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## Floral density, pollen limitation, and reproductive success in *Trillium grandiflorum*

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**Abstract** Decreases in floral density can disrupt mutualistic interactions between plants and their pollinators, and decrease reproductive success. I addressed the relationship between floral density and plant reproductive success using two experimental approaches: a pollen supplementation experiment in 12 populations of *Trillium grandiflorum* that naturally varied in floral density, and a transplant experiment in which floral density was manipulated in plots at four experimental sites. In the pollen supplementation experiments, the degree of pollen limitation, in terms of fruit set and seed set, decreased with floral density. Further, in the experimental sites, plant reproductive success increased asymptotically with floral density. These results demonstrate the value of simultaneously conducting experiments in both experimental sites and natural populations to understand how population density influences plant reproductive success. Factors that reduce the density of this perennial herb, such as habitat fragmentation and herbivory by white-tailed deer (*Odocoileus virginianus*), should be expected to limit its reproduction.

**Keywords** Allee effect · Pollen limitation · Population density · Reproductive success · *Trillium grandiflorum*

### Introduction

The positive relationship between population density and the reproduction and survival of individuals is known as

the Allee effect (Allee et al. 1949). Although Allee effects have traditionally been discussed in the context of social animals (e.g., Courchamp et al. 1999; Stephens and Sutherland 1999), they may be quite widespread among flowering plants (Kunin 1997a). This is because plants, which are immobile, rely on external vectors (e.g., wind, animal pollinators) to achieve mating, and increases in density are likely to increase the probability that the vectors will transmit pollen from one individual to another.

Many species of flowering plants rely on mutualistic interactions with animal pollinators for sexual reproduction (e.g., Buchman and Nabhan 1996). If pollinators fail to deliver an adequate quantity and quality of pollen, then plants will mature fewer seeds. The magnitude of pollen limitation, a reduction in fruit or seed set as a result of inadequate pollen deposition, is often highly variable among populations of a single species (e.g., Dudash and Fenster 1997; Baker et al. 2000; Goodwillie 2001). The floral density of a population is one factor that may predict the extent of pollen limitation; plants in lower density populations may be more pollen limited. Furthermore, anthropogenic disturbances, such as fragmentation, often decrease the floral density of a population. As a consequence, conservation ecologists have become interested in how changes in floral density will affect plant-pollinator interactions, and ultimately plant reproductive success and population viability (Kunin 1997a; Groom 1998; Hackney and McGraw 2001).

There are a number of specific mechanisms that can lead to a positive relationship between female reproductive success and floral density. Individuals occurring at low densities may suffer from insufficient pollen quantity if they attract fewer pollinators and/or receive fewer conspecific pollen grains per pollinator visit (Feinsinger et al. 1986; Klinkhamer and de Jong 1990). They may suffer from insufficient pollen quality if they are surrounded with incompatible individuals, which becomes more likely as the number of individuals in their neighborhood decreases (Ramsey and Vaughton 2000). Plants that are dioecious, self-incompatible and heterostylous may be particularly vulnerable to pollen limitation when floral density

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decreases (House 1993). It should be noted, however, that there are also mechanisms that can lead to a negative relationship between female reproductive success and floral density, such as increased competition among plants for resources and pollinators (Fritz and Nilsson 1994; Larson and Barrett 1999; Baker et al. 2000). Thus, plant populations may experience Allee effects, density dependence, or a combination of the two.

Some observational studies have found positive correlations between floral density and pollinator visits, pollen loads, fruit set and seed set (e.g., Feinsinger et al. 1986; Klinkhamer and de Jong 1990; House 1993; Steven et al. 2003). Such correlations suggest that decreased reproductive success at low floral density results from reduced pollination. However, it is also possible that other factors, such as resource availability, reduce both floral density and plant reproductive success simultaneously. Thus, experimental studies are necessary to determine if populations with lower densities of reproductive plants are more pollen limited.

Two types of experiments have been used to address the influence of plant density on pollen limitation and reproductive success. First, some studies have manipulated pollen receipt through supplemental pollen treatments in populations that naturally vary in floral density. Higher reproductive success of plants in the supplement relative to the control treatment indicates pollen limitation. The relationship between pollen limitation and floral density was highly variable across these studies (Table 1). To date, these studies have only considered a few populations, and are thus limited in their ability to describe whether there is a relationship between floral density and the magnitude of pollen limitation. Second, some studies have explicitly manipulated floral density and observed female reproductive success (Widen 1993; Kunin 1993, 1997b; Mustajarvi et al. 2001). These studies typically show a positive relationship between floral density and reproductive success.

