

Reproductive assurance weakens pollinator-mediated selection on flower size in an annual mixed-mating species

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- **Background and Aims** In animal-pollinated plants, direct and indirect selection for large and small flowers in predominantly outcrossing and selfing species, respectively, is a common consequence of pollen limitation (PL). However, many hermaphroditic species show a mixed-mating system known as delayed selfing, which provides reproductive assurance (RA) only when outcrossing is not realized. Although RA is expected to reduce pollinator-mediated selection towards larger flowers, the consequences of delayed selfing for selection on flower size in mixed-mating species remain overlooked. We investigated whether RA weakens selection on flower size in *Tuberaria guttata*, a mixed-mating annual herb.
- **Methods** We related pollinator visitation rates to flower size and measured seed production in emasculated, hand cross-pollinated and intact (control) flowers in three natural populations. For each population, we estimated variation in PL and RA across individuals differing in flower size and phenotypic selection on this trait.
- **Key Results** Pollinator visitation increased and RA decreased with flower size in all populations. Increasing RA diminished but did not fully alleviate PL, because of early-acting inbreeding depression. In the least-visited and most pollen-limited population, RA increased seed production by >200 %, intensely counteracting the strong pollinator-mediated selection for larger corollas. In the most-visited population, however, RA increased seed production by an average of only 9 %. This population exhibited the largest fraction of individuals that showed a decrease in seed production due to selfing and the weakest pollinator-mediated selection on flower size.
- **Conclusions** The results suggest that the balance between the extent of RA and outcrossing contributes to determine flower size in mixed-mating systems. Pollinator-mediated selection favours larger flowers by increasing outcrossed seeds, but the benefits of RA greatly lessen this effect, especially under severe conditions of pollen limitation. Our findings also indicate that a mixed-mating system can represent an ‘evolutionary trap’ under an adequate pollinator supply.

Key words: Delayed selfing, emasculation, outcrossing, pollen limitation, pollinator visitation rates, seed production, *Tuberaria guttata*.

INTRODUCTION

Reproductive success in seed plants is often limited by pollen resulting from either few or inefficient pollinator visits or a shortage of compatible mates (Lloyd, 1992; Knight *et al.*, 2005; Alonso *et al.*, 2013). Selection on floral traits and the mating system can be particularly strong under pollen limitation. However, the possible evolutionary pathways to alleviate the negative fitness consequences of pollen limitation can be diverse, depending on species characteristics and ecological context (Harder and Aizen, 2010). For example, selection for larger flowers or other correlated traits favouring pollinator attraction and outcrossed pollen transfer has been documented in pollen-limited populations of self-incompatible species (e.g. Galen, 1989; Totland, 2001; Hodgins and Barrett, 2008; Trunschke *et al.*, 2017). Alternatively, pollen limitation can select for increasing ability to self-fertilize independently of

any pollen vector (hereafter, autonomous selfing), thus ensuring reproduction and relaxing selection for floral traits associated with increasing pollinator attraction (Lloyd, 1992; Kalisz *et al.*, 2004; Schoupe *et al.*, 2017). In the case of flower size, selection for increasing self-fertilization can also select for small flowers as the production of showy corollas can be costly, or because of selection on other correlated traits such as the anther–stigma separation (Fishman and Willis, 2008; Goodwillie *et al.*, 2010; Sicard *et al.*, 2016). Therefore, alternative adaptive options under pollen limitation may govern both the evolution of the mating system itself and the nature and strength of selection on flower size, a trait showing high heritability and sometimes high evolvability (Ashman and Majetic, 2006; Opedal, 2019). Ultimately, pollen limitation will favour larger flowers when this or other correlated traits are subjected to pollinator selection, or smaller flowers when indirectly affected by increasing autonomous selfing that ensures reproduction.

