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BRIEF COMMUNICATION

RESOURCE REALLOCATION DOES NOT INFLUENCE ESTIMATES OF POLLEN LIMITATION OR REPRODUCTIVE ASSURANCE IN *CLARKIA XANTIANA* SUBSP. *PARVIFLORA* (ONAGRACEAE)[1]

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- *Premise of study:* Studies of pollen limitation and the reproductive assurance value of selfing are important for examining the process of floral and mating system evolution in flowering plants. Recent meta-analyses have shown that common methods for measuring pollen limitation may often lead to biased estimates. Specifically, experiments involving single- or few-flower manipulations per plant tend to overestimate pollen limitation compared to those involving manipulations on most or all flowers per plant. Little previous work has explicitly tested for reallocation within individual systems using alternative methods and response variables.
- *Methods:* We performed single-flower and whole-plant pollen supplementation and emasculation of flowers of *Clarkia xantiana* subsp. *parviflora* to estimate pollen limitation (PL) and reproductive assurance (RA). We compared levels of PL and RA using the following response variables: fruit set, seeds/flower, and seeds/plant. We also assessed the germination and viability of seeds to evaluate potential variation in pollen quality among treatments.
- *Key results:* Autonomous selfing in *Clarkia xantiana* subsp. *parviflora* eliminates pollen limitation and provides reproductive assurance. Estimates from single-flower manipulations were not biased, closely resembling those from whole-plant manipulations. All three response variables followed the same pattern, but treatments were only significantly different for seeds/flower. Pollen quality, as indicated by seed viability, did not differ among treatments.
- *Conclusions:* Partial plant manipulations provided reliable estimates of pollen limitation and reproductive assurance. These estimates were also unaffected by accounting for pollen quality. Although whole plant manipulations are desirable, this experiment demonstrates that in some systems partial plant manipulations can be used in studies where whole-plant manipulations are not feasible.

Key words: mating system; outcrossing and selfing; plant-pollinator interactions; plant reproduction; pollen quantity and quality; pollination; resource allocation.

Pollen limitation is considered to be a strong force driving the evolution of pollination strategies and mating systems in flowering plants (Haig and Westoby, 1988; Lloyd, 1992; Maurice and Fleming, 1995; Ashman et al., 2004). Pollen limitation (PL) occurs when plants receive an insufficient supply of pollen such that reproductive success is compromised. Several recent and influential papers have highlighted the theoretical aspects of the interface of PL and floral and mating system evolution (Ashman et al., 2004; Morgan and Wilson, 2005), as well as the major patterns of pollen limitation based on reviews of empirical studies (Burd, 1994; Larson and Barrett, 2000; Knight et al., 2005). One evolutionary outcome of chronic pollen limitation is the evolution of self-fertilization because selfing elevates seed production and therefore provides reproductive assurance (RA) in poor pollination environments (Baker, 1955; Stebbins, 1974; Jain, 1976; Morgan and Wilson, 2005). In empirical studies, reproductive assurance is measured by evaluating the extent to which intact flowers (where selfing can occur) have elevated reproduction compared to flowers where anthers have been removed and only outcrossing can occur. Due to their implications for population persistence and floral evolution, pollination biologists often measure PL and RA in field environments. A persistent problem has been to identify reliable field methods for estimating PL and RA to address outstanding ecological and evolutionary questions.

A recent meta-analysis showed that estimates of PL are often biased when floral manipulations are performed on one or few flowers per plant as compared to when most flowers are manipulated (Knight et al., 2006). For example, if a single flower on a plant receives supplemental pollen, resources may be shunted to that developing fruit at the expense of other fruits, the production of additional flowers, or reproduction in subsequent years (Stephenson, 1981; Bawa and Webb, 1984; Zimmerman and Pyke, 1988; Knight et al., 2006). Whole-plant manipulations are often viewed as more accurate because resources cannot be differentially reallocated among fruits according to pollen quantity or quality. The same reallocation bias has been detected by comparing treatments where all flowers on a plant were unmanipulated with treatments where some flowers on the plant were supplemented and others were unmanipulated (Zimmerman and Pyke, 1988; but see Fernandez et al., 2012). Although whole-plant manipulations should be used if possible, it may be untenable for some species or some studies if, for example, plants are very large or studies are carried out over large geographic areas in many

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populations. In these cases, it is beneficial to determine if reliable estimates of PL and RA are obtainable through more manageable single- or few-flower manipulations (Wesselingh, 2007).

