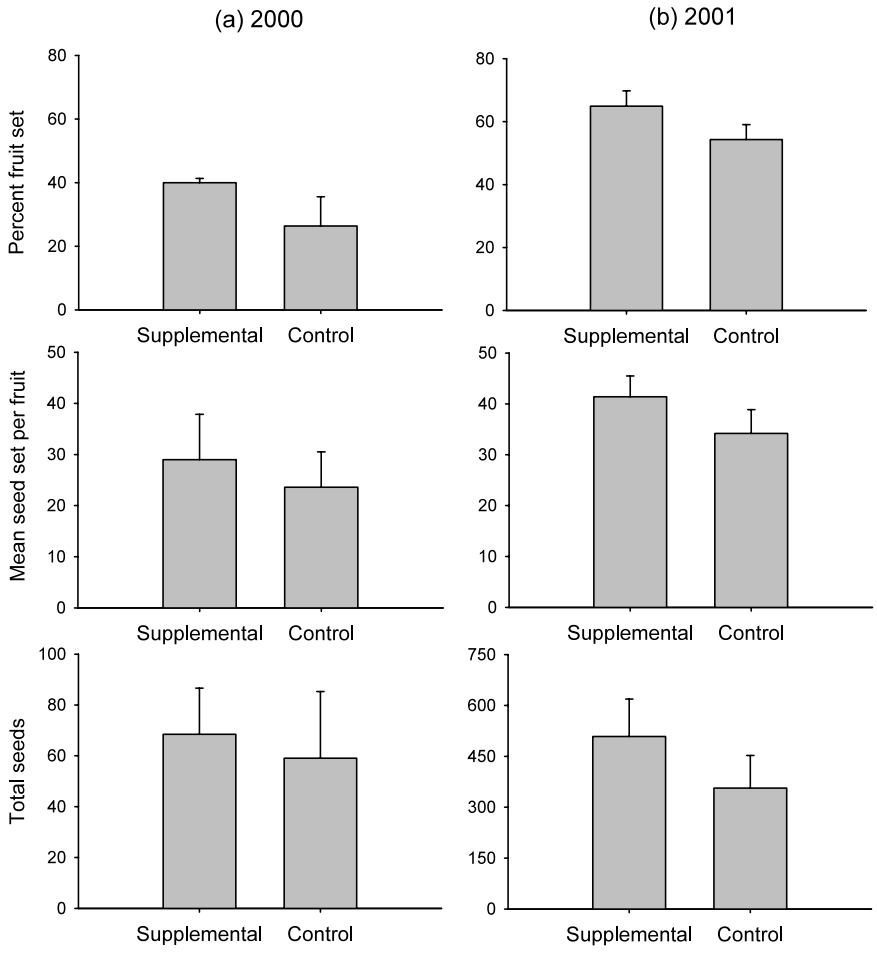


Fig. 2. Supplemental-pollination treatments had only marginal benefits to percent fruit set, seed set per fruit, and total seeds in (a) 2000 and (b) 2001 for *Castilleja linariaefolia*.

encing 47% higher percent fruit set (Fig. 2a). However, pollen supplementation only increased seed set per fruit by 18% and total seed production per plant by 14% (mean difference of 10 seeds per plant between treatments) (Fig. 2a). These differences in seed set per fruit and total seed production per plant between pollen-supplementation and open-pollinated control treatments were not statistically significant (mean seed set per fruit, ANOVA: $F_{1,27} = 1.19$, $P = 0.28$; total seed production per plant, ANOVA: $F_{1,27} = 2.54$, $P = 0.12$; Fig. 2a). To achieve statistical significance between pollination treatments at $\alpha = 0.05$, we would have needed 96 plants per treatment for mean seed set per fruit and 46 plants per treatment for total seed production per plant.

In 2001 across all sites, plants that received supplemental pollen experienced 19% higher percent fruit set, 17% higher seed set per fruit, and 30% higher total seed production compared to open-pollinated control plants (Fig. 2b). However, these differences in female reproduction between plants in the supplemental-pollination treatment and the open-pollinated control treatment were not statistically significant (MANOVA: $F_{3,75} = 0.77$, $P = 0.51$) nor did we find a significant site effect (MANOVA: $F_{6,150} = 0.78$, $P = 0.59$). To find a statistically significant difference between treatments at $\alpha = 0.05$ with a power of 0.50 for each of our three fitness estimates, we would have needed 106 plants for percent fruit set, 291 plants per treatment for seed set per fruit, and 232 plants per treatment for total seed production per plant.