Even though pollen limitation could select alternatively for increasing outcrossing or selfing, many hermaphroditic plant species have mixed-mating systems where reproduction can occur via both autonomous selfing and pollinator-mediated outcrossing (Goodwillie *et al.*, 2005). Although the long-term evolutionary stability of this mating system is a subject of ongoing debate (Goodwillie *et al.*, 2005; Winn *et al.*, 2011; Barrett and Harder, 2017), this reproductive strategy represents the best-of-both-world's response to pollinator unpredictability as it promotes outcrossing when pollinators are present but provides reproductive assurance when absent (Becerra and Lloyd, 1992; Kalisz and Vogler, 2003; Vaughton and Ramsey, 2010a; Goodwillie and Weber, 2018). Delayed selfing is a particular type of mixed-mating system where autonomous selfing occurs at the end of flower life span when chances of cross-pollination are exhausted. This selfing mode involves either changes in floral development promoting contact of anthers and stigma or the breakdown of self-incompatibility late in flower life (Lloyd, 1979, 1992; Kalisz *et al.*, 1999). However, while this mating can diminish pollen quantity limitation, it does not necessarily alleviate quality limitation when either autonomous or pollinator-mediated selfing leads to early-acting inbreeding depression (Aizen and Harder, 2007). Despite inbreeding costs, delayed selfing is particularly beneficial because it minimizes fitness losses through either missing siring opportunities or incomplete ovule fertilization (Eckert *et al.*, 2006). Therefore, the consequences of delayed selfing, or of any other type of mixed-mating system, for the evolution of floral traits are of paramount importance to understand the relative influence of the selective pressures exerted by both pollinators and reproductive assurance under contrasting pollen limitation conditions. However, this process has been largely overlooked and deserves further attention.

The effects of a mixed mating system on the evolution of floral traits are likely to depend on the pollination environment. In particular, it is expected that increasingly pollen-limited populations experience increasing rates of reproductive assurance (Kalisz *et al.*, 2004; Brys and Jacquemyn, 2011; Rodger *et al.*, 2013). Therefore, selection for floral traits related to pollinator attraction, such as corolla size, would be more relaxed in populations where pollinators are scarce. This hypothesis predicts that in a poor-pollinator environment the strength of phenotypic selection for larger flowers is weaker when reproduction is assured by means of autonomous selfing than when it completely depends on pollinator-mediated pollen deposition. Likewise, selection is also expected to be relaxed in populations experiencing high visitation, because most flowers would receive enough pollen from pollinators before any autonomous self-pollen deposition occurs irrespective of corolla size.

Determining the strength and direction of selfing-mediated and pollinator-mediated selection on flower size represents an important first step to understand the effects of pollen limitation on floral trait evolution in mixed-mating plants in general, and in species with delayed selfing in particular. Specifically, it is expected that in species with delayed selfing the fertilization benefits achieved via this reproductive assurance mechanism increase in small-flowered individuals, as their flowers would receive fewer visits than large-flowered individuals (Elle and Carny, 2003; Kennedy and Elle, 2008; Elle *et al.*, 2010). As a consequence, reproductive assurance in these species could

potentially counteract to a large extent pollinator-mediated selection towards larger flowers. To date, the link between pollinator attraction and reproduction assurance, and its consequences for evolution of flower size, remain unexplored.

In this study, we evaluated the relative contributions of reproductive assurance and outcrossing to reproductive output in the hermaphroditic annual herb *Tuberaria guttata* (L.) Fourr. (Cistaceae). Although this species displays chasmogamous flowers, they usually attract few pollinators, and thus reproductive assurance mediated by delayed selfing reduces pollen limitation, increasing seed production (Herrera, 1992, 2004). To achieve our goal, we first compared seed number between intact (i.e. control) and hand cross-pollinated flowers, and between intact and emasculated flowers to evaluate how pollen limitation and reproductive assurance, respectively, vary across individuals differing in flower size in three natural populations with contrasting pollinator visitation rates. We predicted that under severe pollen limitation, as occurs in populations with low visitation rates and/or individual plants bearing unattractive small flowers, delayed selfing alleviates pollen limitation (Aizen and Harder, 2007). Secondly, we estimated phenotypic selection on this trait for each treatment and population. We expected that pollinator-mediated selection favours larger flowers by increasing cross-pollen deposition (i.e. as assessed in emasculated flowers), but that reproductive assurance reduces selection strength (i.e. as assessed in control flowers) especially under conditions of severe pollen limitation.