Researchers also generally only consider one of a set of the commonly measured variables when assessing PL and RA (Knight et al., 2006). The choice of response variable may depend on the specific question addressed by a study with respect to PL and RA but it may also influence the magnitude of PL and RA. For example, seeds per plant may be more relevant for studies of population dynamics whereas seeds per flower may be appropriate for questions about mate acquisition. Knight et al. (2006) found that the effect size of PL differed among response variables, with percentage of fruit set having the highest and seeds/plant or seeds/fruit having the lowest effect size (i.e., higher reproductive success in supplemented manipulations over unmanipulated plants or flowers).

Resource reallocation may occur among fruits because of variation in the quantity or quality of pollen received and subsequent variation in offspring quantity or quality (Zimmerman and Pyke, 1988; Aizen and Harder, 2007). In highly outcrossing taxa, reallocation could occur largely because of pollen quantity (flowers that get more pollen receive disproportionate resources). In partially or highly selfing taxa, resource reallocation may occur because of variation in pollen quality among flowers (e.g., the proportion of selfed vs. outcrossed pollen grains) even when there is no pollen limitation. Recently, it has been noted that pollen supplementation experiments may overlook the potentially confounding effects of pollen quantity and quality as both may affect seed production and offspring quality (Pflugshaupt et al., 2002; Aizen and Harder, 2007). Pollen limitation is generally only assessed for seed production but ideally should be estimated at various life stages as it is possible that the magnitude of pollen limitation may be enhanced or fully negated by offspring genetics. For example, Fernandez et al. (2012) found that pollen supplementation increased seed production but did not further enhance seedling emergence.

Resource reallocation among fruits or between current and future reproduction may bias estimates of PL and RA to a greater or lesser extent depending on life history and costs of reproduction. The bias toward greater pollen limitation was most severe in polycarpic plants but also evident in monocarpic plants (Knight et al., 2006). In polycarpic plants, resources can be reallocated across years whereas in monocarpic plants resources can only be differentially allocated among developing fruits. Long-lived, polycarpic species, such as *Aesculus californica* (Spach) Nutt., that produce costly fruits and abort more than 90% of flowers and young fruits (Newell, 1991) may be particularly problematic in confounding pollen limitation with patterns of resource investment. Since the 2006 publication of the meta-analysis by Knight et al., there has been particular interest in this problem. There has also been debate about whether resource reallocation should cause concern in all systems or is a problem more frequently influencing estimates of PL and RA in longer-lived, iteroparous plants or plants with high costs of reproduction.

We are not aware of studies that have compared whole-plant vs. single-flower manipulations for individual species in a single experiment using multiple PL and RA response metrics. In the meta-analysis by Knight et al. (2006) comparisons had to be made between different manipulation approaches applied to different species, rather than different manipulation approaches applied to single populations of single species. Ideally, individual taxa should be evaluated to determine whether resource reallocation is a phenomenon that might bias estimates of PL and RA prior to larger scale studies. Here, we compare two common approaches to measuring pollen limitation and reproductive assurance in a natural population of *Clarkia xantiana* subsp. *parviflora*. Our objective was to test the hypothesis that resource reallocation biases estimates of PL and RA and to quantify the extent of bias when manipulations involve single flowers per plant vs. many flowers per plant. Additionally, we measured seed germination and viability to determine if there were measureable differences in pollen quality among treatments, which could influence the magnitude of PL and RA even in the absence of differences in pollen quantity.