Fruit and seed production of *C. linariaefolia* may be hindered, in part, by seed predation by plume moth larvae and fly larvae. In both years of study, there were relatively high levels of damage to the fruits of *C. linariaefolia* by the seed predators. In 2000, $31.55\% \pm 7.61\%$ (mean $\pm$ 1 SE) of fruits scored were partially or fully destroyed by the seed predators; while in 2001, $14.73\% \pm 2.74\%$ (mean $\pm$ 1 SE) of fruits were attacked. In 2000, the proportion of undamaged flowers that set fruit was negatively correlated with the proportion of flowers that were damaged ($r = -0.39$, $n = 29$, $P = 0.03$), suggesting that fruit set may be limited by seed predator attack. However, in 2001, there was no signifi-

cant relationship between the proportion of undamaged flowers that set fruit and the proportion damaged ($r = -0.0796$, $n = 89$, $P = 0.46$). Although not statistically significant, plants that received supplemental pollination received 34% more seed predation than plants in the open-pollinated control treatment in 2000 (supplemental vs control mean percent of fruits fully or partially destroyed by seed predators $\pm$ 1 SE: 40.00% $\pm$ 13.33% vs 26.39% $\pm$ 9.20%; $F_{1,27} = 0.74$, $P = 0.40$). However in 2001, there was no distinguishable difference in seed predation between plants in the two pollination treatments (supplemental vs control mean percent of fruits fully or partially destroyed by seed predators $\pm$ 1 SE: 16.37% $\pm$ 3.54% vs 13.04% $\pm$ 4.22%; $F_{1,84} = 2.37$, $P = 0.13$).

Selection by pollinators vs seed predators on plant and floral characters in *C. linariaefolia*

Using SEM, we found that neither the correlation matrix from Model A nor the correlation matrix from Model B deviated significantly from the observed correlation matrix (Table 1). Thus, both models incorporating selection on plant and floral characters via pollination and seed predation appropriately represented the observed data. The correlation matrix from Model C deviated significantly from the observed correlation matrix and had the highest AIC value ($P = 0.03$, $AIC = 3.01$), suggesting that the removal of pollinators as agents of selection on plant and floral traits did not adequately represent the observed interactions. When we compared Model A vs Model B by taking the difference in the goodness-of-fit statistics, we found no statistically significant difference between the two models ($\chi^2 = 1.03$, $df = 1$, $P = 0.75$). However, because Model B had a lower AIC value than Model A and because Model B is simpler than Model A (Model B estimates fewer pathways; Fig. 1), we favor Model B based on the AIC values and on the principle of parsimony (Mitchell 1993) and discuss it further below.

Pollinator visitation

We found no evidence that the plant and floral characters we measured (calyx length, plant height, and number of flowers produced) resulted in changes in pollinator selectively of plants, as none of the path coefficients from plant and floral characters to pollinator visitation were statistically significant (Table 2, Fig. 3, $P > 0.34$ in all cases). Moreover, although the pathway from pollinator visitation to relative seed production was positive, the magnitude of the standardized path coefficient was small ($p = 0.07$) and the pathway was not statistically significant (Table 2, Fig. 3).

Table 1. A comparison of alternative path diagrams using structural equation modeling found that Model B provided the most appropriate fit to the observed data.

Model	χ^2	df	P	AIC
A	4.40	2	0.1110	0.3963
B	5.43	3	0.1430	−0.5716

Table 2. Magnitude of direct effects (path coefficients) of plant and floral characters on pollination, seed predation, and relative seed set. Pathways are depicted in Fig. 3.

	Pollination	Seed predation	Relative seed set
Calyx length	−0.31	−0.28*	–
Plant height	−0.03	0.61**	–
No. flowers	−0.11	0.13	0.83**
Pollination	–	–	0.07
Seed predation	–	–	−0.49**
R^2	0.12	0.50	0.70

*$P = 0.07$; **$P \leq 0.001$.

Seed predation

Plant and floral characters had a significant effect on seed predator attack, plume moth larvae and fly larvae combined. The path coefficient from calyx length to seed predation was negative ($p = -0.28$) and marginally statistically significant (Table 2, Fig. 3), and the path coefficient from plant height to seed predation was positive ($p = 0.61$) and statistically significant (Table 2, Fig. 3). Flower production had a positive effect on seed predation ($p = 0.13$); however, this pathway was not statistically significant (Table 2, Fig. 3). Levels of seed predation had a significant negative effect on relative seed set ($p = -0.49$, Table 2, Fig. 3), suggesting that changes in seed predation influence relative female plant-reproductive success.