MATERIALS AND METHODS

Species and study area

Tuberaria guttata is an annual herb that occurs in western Mediterranean oak forests, meadows and open shrub habitats with acidic soils (Arrington and Kubitzki, 2003). It flowers from March to June, and each plant produces one straight inflorescence bearing a total of 10–20 yellow, disc-shaped flowers opening sequentially over its flowering period (Herrera, 2004). Within populations, flowers open synchronously across individuals each day at dawn over several weeks. Most plants open just one flower per day (mean = 1.3 flowers; range = 1–3 flowers) and each flower lasts about 6 h, dropping their petals by noon. On average, flowers contain about 20 anthers and one ovary with 50–100 ovules (Herrera, 1992, 2004). The stigmatic surface remains clean of self-pollen during the first 4–5 h after anthesis, but delayed selfing takes place when flowers drop their petals and the stamens are pressed against the gynoecium (Herrera, 1992). Because of delayed selfing, approx. 80–100 % of flowers set fruit when pollinators are excluded (Herrera, 1992). Fruits are globular woody capsules and contain between 20 and 100 seeds 0.5 mm in length (Herrera, 2004).

The study was conducted between April and June 2013 in three populations in Madrid province, central Spain (725–741 m a.s.l.; 40°32'–40°34'N, 3°41'–3°42'W; Table 1). The study populations (i.e. El Goloso, Valdelatas and Tres Cantos) were about 1–2 km apart and located in open forests with scattered trees of *Quercus ilex* L. (Fagaceae) and *Pinus pinea* L. (Pinaceae) interspersed in a shrub matrix dominated by *Cistus ladanifer* L. (Cistaceae). These were the most accessible

TABLE 1. Mean (s.d.) flower size and percentage of variation (%) in flower size among and within plants, along with mean (s.d.) pollinator visitation rates recorded in control and emasculated flowers

Population	Flower size (mm)	Variation (%)		Visitation rate (no. of visits per flower 10 min ⁻¹)		PL	RA
		Among	Within	Control	Emasculated		
El Goloso	13.9 (2.9)	92.22	7.78	0.2 (0.4)	0.2 (0.4)	0.47 (0.24)	0.77 (0.36)
Valdelatas	15.4 (2.2)	88.27	11.73	0.4 (0.5)	0.3 (0.6)	0.14 (0.33)	0.60 (0.37)
Tres Cantos	12.2 (1.5)	75.39	24.61	0.8 (0.8)	0.7 (0.6)	0.18 (0.24)	0.21 (0.51)

Mean estimates (s.d.) of total pollen limitation (PL), and reproductive assurance (RA) of *Tuberaria guttata* for the three study populations are provided.

and nearest existing populations in the study area, which facilitated the logistics of this study. Substrates are predominantly clay and sand, and the climate is dry, typical Mediterranean, with a mean annual temperature and precipitation of 14 °C and about 530 mm, respectively (Ninyerola *et al.*, 2005). Each population was characterized by a high density of individuals (up to about 5–6 m⁻²) and included several hundred flowering plants.

Experimental design

During the flowering peak, we selected and tagged all plants with synchronous flowering that showed >10 flower buds at each population. Ultimately, 50, 54 and 43 plants were sampled at El Goloso, Valdelatas and Tres Cantos, respectively. The single flower opening daily in each plant was assigned to one of the three treatments: naturally-pollinated intact flowers (hereafter control flowers), emasculated flowers that were hand-pollinated with outcross pollen (hereafter hand-pollinated flowers) and emasculated flowers that were just pollinated by flower visitors (hereafter emasculated flowers). Hand-pollinated flowers provide an estimate of (maximum) reproductive success in the absence of pollen limitation (Vaughton and Ramsey, 2010a). Therefore, we compared seed production between hand-pollinated and control flowers to estimate total pollen limitation (Aizen and Harder, 2007). Also, under the assumption that emasculation does not affect pollinator visitation, this technique can be successfully used to estimate reproductive success in the absence of any reproductive assurance mechanism (Schoen and Lloyd, 1992; Vaughton and Ramsey, 2010b; Rodger *et al.*, 2013). More specifically, since emasculation eliminates any possibility of intrafloral selfing, both autonomous and pollinator mediated, the relative difference in seed production between control and emasculated flowers provides an upper-bounded estimation of the extent to which reproductive assurance alleviates pollen limitation.