In this study, I combine both approaches to determine the influence of floral density on the pollination biology of *Trillium grandiflorum*, a perennial herb. Studies on this species have shown variable levels of pollen limitation

(Kalisz et al. 1999; Irwin 2000; Griffin and Barrett 2002). Furthermore, if the degree of pollen limitation depends on population density, then recent anthropogenic factors including forest fragmentation and heavy browsing by white-tailed deer (*Odocoileus virginianus*), which reduce the density of this species (Anderson 1994; Augustine and Frelich 1998; Jules 1998; Knight 2003a), may increase the degree of pollen limitation.

I first conducted replicated pollen supplementation experiments in 12 different populations of *T. grandiflorum* that were highly variable in their floral density, possibly as a result of variation in the intensity of deer herbivory (Knight 2003a). Next, in areas adjacent to four of the natural populations, I experimentally manipulated floral density and quantified reproductive success. The combination of these experimental approaches has several advantages over previous studies investigating the role of floral density on plant reproductive success. First, this would be the first study to attempt quantify a statistical relationship between floral density and the degree of pollen limitation among natural populations. Second, the manipulation of density in experimental sites allows me to explore the role of density when other co-varying factors are eliminated. Finally, it was logistically difficult to find low-density natural populations where I could perform pollen supplementation experiments with adequate sample size, and thus the manipulations of floral density in experimental sites allowed higher representation of low floral densities.

## Materials and methods

### Study system

*Trillium grandiflorum* (Lilaceae) is a non-clonal herb that occurs in the understory of deciduous forests throughout eastern North America (Case and Case 1997). Reproductive plants consist of a single stem, a whorl of three leaves and a single terminal hermaphroditic flower with three white petals. Flowers bloom for 2–3 weeks in the early spring (late April to mid-May in northwestern Pennsylvania), before forest canopy leaves appear, and are primarily bumble-bee (*Bombus* spp.) pollinated (Irwin 2000). The mating system of *T. grandiflorum* varies across its range

**Table 1** A list of studies where pollen supplementation experiments were conducted in more than one population, and the densities of the populations were reported. Most of these studies were done in only a couple of populations. The relationship between pollen limitation

and densities were highly variable. In some studies, the denser population was less pollen limited (–), whereas in others, the denser population was more pollen limited (+) or equally pollen limited (0) to the less dense population

Plant	Number of populations	Relationship between pollen limitation and density	Reference
<i>Oenothera macrocarpa</i>	3	–	Moody-Weis and Heywood 2001
<i>Pedicularis palustris</i>	2	–	Karenberg and Jensen 2000
<i>Solanum carolinense</i>	2	–	Steven et al. 1999
<i>Narcissus assoarius</i>	2 <sup>a</sup>	+	Baker et al. 2000
<i>Rhexia virginica</i>	2	+	Larson and Barrett 1999
<i>Agalinis strictifolia</i>	2	0	Dieringer 1992
<i>Erica multiflora</i>	2	0	Santandreu and Lloret 1999

<sup>a</sup>actually 5 populations, but only 2 different densities

from self-compatible to self-incompatible (Broyles et al. 1997; Irwin 2000, 2001; Sage et al. 2001; Steven et al. 2003). At my study populations, *T. grandiflorum* was found to be completely self-incompatible (Knight 2003b).

I studied 12 populations of *T. grandiflorum* that occur in northwest Pennsylvania (USA) near the Pymatuning Laboratory of Ecology (Crawford County, Penn.). All populations occurred within deciduous forests and were separated from each other by an average of 15 km. I quantified the density of reproductive plants in each population by running a single transect through the spatial extent of the population, and every 10 m counting the number of reproductive plants within a 1-m<sup>2</sup> plots. The density of reproductive individuals was highly variable across these populations (ranging from 0.2 to 14.0 flowering plants per m<sup>2</sup>).