MATERIALS AND METHODS

Study system—*Clarkia xantiana* subsp. *parviflora* (Eastw.) H. F. Lewis and P. H. Raven (hereafter *parviflora*) is an annual plant found primarily in Kern and Tulare counties, California, USA. It occurs in the foothills of the southern Sierra Nevada Mountains in oak woodlands, xeric scrub, or pinyon-juniper woodland. Plants produce a single inflorescence producing typically ~1-20 flowers that open sequentially on the flowering spike. Flowers are perfect, 4-merous, rotate, and can be pink or white. *Parviflora* is self-compatible, partially selfing, and recently diverged from its self-compatible but primarily outcrossing sister taxon, *Clarkia xantiana* A. Gray subsp. *xantiana* (Pettengill and Moeller, 2012a, 2012b), with which it has a parapatric distribution (Eckhart and Geber, 1999). Selfing can occur in *parviflora* because anthers dehisce at anthesis allowing contact with receptive stigmatic lobes; however, flowers are typically open for several days and sometimes receive visits from pollinators (Moeller and Geber, 2005, unpublished data) causing at least occasional outcrossing (Moeller, 2006). *Parviflora* populations also exhibit some heterozygosity in DNA sequence and microsatellite data suggesting that outcrossing occurs at some frequency (Pettengill and Moeller, 2012b). We quantified the extent to which seed set in *parviflora* is limited by total pollen receipt (overall PL) and pollen received only through pollinator visitation (pollinator-mediated PL). Pollinator-mediated PL quantifies the potential for outcrossing relative to maximum seed production and is important for quantifying pollinator availability and its role in driving mating system evolution. Overall pollen limitation can be affected by both outcrossing and selfing and does not suitably assess the contribution of pollinators per se to reproduction. This metric has also been called “pollinator failure” (Kalisz et al., 2004). We also assessed the extent to which selfing elevates reproductive success over outcrossing, alone, thus providing reproductive assurance (RA). Given the increased recognition that studies measuring PL and RA can be biased due to the experimental approach taken, we quantified the extent to which the level of treatment application (whole plant vs. single flower) and alternative outcome variables influenced the results.

Floral manipulations—We conducted floral manipulations in a natural population of *Clarkia xantiana* subsp. *parviflora* (Yankee Canyon, Kern Co., California, USA; latitude: 35°39’ 21.14” N; longitude: 118°26’ 20.60” W, elev. 823 m (2700 ft)). Our study included most plants in the population. Plants were assigned to one of six treatment groups: 1) single flower supplemented; 2) single flower unmanipulated; 3) single flower emasculated; 4) all flowers supplemented; 5) all flowers unmanipulated; or 6) all flowers emasculated. We marked supplemented flowers with a small piece of green tape on the stem and pollinated them by gently swiping field-collected anthers across the experimental flower; with the pollen coming from two independent sires that were at least 3 meters away from the manipulated plant. Unmanipulated flowers were marked in the same manner but were left untouched. For emasculation treatments, we emasculated a fully formed bud just prior to opening by gently splitting open sepals and removing anthers using a pair of fine-tipped forceps, while avoiding damage to petals. Previous experiments have shown that the emasculation procedure does not damage reproductive organs and influence seed set (Moeller, 2006). We did not quantify the effect of emasculation on pollinator visitation but we have observed pollinators visiting emasculated flowers and inflorescences. In a study of *parviflora*’s sister taxon, subspecies *xantiana*, we observed no difference in reproductive success between emasculated and unmanipulated flowers despite over 1000 flowers in each treatment (Moeller et al., 2012). Since autonomous selfing is very rare in subspecies *xantiana*, this result suggests that pollinators readily visit both emasculated and intact flowers.