Because most individual plants open one flower per day, in each population all three treatments were applied to different plants in a given sampling day to avoid any confounding effect of daily variation in climate and pollination treatment on seed production. The three treatments were assigned sequentially to each target plant, so that a complete round of treatments was typically completed during a 3 d period. We repeated this treatment sequence three times, thus treating a total of nine flowers per focal plant, which represents a large fraction (>50 %) of all the flowers produced by each individuals plant. In total, we

sampled 1323 flowers across all three populations, and between 387 and 486 flowers per population. The assignment of all three treatments to flowers of each focal plant allows us to account for interindividual variation in our experimental design, thus increasing precision in treatment comparisons. However, resource translocation between the best and poorest pollinated flowers can unduly accentuate differences in reproduction success attributed to pollination alone (Knight *et al.*, 2005). All else being equal, this hypothesis would predict negative covariation in seed output between treatments, a pattern we did not find (see the Results).

Using tweezers and small manicure scissors, emasculation was conducted by removing anthers immediately after anthesis and before they dehisced. In removing anthers, we were careful not to inflict mechanical damage to the rest of floral structures. To ensure maximum seed output, hand pollination was carried out using a fresh pollen mixture collected from five donors located approx. 30 m distant from the recipient (Vaughton and Ramsey, 2010b). Outcross pollen was deposited on the stigma with a small make-up brush about 2 h after anthesis and emasculation, the time of maximum stigmatic receptivity (Herrera, 1992). Corolla diameter of all sampled flowers was recorded (to the nearest 0.1 mm) as a proxy for flower size using a caliper. Then, mean flower size per treatment, plant and population was calculated.

Pollinators

We monitored pollinator visitation rates to both control and emasculated flowers to evaluate three different assumptions: (1) floral emasculation does not affect attractiveness to pollinators and visitation frequency, a stringent assumption because *T. guttata* flowers are basically nectarless (Hidalgo and Cabezudo, 1995); (2) differential pollinator visitation is the main factor determining the extent of pollination limitation and thus of reproductive assurance (Knight *et al.*, 2005); and (3) there is a positive relationship between pollinator visitation rates and flower size. At each population, we evaluated the number of pollinators visiting flowers in relation to treatment and flower size over a 4 d period. In each population, 20 experimental plants, ten with a control and ten with an emasculated flower, were observed during a single day. We alternated the observed treatments within the experimental plants between consecutive observation days, and each treatment was repeated twice in each plant. Observations were conducted on sunny days to avoid any effect of daily climatic variation on pollinator visitation rates

per treatment. Flower visitors to each plant were censused during 10 min each day, totalling approx. 13 h of visitor observation per population over the 4 d of pollinator sampling. Observations were conducted between 09.00 and 13.00 h, corresponding to the peak of pollinator activity and before flower senescence. During each census, we counted visitors belonging to four large taxonomic/functional groups (i.e. muscoid flies, hover flies, solitary bees and beetles). A legitimate visit was defined as one in which the visitor's body contacted the stigma. At each treatment and plant, we calculated visitation rate as total number of visitors per flower per 10 min.

Seed production, pollen limitation and reproductive assurance

At 2–3 weeks after flowering, all ripe fruits from previously tagged flowers were collected before seed dispersal, and the total number of seeds produced per flower was counted (hereafter seed production). Seed production is the most appropriate response variable for questions related to the study of pollen limitation, because it is one of the most important components of maternal fitness of an individual plant (Knight *et al.*, 2005, and references therein). For each individual plant, we summed seed production across like-treated flowers. Then, we first estimated total pollen limitation as $PL = 1 - (\text{seed}_{\text{ctrl}} / \text{seed}_{\text{hand}})$, where $\text{seed}_{\text{ctrl}}$ and $\text{seed}_{\text{hand}}$ are the mean number of seeds per flower in control and hand-pollinated flowers, respectively (Larson and Barrett, 2000). Therefore, PL represents the expected relative reduction in fecundity due either to a lack of ovule fertilization, most probably associated with insufficient pollen deposition (i.e. pollen quantity limitation), or to embryo abortion, most probably associated with early-acting inbreeding depression (i.e. pollen quality limitation) (see Aizen and Harder, 2007). PL is expected to vary from 0 when there is no pollen limitation to 1 when there is a complete reproductive failure. However, besides sampling errors, this index can have negative values because of reduced seed set due to overpollination including, for instance, pollen tube crowding (Young and Young, 1992; Harder *et al.*, 2016). Secondly, we calculated an index for the overall reproductive assurance benefit of selfing (RA) estimated as $RA = 1 - (\text{seed}_{\text{emas}} / \text{seed}_{\text{ctrl}})$, where $\text{seed}_{\text{emas}}$ and $\text{seed}_{\text{ctrl}}$ are the mean number of seeds per flower in emasculated and control flowers, respectively (Kalisz and Vogler, 2003; Eckert *et al.*, 2006; Rodger *et al.*, 2013). Therefore, RA represents the expected relative reduction in fecundity when selfing is prevented. Positive values of RA between 0 and 1 indicate that reproductive assurance benefits via selfing. However, this index can have negative values when selfing, which leads to high embryo mortality due to early-acting inbreeding depression, is mediated by pollinators (i.e. pollinator-facilitated selfing) and ovules fertilized by self-pollen are prevented from being fertilized by outcross pollen (Lande and Schemske, 1985; Lloyd, 1992; Herlihy and Eckert, 2005; Aizen and Harder, 2007; Vaughton and Ramsey, 2010a).