Relative seed set

Flower number had the strongest direct positive effect on seed set ($p = 0.83$, Table 2, Fig. 3). The positive effects of flower number on relative seed set greatly outweighed the negative indirect effects of flower number mediated through increased seed predation and reduced pollination (Table 3). Plant height had a negative indirect effect on relative seed set through both pollinators and seed predators (Table 3, Fig. 3). Conversely, larger calyces had a positive indirect effect on seed set by reducing seed loss to seed predators (Table 3, Fig. 3). Calyx length experienced opposing selective forces by pollination vs seed predation, as both pollination and seed predation were highest on plants with shorter calyces (Fig. 3). The magnitude of selection via the seed predation pathway exceeded selection via the pollination pathway by 86% (Table 3). And in general, the effects of plant and floral characters on relative seed set through seed predation were consistently stronger than through pollinator visitation (Table 3).

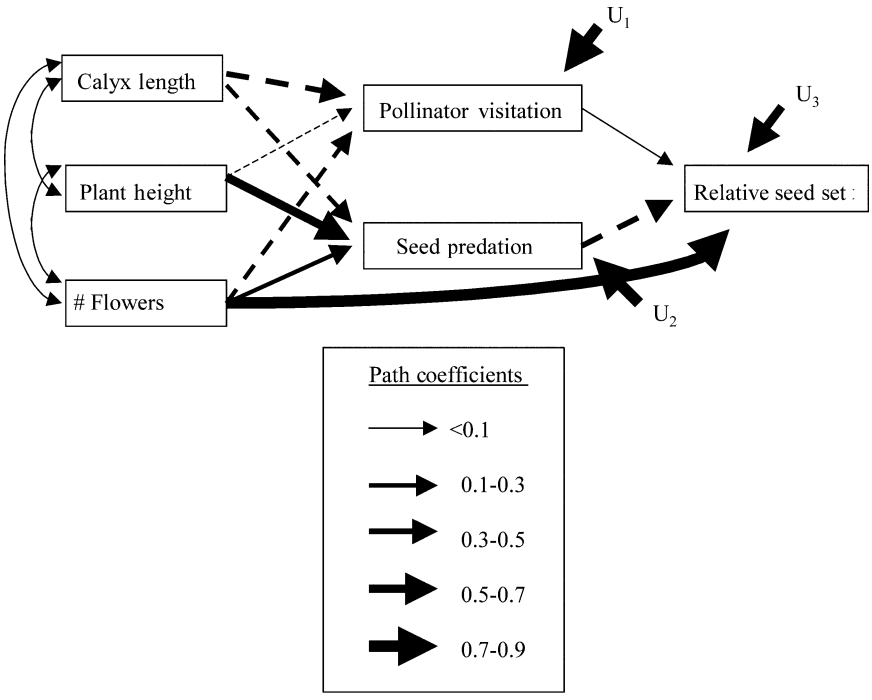


Fig. 3. Path diagram for the effects of plant and floral characters on pollination (estimated as pollen receipt to stigmas), seed predation, and relative seed set in *Castilleja linariaefolia* that best fit the observed correlation matrix, identified using SEM. Positive effects are indicated by solid lines, and negative effects by dashed lines. Double-headed arrows indicate weak positive correlations between plant and floral characters. The widths of the arrows indicate the magnitude of the standardized path coefficients. Significant pathways are shown in Table 2. The residual variables for pollinator visitation (U_1), seed predation (U_2), and relative seed set (U_3) indicate unmeasured factors. In this model, plant and floral characters have weak negative effects on pollinator visitation, and pollinator visitation has a weak positive effect on relative seed set. Plant and floral characters have stronger effects on seed predation than on pollination, and seed predation has a strong negative effect on relative seed set. Finally, number of flowers produced has a direct positive effect on relative seed set.