We visited the population every 3-4 d throughout the entire flowering season from 5 May – 16 May 2012 to apply treatments (4 visits). We treated 183 experimental plants for a total of 367 experimental fruits (average 3.8 +/− 0.11 fruits/plant on whole-plant manipulations). Fruits were allowed to ripen on plants and were collected at the end of the season just prior to dehiscence and all experimental fruits were collected individually. We obtained estimates of fruit set and counted all fully formed seeds to obtain estimates of seeds per flower (for all experimental plants); we also obtained estimates of fruit set and seeds per plant for experimental plants where all flowers were treated (whole-plant manipulations).

Seed germination and viability—We tested for potential differences among treatments in seed quality through germination of all fully formed seeds from the collected fruits of single-flower manipulations. Seeds were sown onto filter paper in petri dishes wetted with 2 mL of ultra-pure water. Petri dishes were then wrapped in parafilm and placed in a cold-room at 10°C under 24h light for 14 d. We then counted and removed germinants for each fruit and left the remaining seeds for an additional 21 d. We assessed viability of up to 10 of the remaining ungerminated seeds to estimate the proportion of remaining seeds that were viable. Ungerminated seeds were dried, sliced in half, and placed in 96-well plates with 0.2% tetrazolium chloride. Plates were wrapped in aluminum foil and left at room temperature and checked after 4 d. Seeds that are viable stain dark red.

Data analysis—*Comparison of whole-plant and single-flower manipulations*—We tested whether estimates of pollen limitation and reproductive assurance were dependent on the number of flowers manipulated per plant. Greater reproductive success in supplemented than unmanipulated treatments indicates that plants are subject to pollen limitation. Greater reproductive success in supplemented than emasculated treatments indicates pollinator-mediated pollen limitation. Greater reproductive success in unmanipulated than emasculated treatments indicates that selfing provides reproductive assurance. We used mixed model ANOVA analyses with seeds per flower as the response variable. Treatment (supplemented, unmanipulated, or emasculated), manipulation (single-flower or whole-plant), and their interaction were treated as fixed factors and individual plants were treated as a random factor to account for nonindependence of fruits collected from the same plant in whole plant manipulations. Analysis was carried out in the R statistical environment (R Core Development Team, 2012) using the lmer function in the lme4 package for mixed model analysis and the pamer.fnc function in the LmerConvenienceFunction package for estimation of parameter significance (Tremblay and Ransijn, 2012). We estimated the significance of fixed factors using a conservative estimate for denominator degrees of freedom. We excluded one outlier fruit that produced greater than 50% more seeds than any other fruit in the experiment (results were still significant in the same direction with the outlier included). We tested for significant differences among treatments using post hoc Tukey tests.

Comparison of alternative response variables—We conducted the analysis of PL and RA on alternative response variables to better understand how their use influences estimates of PL and RA in *parviflora*. For whole-plant manipulations, we estimated fruit set, seeds per flower, and seeds per plant. For fruit set, we used a generalized linear model with a binomial error structure and logit link function with successful fruits out of total fruits as the binomial response variable and treatment as a fixed factor. Significance of the model was assessed using a χ^2 distribution ($df = 2$). For seeds per flower, we used a linear mixed model with treatment as a fixed factor and plant as a random factor. For seeds per plant, we used a one-way ANOVA with treatment as a fixed factor. We tested for significant differences among treatments using post hoc Tukey tests.

Seed germination and viability—We analyzed the proportion of germinating seeds and the proportion of total viable seeds (germinated + viable ungerminated seeds) from fruits of single-flower treatments to test for differences among treatments. Both dependent variables were arcsin-square root transformed to improve the normality and homogeneity of variance. We tested for significant differences among treatments using post hoc Tukey tests.