Lastly, we assessed whether PL and RA vary with mean individual flower size. A significant positive relationship of PL and a negative relationship of RA to flower size would indicate lower levels of pollination limitation due to higher rates of reproductive assurance in small- than large-flowered individuals. However, the relationship of PL to flower size can also be negative if RA is able

to overcome quantity but not quality limitation. Additionally, a higher frequency of negative values of RA as flower size increases would indicate that reproductive benefits of cross-pollination are reduced because of pollinator-mediated selfing.

Statistical analysis

Differences in flower size among and within-populations were assessed by means of a mixed-model analysis of variance (ANOVA), which included population and treatment as fixed factors and plant within population as a random factor. The assumptions of normality and homogeneity of variance were tested using Shapiro–Wilk's and Levene's tests, respectively.

To determine whether pollinator visitation rates and seed production significantly differed between populations and treatments, we fitted generalized linear mixed models (GLMMs) including population, treatment and the population \times treatment interaction as fixed factors, and plant within populations as a random factor. A significant interaction indicates a different effect of the treatment on pollinator visitation rates or seed production among populations. Due to overdispersion in our count data, we ran quasi-Poisson linear models to account for an inflated variance for both pollinator visitation rates and seed production (Ver Hoef and Boveng, 2007). Main effects were estimated using the restricted maximum likelihood estimator (REML). For each population, we additionally evaluated whether pollinator visitation rates significantly increase with flower size by means of quasi-Poisson regressions. In this case, pseudo-coefficients of determination (r^2) were estimated as $1 - (D_{\text{model}} / D_{\text{null}})$, where D_{model} and D_{null} are the residual deviances of the regression model and of the corresponding null model, respectively. All GLMMs were conducted using the MASS package (Venables and Ripley, 2013) in R software (R Development Core Team, 2016).

We compared the indexes PL and RA among populations by means of a Kruskal–Wallis test. Subsequently, for each population, we regressed PL and RA vs. mean individual flower size. In each case, we compared the fit of linear and quadratic regression models, and chose the latter when the quadratic term significantly increased the coefficient of determination (Sokal and Rohlf, 1981).

We estimated and compared phenotypic selection on flower size through seed production in control, hand-pollinated and emasculated flowers per population following Lande and Arnold (1983). For each population, we first regressed relative fitness (w), calculated as individual number of seeds/mean number of seeds across individuals for each pollination treatment separately, against standardized flower size (with a mean of 0 and an s.d. of 1). Additionally, we estimated non-linear selection coefficients (γ) to further estimate stabilizing/disruptive selection by including the quadratic deviations from the mean of flower size (Lande and Arnold, 1983). Therefore, we used (standardized) flower size and squared (standardized) flower size in a second set of regression models. Quadratic regression coefficients were doubled for proper estimation of stabilizing/disruptive selection gradients (Lande and Arnold, 1983; Stinchcombe *et al.*, 2008). In both the linear and polynomial regression models conducted for each population, we included treatment and the interaction between treatment and flower size as further predictors. To determine whether the form and magnitude of phenotypic selection vary among treatments within

each population, the estimated linear and quadratic selection coefficients were compared among treatments using the ‘testInteractions’ function of the *phia* package v. 0.2-1 (De Rosario-Martínez, 2015) for R (R Development Core Team, 2016).