#### Pollen supplementation experiments

I performed pollen supplementation experiments in 8 populations in 2000 and 12 populations in 2001. In each population, I identified 30–40 pairs of reproductive plants. Individuals in each pair were chosen based on their similarity in size and proximity to each other, to control for plant resource and micro-site conditions. Each plant of a pair was randomly assigned to either supplement or control pollination treatments. I applied fresh pollen collected from 30 different plants within a large pollen donor population to the stigmas of flowers in the supplement treatment with a fine paintbrush. The donor population was less than 100 km away from all other populations. Control plants were not manipulated. Both supplement and control plants were open to subsequent natural pollination. Because excess pollen from 30 donor plants was applied to the stigmas of plants in the supplement treatment, these plants may have augmented quantity and quality of pollen relative to the control plants. Different plants were used in the 2 years of study.

I determined whether each flower had set a fruit, and collected all fruits in late June. I counted seeds and unfertilized ovules of each fruit under a dissecting microscope. I calculated seed set (number of seeds/number of ovules) per fruit and per flower; the first value only considered the subset of flowers that set fruit, while the latter value considered the seed set of all flowers (i.e. flowers that failed to fruit had zero seed set).

The frequency at which flowers set fruit or failed to set fruit was compared between supplement and control treatments using a  $\chi^2$  analysis. Values for seed set were arcsine transformed. The effects of pollination treatment, population, year and their interactions on seed set per fruit and seed set per flower were tested in a factorial design using ANOVA. Because this experiment was not replicated in both years for all 12 populations, this analysis only considered the 8 populations that were experimentally manipulated in both 2000 and 2001.

To determine the role of floral density on the degree of pollen limitation for these 12 populations, I calculated an effect size (average difference between supplement and control treatments) of each response variable (fruit set, seed set per fruit, seed set per flower) for each population. The mean density of reproductive plants in each population was log-transformed, and linear regression was used to determine if floral density of the populations predicted the effect size of either fruit or seed set.

#### Experimental sites

To determine if floral density affected plant reproductive success, I transplanted reproductive *T. grandiflorum* into four experimental sites (Site A in 2000 and Sites B, C, and D in 2001), each in a different forest. These sites were all approximately 250 m away from natural *T. grandiflorum* populations. This distance was chosen to minimize the effect that other *T. grandiflorum* in the forest could have on pollinator attraction while ensuring that bumblebee pollinators were in the area. Site A was near Wallace Woods, a population of *T. grandiflorum* occurs there, but reproductive

individuals were not numerous enough for pollen supplementation experiments to be conducted. The other sites were near populations where pollen supplementation experiments were conducted, and the populations are abbreviated in this study by two-letter codes; Site B was near population DC, Site C was near WC, and site D was near WH.

Within each experimental site, I established 14–17 plots that were 4×4 m in size in random locations, but separated from each other by at least 100 m to ensure that pollinators would perceive these plots as separate patches. Each plot received a randomly selected density treatment, which were similar to the range of densities naturally observed in *T. grandiflorum* populations. Specifically, in Site A, treatments were [reproductive plants per m<sup>2</sup> (replicates)]: 0.06(6), 0.25(3), 0.5(1), 1(1), 2(1), 3(1), 4(1), 6(1), 8(1), 10(1). In sites B, C, and D, treatments were: 0.06(8), 0.25(1), 0.5(1), 1.4(1), 2.9(1), 4.8(1) and 7.3(1). The plants were uniformly placed within plot in locations that maximized nearest-neighbor distance.

Transplants were excavated from two large natural populations (populations DC and WH) in late April while in the floral bud stage, and transplanted immediately into experimental plots. All transplanted individuals were randomized with respect to source populations to eliminate genetic structure from being introduced into the experimental plots. Each plant was watered 3 times in the first week of this experiment to reduce transplant shock (>95% survived). Plants consumed by herbivores (1–35% of plants per site), primarily white-tailed deer, were eliminated from all analyses.