Discussion

Flowering plants are besieged by a variety of floral visitors, conferring a continuum of positive to negative effects on plant-reproductive success and potentially impacting the evolution of plant and floral traits. *Castilleja linariaefolia* is self-incompatible and relies on hummingbirds for pollination service but also incurs seed loss through attraction of pre-dispersal seed predator moth and fly larvae. Thus, plants may face trade-offs in attracting pollinators while also luring pre-dispersal seed predators. Here we found that the floral traits we measured had weak effects on estimates of pollinator visitation, and pollinator visitation had a weak positive effect on seed set, suggesting pollinators exert only weak selection on the traits measured. Indeed, pollen-supplementation experiments exhibited no significant positive effects on seed production. In contrast, pathways from plant and floral characters through seed predators had stronger effects on relative seed set than through pollinators. These results suggest that pre-dispersal seed predators exert stronger selection than pollinators, likely because of their strong negative effects on seed set. Because we did not measure oviposition events by the seed predators, we do not know whether increased seed predation was the result of differential oviposition events and/or differential egg and larval survival. The mechanism warrants further attention.

Table 3. Magnitude of direct and indirect effects of plant and floral characters on relative seed set via pathways involving pollination vs seed predation. DE refers to direct effect, IE to indirect effect, and TE to total effect. Pathways are depicted in Fig. 3.

	DE	IE via pollination	IE via seed predation	TE
Calyx length	–	−0.02	0.14	0.12
Plant height	–	−0.002	−0.30	−0.30
No. flowers	0.83	−0.01	−0.06	0.76

Pollen limitation of plant reproduction is common among flowering plants (reviewed by Burd 1994). Although we found a trend for pollen limitation of female plant reproduction, the difference in seed production between pollen-supplementation and control treatments was not statistically significant. One caveat about interpretation of these results is that *C. linariaefolia* is a

long-lived perennial, and two single-season pollen-supplementation experiments do not demonstrate that lifetime seed production of these plants is not limited by pollinators. Yet, our results are similar to other short-term studies of *Castilleja* spp. showing no evidence of pollen limitation to female plant reproduction (Carpenter 1988, L. S. Adler, unpubl.).

Even with the application of excess pollen to stigmas in both years of this study, many hand-pollinated flowers failed to set fruit, suggesting that nutrient and/or water resources may limit, in part, female reproduction in this hemi-parasitic plant. Hemi-parasitic plants experience a widely variable environment with respect to host plants, from which they acquire carbon, nitrogen, water, and secondary compounds via root haustoria connections to the host vascular system (reviewed by Adler 2000). Variation in host-plant quality may limit the degree to which plants can respond to the positive effects of supplemental pollination (Marvier 1996, 1998, Adler 2000). The possible beneficial effects of pollen supplementation to seed set could also have been masked by the high prevalence of seed predation. Because non-pollinating pre-dispersal seed predators rely on pollinators to provision their larvae, the larvae of ovipositing females may gain an advantage if females oviposit on flowers that are more likely to set fruit. We did find a 34% increase in seed predation in plants in the pollen-supplementation treatment compared to plants in the open-pollinated control treatment in 2000 and a 19% increase in 2001. Similarly, Herrera (2000) found that hand-pollination of flowers increased mammalian herbivory to plants, negating the beneficial effects of increased pollination (Herrera et al. 2002). That we didn't find a larger difference in seed predation between pollen-supplementation and control treatments may be due, in part, because plume moth larvae feed on flowers as well as developing seeds. Therefore, these larvae do not rely solely on seeds, as opposed to the fly larvae in this study.

Path analysis in combination with structural equation modeling demonstrated that seed predation had a stronger direct effect on relative seed set than did pollination. In addition, the magnitude of the effect of floral and plant traits on relative seed set was stronger through pathways involving seed predators than those involving pollinators. Other studies have found similar results. For example, pre-dispersal seed predators responded to petal size manipulation in *Hibiscus moschetus* more so than did pollinators (Kudoh and Whigham 1998). Ungulate herbivory had a stronger effect on female plant reproduction and selection on plant and floral characters of *Hormathophylla spinosa* than did pollinators in areas of high herbivore pressure (Gómez and Zamora 2000). Despite our supposition that plants should face trade-offs in attracting pollinators as well as seed predators, we found that only one floral character (calyx length) experienced weak but conflicting selection pressures by pollinators vs seed predators. The expression of trade-offs may only be apparent in systems where plants experience strong limitation of plant reproduction via seed predators as well as pollinators, which was not the case here.