RESULTS

Flower size and pollinators

Flower size ranged from 8.8 and 19.8 mm in diameter, averaging 13.9 mm (s.d. = 2.6 mm). Within populations, most (>75 %) of this variation occurred among individual plants (Table 1). Plants at Valdelatas and El Goloso bore flowers that were 26 and 14 % larger than at Tres Cantos, respectively (Table 1). Additionally, Tres Cantos showed the lowest variation in flower size (Table 1). Flower size differed significantly among populations, and greatly varied among plants within populations (Table 2). Flowers assigned to the different treatments did not differ in size (Table 2), averaging 13.95, 13.96 and 13.93 mm for control, hand-pollinated and emasculated flowers, respectively.

On average (mean \pm s.d.), pollinators at each population were mainly represented by nectar-feeding muscoid (54 \pm 7 %) and hover flies (24 \pm 2 %) and, to a lesser extent, beetles (12 \pm 12 %) and solitary bees (10 \pm 9 %). Overall, we estimated an average visitation rate of about 0.4 visits per flower 10 min⁻¹. Visitation rates differed among populations and plants within populations, but did not differ between emasculated and control flowers across populations (Tables 1 and 3). Muscoid flies, the most frequent pollinators, also visited flowers independently of treatment ($t_{1,42} = 1.03$, $P = 0.31$). Therefore, emasculation did not affect pollinator visitation and frequency to any large extent. Among populations, El Goloso experienced the lowest mean visitation rate, whereas rates at Valdelatas and Tres Cantos were about twice and four times higher, respectively (Table 1). In all populations, pollinator visitation significantly increased with flower size. However, visitation rate seems to increase faster with flower size at Tres Cantos than at El Goloso (Fig. 1). No visitation was recorded to plants with small flowers at El Goloso and, to a lesser extent, at Valdelatas. On the other hand, even plants with small flowers were visited at Tres Cantos (Fig. 1).

Seed production, pollen limitation and reproductive assurance

Seed production was significantly greater in hand-pollinated than in control flowers, and in control than in emasculated flowers in all three populations (Fig. 2). However,

differences in seed production among treatments depended on population (i.e. a significant population \times treatment interaction; Fig. 2; Table 3). In agreement with differences in pollinator visitation rates, El Goloso (the least-visited population) showed the highest differences in seed production among treatments (Fig. 2). Pearson’s correlations in seed production between treatments and across populations ranged from close to 0 to highly positive, but they were never negative (range of r -values = 0.081–0.814; $n = 50, 54$ and 43 for El Goloso, Valdelatas and Tres Cantos, respectively). Absence of negative associations lessens the possibility of resource translocation between differently treated flowers within plants (see the Materials and Methods).

Differences in visitation rate translated into strong differences among populations in the extent of pollen limitation (Kruskal–Wallis $\chi^2 = 35.5$, $P < 0.001$). Accordingly, El Goloso, the least pollinator-visited population, exhibited the highest PL index followed by Tres Cantos and Valdelatas (Table 1). Actually, plants at Valdelatas were more pollen limited than at Tres Cantos, when the same overlapping range of flower sizes were compared (PL = 0.36 vs. 0.13, respectively; Kruskal–Wallis $\chi^2 = 13.6$, $P < 0.001$). Therefore, the extent of pollen limitation across populations was inversely related to visitation rate. Interestingly, the relationship between PL and flower size was positive, with a slight decrease after a maximum at El Goloso, whereas it was strongly negative at Valdelatas and Tres Cantos (Fig. 3). Overall, the proportion of all individuals exhibiting negative values of PL was 20, 24.5 and 18.6 % at El Goloso, Valdelatas and Tres Cantos, respectively ($\chi^2 = 10.5$, $P < 0.01$).