RESULTS AND DISCUSSION

Self-fertilization can serve as a mechanism to ensure reproduction in the absence of sufficient pollinators or mates (Lloyd, 1992). In *parviflora*, we found significant pollinator-mediated pollen limitation and reproductive assurance regardless of the level of experimental manipulation (single-flower (SF) or whole-plant (WP)). We found a significant overall treatment effect (e.g., supplemented, unmanipulated, or emasculated) on seeds set per flower ($F_{2,178} = 35.7$; $P < 0.001$) and no significant effect of manipulation (SF vs. WP) ($F_{1,178} = 2.3$; $P = 0.132$) or the interaction term ($F_{2,178} = 1.4$; $P = 0.260$) (Fig. 1) (Appendix S1, see Supplemental Data with the online version of this article). Further, in a post hoc Tukey test, we found that emasculated flowers set significantly fewer seeds per flower than unmanipulated and supplemented flowers in both single-flower and whole-plant manipulations (Fig. 1). There were no other significant differences among treatments or between manipulations. These patterns were also consistent with the population-level estimate of pollinator-mediated PL and RA (seeds per flower) where, for single-flower manipulations, emasculated flowers produced only 47% and 52% of the seeds produced by supplemented flowers and unmanipulated flowers, respectively. For whole-plant manipulations, emasculated flowers produced only 37% and 25% of the seeds produced by supplemented and unmanipulated flowers, respectively (Appendix S2, see Supplemental Data with the online version of this article).

Autonomous self-pollination in *parviflora* also effectively eliminates overall pollen limitation for both WP and SF manipulations; supplementation of flowers with outcross pollen did not lead to appreciable increases in seed set when compared to unmanipulated flowers (Fig. 1) and population-level estimates of overall PL were low (SF: −10%; WP: 15%) (Appendix S2, see Supplemental Data with the online version of this article).

Taken together, our results along with previous field studies (Fausto et al., 2001; Moeller and Geber, 2005; Moeller, 2006) suggest that selfing is advantageous and provides reproductive

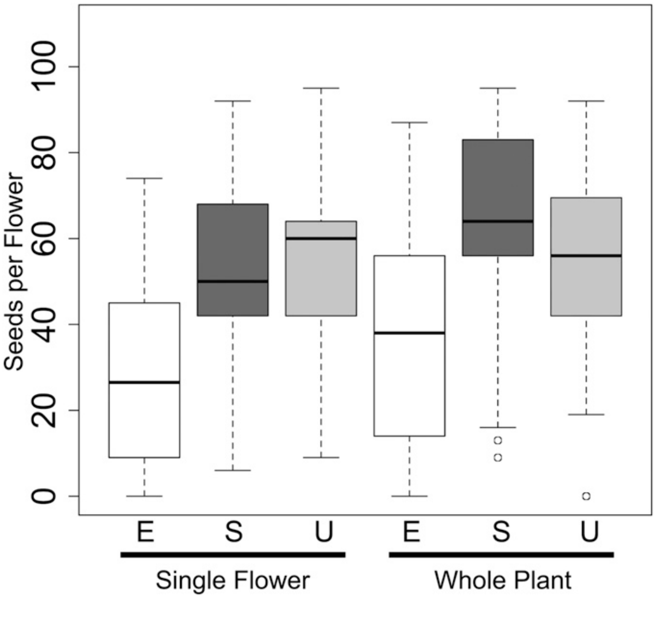

Fig. 1. Standard boxplots of *parviflora* seed set per fruit for treatments applied to whole plants (WP) and to a single flower (SF) on a plant. White bars signify emasculation (E) treatments [SF: N = 32; WP: N = 32 (94 fruits)], dark gray bars signify supplementation (S) treatments [SF: N = 29; WP: N = 30 (85 fruits)], and light gray bars signify unmanipulated (U) treatments [SF: N = 30; WP: N = 30 (87 fruits)]. In a post hoc Tukey test of treatment differences, the emasculated treatment was significantly less than unmanipulated and supplemented treatments for both types of manipulations.

assurance in *parviflora* because of a limited supply of outcross pollen. Plant density was high for the Yankee Canyon population in 2012 (~0.2 plants/m^2 averaged across the rocky terrain) and the flowering periods of individuals were highly overlapping, suggesting that the reduced seed set of emasculated flowers compared to unmanipulated and supplemented flowers was likely caused by limited pollinator visitation rather than mate availability.