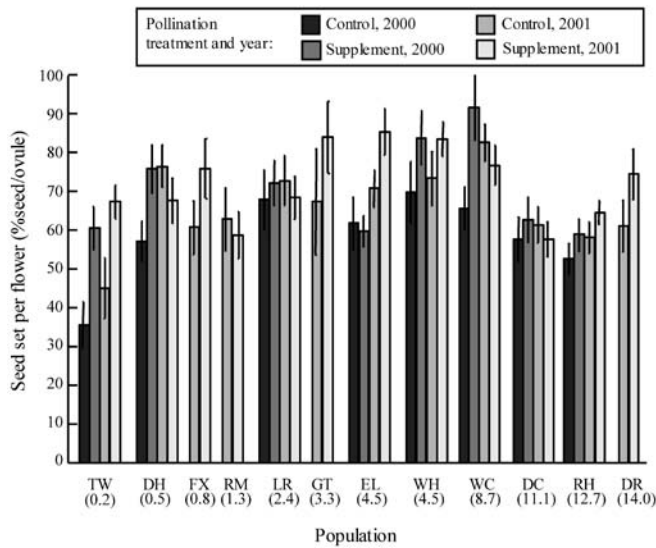
Two months later, just prior to natural fruit release, I assessed whether or not each plant set fruit. All fruits, or 30 randomly chosen fruits from plots with >30 transplants, were collected from each plot. I counted seeds and unfertilized ovules in each fruit under a dissecting microscope. I used linear regression to estimate the relationship between floral density and fruit set, seed set per fruit and seed set per flower. In addition, I fit the data to the asymptotic formula,  $y=ax^b$ . If the adjusted  $r^2$  value was higher, then the asymptotic relationship was considered a better fit (Hillborn and Mangel 1997). The four experimental sites did not significantly differ in the linear and asymptotic parameter estimates ( $t$ -tests;  $P>0.1$ ), and were therefore pooled. All of the results presented represent analyses of this pooled data. Floral density was log-transformed and all response variables were arcsine transformed. All analyses were done using SYSTAT (1999).

## Results

#### Pollen supplementation experiments

Across all populations, more flowers set fruit in the supplemental pollination treatment relative to the control treatment ( $\chi^2=16.19$ ,  $P<0.001$ ). However, fruit set was generally high among flowers in both treatments (98.9% in supplemental treatment, 93.8% in control). While, in general, the seed set per fruit and the seed set per flower of plants in the pollen supplementation treatment were greater than those of control plants, this varied across populations and among years, as indicated by the marginally significant three way interactions (Fig. 1, Table 2).

Pollen limitation was negatively related to floral density. The difference in fruit set between supplemental and control plants decreased linearly with the floral density of the population in both 2000 and 2001 (Fig. 2A). However, the difference between supplemental and control plants in seed set per fruit did not vary with the floral density of the population in either year (Fig. 2A). The difference between pollination treatments in seed set per flower

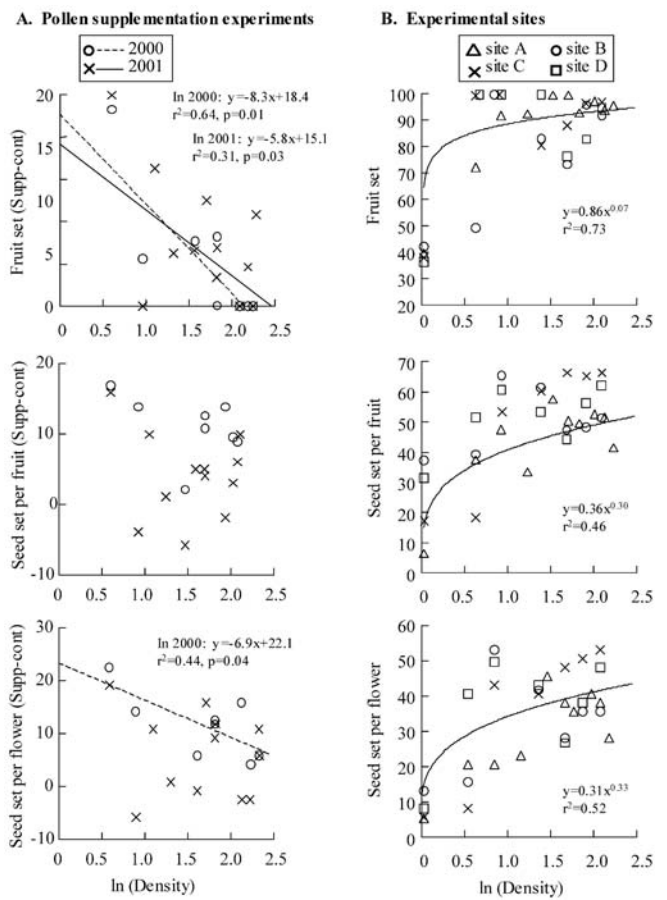


**Fig. 1** Seed set per flower ( $\pm 1$  SE) of *Trillium grandiflorum* across populations, years, and pollen supplementation treatments (supplement, control). The populations are abbreviated by a two-letter code and are shown in order of floral density; density is given in parentheses (number of flowering plants per m<sup>2</sup>)