On average, reproductive assurance greatly differed among populations (Kruskal–Wallis $\chi^2 = 35.4$, $P < 0.001$), decreasing with pollinator availability and subsequently increasing with pollen limitation (Table 1). Accordingly, El Goloso, the least pollinator-visited and most pollen-limited population, was the one that exhibited the largest mean RA index, whereas Tres Cantos, the most pollinator-visited and least pollen-limited population, exhibited the smallest mean RA index (Table 1). RA increased plant reproductive success by an average of 215, 132 and only 9 % at El Goloso, Valdelatas and Tres Cantos, respectively. The RA index varied greatly among individuals within populations and was negatively related to flower size in all three populations (Fig. 4). At El Goloso, practically all individuals with flower sizes below the population mean had RAs = 1, whereas only a few of the largest flowered individuals had RA values of around 0. At the other extreme, at Tres Cantos, only two of the smallest-flowered sampled individuals had RAs = 1, whereas most individuals above the flower mean

TABLE 2. Results of ANOVA for the effects of population, plants within populations and treatment on flower size of *Tuberaria guttata*

Effect	d.f.	MS	F	P
Population	2	1102.56	23.13	<0.001
Plant (population)	144	47.67	555.89	<0.001
Treatment	2	0.02	0.28	0.757
Population \times treatment	2	0.00	0.06	0.901
Error	1174	8.58		

Population and treatment are fixed factors and plant is a random factor. Significant P -values are shown in bold.

TABLE 3. Summary ANOVA tables of the results of the general linear mixed models for differences in pollinator visitation rates and seed production of *Tuberaria guttata* among populations and treatments (fixed factors) and plants (random factor nested within population)

Variable	$\sigma^2 \pm$ s.e.	d.f.	χ^2	P
Pollinator visitation rates				
Population		2	14.28	<0.001
Treatment		2	0.43	0.806
Population \times treatment		2	0.08	0.961
Plant (population)	0.86 \pm 0.64			
Seed production				
Population		2	17.81	<0.001
Treatment		2	211.57	<0.001
Population \times treatment		4	122.41	<0.001
Plant (population)	0.24 \pm 5.62			

Both pollinator visitation rates and seed production were assumed to follow a Poisson error distribution but were analysed with a quasi-Poisson model because of overdispersion.

Sample size for pollinator visitation rates: a total of 120 visitor censuses, one on a control and one on an emasculated flower of each of 60 individuals from three populations.

Sample size for seed production: a total of 441 seed production values, three per plant and one for each of the three different flower treatments (i.e. control, hand pollination and emasculation) per plant for a total of 147 plants distributed among the three study populations.

$\sigma^2 \pm$ s.e. shows the mean residual variance \pm the standard error of the random effect.

Significant *P*-values are given in bold.

size in that population had negative values of RA. Overall, the proportion of all individuals exhibiting negative values of RA was 4.1, 5.6 and 41.9% at El Goloso, Valdelatas and Tres Cantos, respectively ($\chi^2 = 37.3$, $P < 0.001$).

Phenotypic selection

Flowers in all population–treatment combinations showed significant positive directional phenotypic selection for larger corollas via seed production, except for two particular cases: control flowers at El Goloso and hand-pollinated flowers in Valdelatas (Fig. 5). In general, positive linear selection coefficients were much larger for emasculated than for control and hand-pollinated flowers. El Goloso, the population with the least pollinator-visited and most pollen-limited flowers, exhibited the largest difference in selection strength, whereas Tres Cantos, the population with the most-visited and least pollen-limited flowers, exhibited the smallest difference. Directional selection on flower size among control flowers was stronger than among hand-pollinated flowers at Valdelatas and Tres Cantos, whereas no significant differences were found at El Goloso (Fig. 5). Significant positive quadratic coefficients for emasculated flowers at El Goloso and Valdelatas showed that fitness benefits accelerated with increasing flower size in the absence of selfing. On the other hand, a significant negative quadratic coefficient for the control flowers at Tres Cantos indicated that fitness benefits tended to decelerate with flower size in this population (Fig. 5).

DISCUSSION

Here we report novel findings on the consequences of the variation in pollinator visitation rates, pollen limitation and subsequent reproductive assurance levels achieved through delayed selfing on flower size in a mixed-mating plant. Among relevant

results, we found that the extent of reproductive assurance is correlated with the extent of pollinator failure and subsequent pollen limitation, and decreases with increasing flower size. We also found that reproductive assurance via delayed selfing greatly lessens selection towards larger flowers compared with a pure outcrossing mating system. Interestingly, average benefits of reproductive assurance, in terms of seed production, were meagre in the least pollen-limited population overall, whereas selfing, most probably mediated by pollinators, was even maladaptive for large-flowered individuals. Therefore, a mixed-mating system, even one involving delayed selfing, can represent an ‘evolutionary trap’ (Vaughton and Ramsey, 2010a) when an adequate pollinator supply can provide high outcrossing.