Recent meta-analyses (e.g., Knight et al., 2006) have suggested that single-flower manipulations potentially bias estimates of PL due to reallocation of resources. In *parviflora*, if resource reallocation biases estimates of PL in single-flower manipulations, we expect plants to differentially shunt resources to supplemented flowers at the expense of unmanipulated flowers because the former is more likely to result in higher quality, outcrossed offspring compared to the latter. If resource reallocation biases estimates of RA, we similarly expect plants to differentially shunt resources to the fruits of emasculated flowers at the expense of unmanipulated flowers because the former is more likely to produce outcrossed offspring. When we compared estimates of PL and RA between single-flower and whole-plant manipulations, we found that the level of manipulation did not significantly affect our estimates. In fact, estimates of PL from single-flower manipulations were somewhat conservative (lower effect sizes in single-flower than in whole-plant manipulations) and estimates of RA were approximately equal between the two approaches. It is therefore feasible to use single-flower manipulations to estimate PL and RA across geographically wide-ranging *parviflora* populations where it would be untenable to conduct whole-plant manipulations at such geographic scales.

Our results indicate that plants do not differentially allocate resources to fruits according to the quantity or quality of pollen received by flowers. Because *parviflora* is an annual, resources cannot be reallocated between years and thus will not affect estimates of lifetime pollen limitation. In addition, an individual plant in the field produces virtually all of its flower buds before flowering, i.e., once flowering has begun, few, if any, new flower buds are produced. This pattern minimizes the possibility that resources can be reallocated to future flowers. A field study of pollen limitation in reciprocal transplants of *Clarkia xantiana* subsp. *parviflora* and *C. x. xantiana* (Geber and Eckhart, 2005) showed that plants receiving no pollen supplementation fail to produce additional flowers compared to plants where all flowers received supplemental outcross pollen (M.A. Geber, Cornell University, Ithaca, NY, USA, personal communication). These results similarly suggest that plants are unable to divert resources to new flowers at the expense of early flowers and fruits, even when pollen limitation is detectable. Finally, *parviflora* plants rarely abort fruits even if those fruits ultimately produce very few seeds (11 fruits from emasculated treatments produced 1-10 seeds, although there are generally ~80-100 ovules). These reproductive patterns observed in *parviflora* are not uncommon in other annual and short-lived plants. Because there is a single opportunity to reproduce, there may be relatively little selection favoring mechanisms that promote dynamic resource allocation or selective abortion. In this system, and potentially other plants, single-flower manipulations appear to be sufficient for obtaining accurate estimates of PL and RA.

Estimates of pollen limitation and reproductive assurance are possible using a variety of response metrics. In *parviflora*, we were able to estimate fruit set per plant, seeds per plant, and seeds per flower for plants where whole plant manipulations