**Table 2** ANOVA of seed set (no.seeds/no. ovules) per fruit and seed set per flower across two pollination treatments (supplement, control), in eight populations of *Trillium grandiflorum* over 2 years (2000, 2001). The eight populations included in this analysis were those that were included in the pollen supplementation experiment both in 2000 and 2001

Source of variation	df	SS	MS	F	P
<b>Seed set per fruit</b>					
Treatment	1	0.443	0.443	7.051	0.008
Population	7	4.955	0.708	11.270	0.001
Year	1	0.434	0.434	6.915	0.009
Treatment $\times$ population	7	0.453	0.065	1.031	0.408
Treatment $\times$ year	1	0.311	0.311	4.949	0.026
Population $\times$ year	7	0.508	0.073	1.155	0.326
Treat $\times$ popn $\times$ year	7	0.834	0.119	1.898	0.067
Error	677	42.519	0.063		
<b>Seed set per flower</b>					
Treatment	1	1.063	1.063	15.444	0.001
Population	7	5.693	0.813	11.815	0.001
Year	1	0.406	0.406	5.898	0.015
Treatment $\times$ population	7	0.822	0.117	1.706	0.104
Treatment $\times$ year	1	0.286	0.286	4.148	0.042
Population $\times$ year	7	0.464	0.066	0.963	0.457
Treat $\times$ popn $\times$ year	7	0.946	0.135	1.963	0.058
Error	693	47.702	0.069		

decreased linearly with the floral density of the population in 2000 but not 2001 (Fig. 2A).



**Fig. 2. A** The difference between plants in supplement and control pollination treatments in fruit set, seed set per fruit, and seed set per flower across populations of *T. grandiflorum* which vary in the density of reproductive plants. Each data point represents a population. Eight populations were considered in 2000 and 12 populations in 2001. A value of 0 indicates that there was no difference between the reproductive success of plants in the supplement and control pollination treatments, and thus the population was not pollen limited. **B** The effects of floral density manipulation on fruit set, seed set per fruit and seed set per flower in four experimental sites (A, B, C, D)

**Experimental sites**

There was a positive relationship between experimental floral density and fruit set, seed set per fruit and seed set per flower (Fig. 2B). Although both linear and non-linear regressions were significant, the non-linear asymptotic equation,  $y=ax^b$ , provided a better fit to the data than a linear regression. The adjusted  $r^2$  values for fruit set, seed set per fruit and seed set per flower were 0.45, 0.39 and 0.44 for the linear regressions, respectively, and 0.73, 0.46 and 0.52 for the asymptotic equation.

**Discussion**

Results from this study provide evidence for a positive relationship between floral density and reproductive success of *T. grandiflorum*. Across 12 natural populations,

plants in denser populations were less pollen limited (Fig. 2A). As such, to my knowledge this is the first study that statistically relates patterns of variable pollen limitation to variation in plant population density. However, the degree of pollen limitation varied across years, whereas the density of these populations remained relatively constant across years. Therefore, while density appears to play a role in determining the degree of pollen limitation among populations, other factors that vary among populations and years also play a role in this effect. While these populations occur in relatively similar forest types in the same region, there are a variety of factors that may vary across these populations, such as the density of pollinators and other plant species. Further, a variety of factors may cause yearly variation in the magnitude of pollen limitation. For example, variation in spring weather may limit the number of pollinator flying days.

The significance of the relationship between population density and pollen limitation depended on the only population of very low density. Indeed, when I removed this population from the analyses, the regressions were not significant. Populations containing very low floral density, yet containing enough reproductive plants to conduct a pollen supplementation experiment with a reasonable sample size, are very difficult to find in nature. Plants in this lowest density population had much greater seed set when pollen was supplemented in both years, and was thus severely pollen limited, while none of the other seven populations studied over multiple years showed consistent pollen limitation. This suggests that density-dependent increases in pollen limitation may only become important to natural populations when they reach very low floral densities. If there is a threshold density in which populations become pollen limited, as would be suggested by the density manipulations within experimental sites (discussed below), then it is possible that the natural populations considered in this study consisted of one population below the threshold density (where pollen limitation occurs), and 11 populations near and above this threshold.