Total flower production had the strongest direct effect on relative seed set. Such a result is likely because increased flower number results in increased number of ovules for producing seeds, and the positive relationship between flower number and seed set is fairly common (Herrera 1993, Brody and Mitchell 1997, Ehrlén 1997, Gómez and Zamora 2000, Ohashi and Yahara 2000, Adler et al. 2001). Surprisingly, increased flower production did not result in increased estimates of pollinator visitation, measured as mean pollen receipt per stigma. Plants with lower flower production experienced higher estimates of pollen receipt. The mechanism behind this result is unknown; however, increased flower production may be correlated with decreased resources for other floral attractive features, such as decreased nectar production rate, if resources are limiting. Conversely, reduced estimates of pollinator visitation to plants that produced more flowers may be a function of estimating pollinator visitation as pollen receipt per stigma, a per-flower estimate. Plants with larger floral displays may receive more total visits but fewer visits per flower than plants with smaller floral displays (Brody and Mitchell 1997). Finally, by using stigma-pollen loads as indices of pollinator visitation, we measured both pollinator visitation as well as pollinator effectiveness, the former likely being affected by floral attractive characters (such as floral display size), the latter by the size and shape of flowers. The relationship between flower production and floral shape is unknown in this system.

Despite the direct benefits of increased flower production to relative seed set, plants may experience trade-offs between increased flower production and increased seed predation. The positive effects of flower production on the intensity of seed predation are well-documented (Augsperger 1981, Hainsworth et al. 1984, Molau et al. 1989, Brody and Mitchell 1997, Ohashi and Yahara 2000, Fenner et al. 2002). Because we did not measure the phenology of flower production in this study, we do not know to what degree the timing of flower production and the size of the floral display impact plant susceptibility to seed predation (Pilson 2000). What remains clear, however, is that the relative importance of the positive direct effects of flower production on seed set vs the indirect effects of flower production through increased seed predation will ultimately depend on the magnitude of seed predation in a given year or site. Temporal and geographic variation in seed predation, along with variation in the magnitude of pollen limitation, will likely drive variation in the plant and floral characters measured (Thompson 1994, Ehrlén 1996, Ehrlén et al. 2002).

One warning about interpretation of the path analysis results is that path analysis assumes the most relevant characters are included in the model (Mitchell 1992, 1993). Obviously, this can never be fully known for any natural system, but we included characters likely to affect pollination and/or seed predation as shown in other systems (Hainsworth et al. 1984, Campbell 1989, Brody and Mitchell 1997, Adler et al. 2001, Caruso 2001, Campbell et al. 2002). Yet, each of our response variables (pollination, seed predation, and relative seed set) had relatively large sources of unexplained variation (Fig. 3). Therefore, it is likely that our model did not fully represent all of the important characters and/or had some amount of measurement error (Mitchell 1992, 1993), as is common in other studies using path analysis to disentangle selection by mutualists vs antagonists (Schemske and Horvitz 1988, Gómez and Zamora 2000, Mothershead and Marquis 2000). For example, both nectar production rate and plant secondary compounds may have strong direct and indirect effects on pollination, seed predation, and relative plant reproduction (Adler 2000). These characters were beyond the scope of this study, but their effects may be important and may have influenced our results. Moreover, because we measured plant reproduction via female-reproductive success only, we remain ignorant of the effects each floral trait, as well as seed predators acting as florivores, had on male-fitness components. Pollinators could still be selecting on traits that influence pollen export or pollen donation, especially since estimates of male fitness are often more sensitive to changes in pollinator visitation than female function (Young and Stanton 1990).

Despite these caveats, the results from the experimental pollen-limitation study in combination with the path analysis suggest that seed predators acted as stronger selective agents than pollinators on plant and floral characters in *C. linariaefolia*. Our path analysis identified correlations among variables and possible targets of selection to further test using an experimental approach (Kingsolver and Schemske 1991, Mitchell 1992). Although we found only weak evidence of pollinator-mediated selection on floral traits, *Castilleja linariaefolia* is a long-lived perennial and it is unknown how this short-term study reflects life-time selection on plant and floral traits. The remarkable intraspecific variation in plant and floral characters exhibited by most flowering plants is likely the result of selection driven by mutualists in concert with antagonists (Galen 1999a, Galen and Cuba 2001), albeit some of this variation in floral form is likely neutral and does not reflect adaptation. Ultimately, the relative importance of antagonists vs mutualists to selection on plant and floral characters will depend on their relative abundance in space and time and the magnitude of their direct and indirect effects on male and female plant fitness. Taken together, these results highlight the subtle but complex interactions that shape floral and vegetative design in natural ecosystems.

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