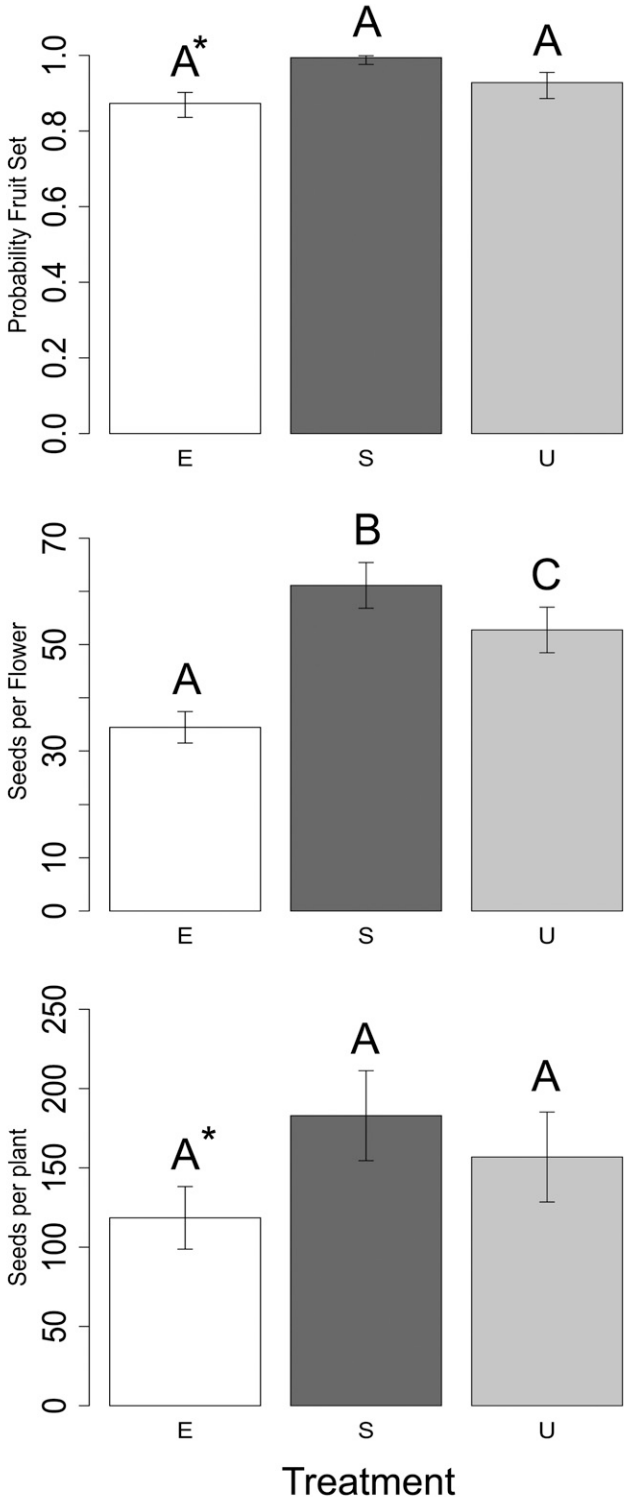

Fig. 2. Comparison of response variables for whole-plant manipulations. Response variables are the probability of fruit set (top panel), seeds per flower (middle panel) and seeds per plant (bottom panel). Estimates (Least squares (LS)-means ± SE) for emasculated flowers (E) are white bars. Estimates (LS-means ± SE) for supplemented flowers (S) are in dark gray and estimates (LS-means ± SE) for unmanipulated flowers are in gray (U). Letters represent post hoc Tukey groupings and the asterisk denotes cases where the reduced probability of fruit set in emasculated flowers was marginally significant ($0.05 < P < 0.08$).