In the experimental sites, where other factors that might influence pollination biology other than density (e.g., resources, pollinator abundance) were controlled for, there was positive relationship between experimental floral density and seed set. The shape of this relationship was asymptotic, and reductions in reproductive success were only seen at very low floral densities. I found a much stronger relationship between floral density and seed production in the experimental sites than between density and pollen limitation across natural populations. This may be because (1) I had higher replication of low-density plots in the experimental sites or (2) there less background variation across plots in an experimental site than across natural populations.

In the pollen supplementation experiment across populations, fruit set, but not seed set per fruit, was significantly related to density (Fig. 2A). There are several possible explanations for this, including: (1) flowers in low-density populations may not have been visited at all,

while others received enough pollen to fertilize many of their ovules, perhaps even from a single pollinator visit, (2) flowers must receive a critical amount of pollen in order to set fruit at all (e.g., Campbell 1986; Mitchell 1997), or (3) plants may abort poorly pollinated ovaries in order to save resources for future reproduction (Stephenson 1981). Data on pollinator visitation, or an experiment where the amount of pollen added to the stigma was varied, would allow greater understanding of the mechanisms that may be responsible for this effect.

The two experimental approaches allowed me to examine the role of floral density on plant reproductive success and the degree of pollen limitation in two different contexts. In the pollen supplementation experiments across natural populations, the density of *T. grandiflorum* is only one of many factors that varies among these sites. Thus, perhaps it is not very surprising that there was variation in the effect of density on the degree of pollen limitation. Alternatively, in the experimental sites, the plots occur within the same forest, co-occur with the same other species of plants. Thus, in this scenario, density is likely to be the main factor varying, and the effect of density on plant pollination and seed set is stronger and easier to detect.

Population size (the number of plants in the populations) is typically correlated with population density (the number of plants per unit area). However, in some populations, population size and population density may not correlate. For example, on islands or habitat fragments of varying size, density can be the same, while the total number of individuals is quite different. In such cases, population size, independent of density, can also influence pollen limitation and female reproductive success. Several mechanisms can cause reduced reproductive success in small populations, including a reduction in the diversity of self-incompatibility alleles (e.g., Byers and Meagher 1992) and uneven sex ratios (e.g., Carlsson-Graner et al. 1998). Agren (1996) studied the role of population size on the degree of pollen limitation in 14 island populations of the tristylous plant, *Lythrum salicaria*. Populations varied in the number of reproductive plants because the size of the islands varied, and so population size was independent of population density. The population size was negatively related to pollen limitation among those island populations, most likely because populations with fewer individuals were more likely to contain individuals of incompatible mating morphs.

In my 12 *T. grandiflorum* populations, floral density was positively correlated with the number of reproductive plants in the population (Pearson's correlation = 0.647). However, this correlation was much less than 1, suggesting that factors other than density also influence population size among these populations; most likely the size of the habitat fragment. I found no relationships between pollen limitation (fruit set, seed set per fruit or seed set per flower) and population size in either year of the pollen supplementation experiment across all 12 natural populations ( $P > 0.15$  for all linear regressions). Thus, population density appears to be a better predictor than population

size for determining the degree of pollen limitation among these populations. Population size and density were simultaneously manipulated within each experimental site (because the plot size was constant), and thus both predicted the observed positive relationships with seed set.

Because many plants require pollinators for reproduction, and pollination rates may be determined by plant density, as suggested here, Allee effects are likely to be quite common and important for many plant species (see also Kunin 1997a; Groom 1998; Hackney and McGraw 2001). This has important implications for plant conservation. It is widely known that small populations are more vulnerable to extinction. Even though conservation biologists and land managers take into account their stochastic risks of extinction when they plan re-introductions and set up preserves, deterministic risks such as Allee effects are often not considered (Courchamp et al. 1999). Understanding these deterministic factors would allow more realistic estimates of minimum viable population size (Hackney and McGraw 2001). *T. grandiflorum* is particularly reliant on its pollinators, because it is self-incompatible. Successful management of plants with breeding systems that make them susceptible to Allee effects may require the simultaneous management of their pollinating animals.

Pollen supplementation experiments across a range of natural populations that vary in plant density, combined with explicit experimental manipulations of plant density, can provide an important index of the influence of density on plant fitness. Such studies are particularly important in a modern context, as a wide variety of plant species are becoming increasingly rare as a result of a variety of anthropogenic influences.

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