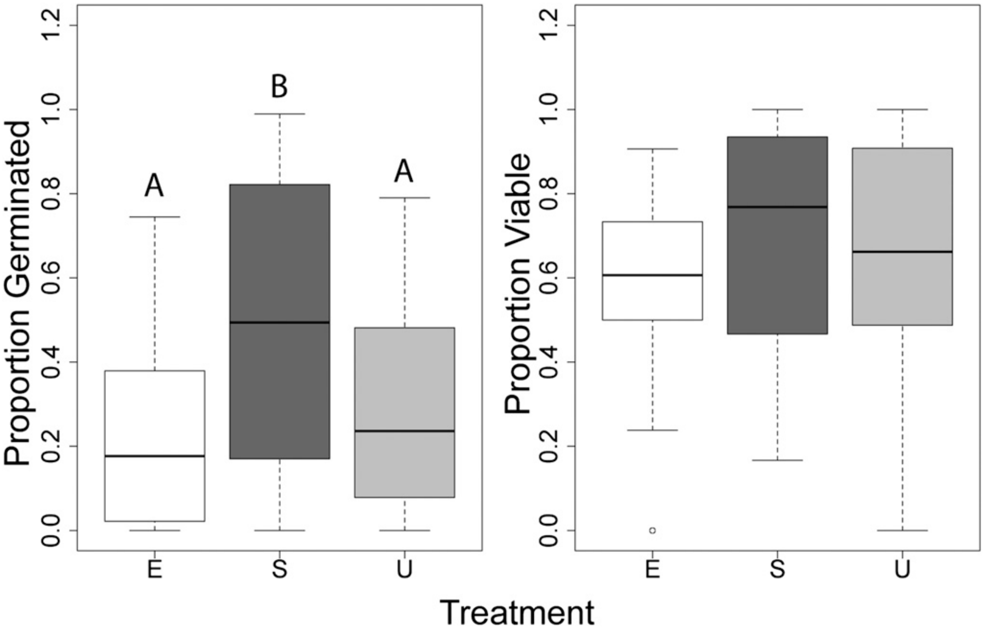

Fig. 3. Standard boxplots of the proportion of germinated seeds and the proportion of total viable seeds (germinated + viable ungerminated) for single-flower treatments. White bars signify emasculated (E) treatments ($N = 32$), dark gray bars signify supplementation (S) treatments ($N = 29$), and light gray bars signify unmanipulated (U) treatments ($N = 30$). Letters represent results of post hoc Tukey groupings where germination was significantly higher for seeds from supplementation treatments. There were no significant differences between treatments for seed viability.

were implemented. There was a significant treatment effect for fruit set ($\chi^2_{2,181} = 15.2$; $P < 0.001$) and seeds per flower ($F_{2,181} = 20.4$; $P < 0.001$) and a marginally significant treatment effect for seeds per plant ($F_{2,181} = 2.6$; $P = 0.078$) (Appendix S3, see Supplemental Data with the online version of this article). The pattern for PL and RA was the same using all three response variables (Fig. 2), but the magnitude of the differences varied widely depending on which response variable was used. Pollinator-mediated PL was only significant for seeds per flower and marginally significant for seeds per plant and fruit set. Similarly, RA and overall PL were only significant for seeds per flower (Fig. 2).

Response variables also have a relationship with the magnitude of pollen limitation measured. Knight et al. (2006) found the largest effect size for PL using fruit set and the smallest using seeds per plant. In *parviflora*, contrary to this expectation, we found the largest effect size using seeds per flower and the smallest using fruit set. *Parviflora*'s propensity to set fruits even under strong pollen limitation is consistent with our finding that fruit set differed to the least extent among treatments. The pattern for seeds per plant had larger variances but was much the same as seeds per flower (Fig. 2). This finding is consistent with the results of Knight et al. (2006).

Outcome variables relating purely to seed production may not appropriately capture pollen limitation due to differences in pollen quality (Aizen and Harder, 2007). Therefore, more detailed studies of variation among treatments in either postpollination, prefertilization mechanisms, or seed quality may be important additional steps to studies of pollen limitation. In this study, seeds from different treatments showed no overall difference in the proportion that were viable ($F_{2,81} = 2.22$; $P = 0.1145$) although there was higher germination in seeds from supplemented fruits ($F_{2,81} = 7.06$; $P = 0.0015$) (Fig. 3). In this regard, our results suggest that treatment differences in seed production are not further enhanced or dampened due to pollen quality differences that might be detected though differences in overall seed viability. The increased germination rate of seeds from supplemented fruits indicates that treatments did affect seed dormancy. It is difficult to determine the cause of differences in dormancy between treatments; the only systematic difference for supplemented flowers was the addition of pollen from a minimum of three meters distance whereas the parentage of seeds from emasculated and unmanipulated fruits is not well-characterized.

In this study, we show that partial plant manipulations do not bias estimates of pollen limitation or reproductive assurance contrary to the findings of broader meta-analyses. More experimental field studies that explicitly test for resource reallocation are needed to understand in detail how common the phenomenon is and how it is related to life history and the costs of reproduction. Although we view whole-plant manipulations as preferable, they are not feasible in many large-scale geographic studies or in species producing many flowers. In those situations, we advocate that researchers use more focused studies to identify potential biases prior to scaling up to larger studies involving partial plant manipulations.

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