Our results support previous findings on the importance of the pollinator environment for the extent of pollen limitation in natural plant populations. However, in *T. guttata*, reproductive output seems to be limited not only by pollen quantity but also by pollen quality (*sensu* Aizen and Harder, 2007). More specifically, in this species, selfing seems to lead to a decrease in seed output via early-acting inbreeding depression, as suggested by several individuals showing negative values of RA, particularly at Tres Cantos, and a high incidence of PL despite high rates of RA (Figs 3 and 4). This latter finding indicates that, in most cases, while delayed selfing provides reproductive assurance, it does not necessarily maximize seed production because it alleviates pollen quantity but not quality limitation. However, an important caveat of our study is that we lack data regarding individual variation in the effectiveness of autonomous selfing. For example, variation among and within plants in herkogamy associated with flower size could influence selfing rates (Camargo *et al.*, 2017). This feature could in turn modulate the levels of reproductive assurance in our study species regardless of pollinator availability. Nevertheless, the fact that variation among and within populations in reproductive assurance in *T. guttata* was related so closely to pollinator

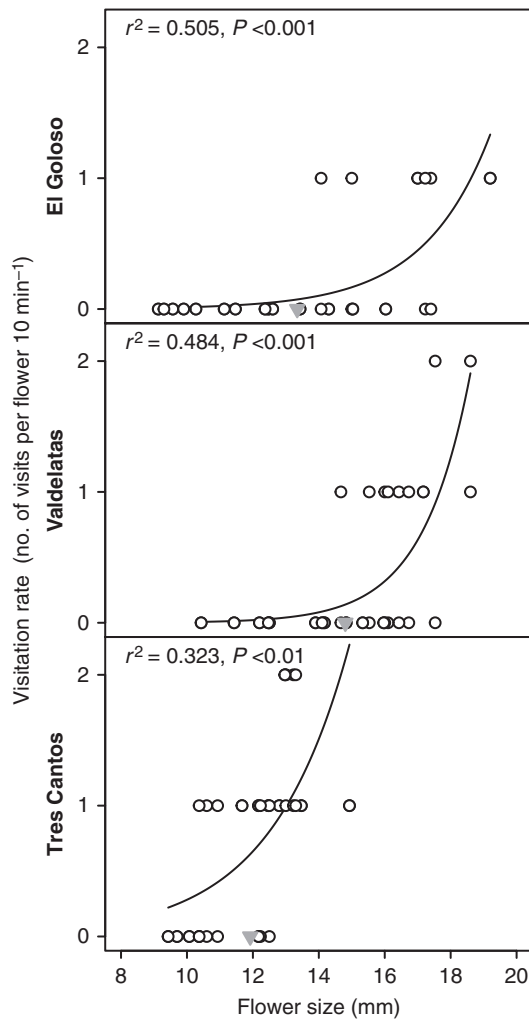


FIG. 1. Quasi-Poisson regressions of the pollinator visitation rates as a function of mean individual flower size in the three study populations. Grey arrows above the x -axis indicate the mean flower size at each population. Populations were ordered according to increasing pollinator visitation.

visitation rates lessens the possibility that changes in RA can be explained solely or even mainly by flower size-correlated variation in autonomous selfing. For instance, RA rates were much greater at Valdelatas than at Tres Cantos, even though the plants of these populations produced, on average, the largest and smallest flowers, respectively (Fig. 4).

Variation in pollinator availability and subsequent pollen limitation levels dictated the magnitude of RA found in the different populations. In this regard, El Goloso was the population in which we found the lowest visitation rates and highest levels of PL and, thus, of RA (Table 1). Similarly to our findings at El Goloso and, to a lesser extent, Valdelatas (Table 1), reproductive assurance via autonomous selfing largely increased seed production in *T. guttata* in other Spanish populations (Herrera, 1992) as well as in different plant species in which pollinator visitation was extremely low (e.g. 92 % in *Centaurium pulchellum*: Brys and Jacquemyn, 2011; 45–90 % in *Lilium formosanum*: Rodger *et al.*, 2013; 67–74 % in *Centaurium* species: Schoupe *et al.*, 2017). Therefore, our

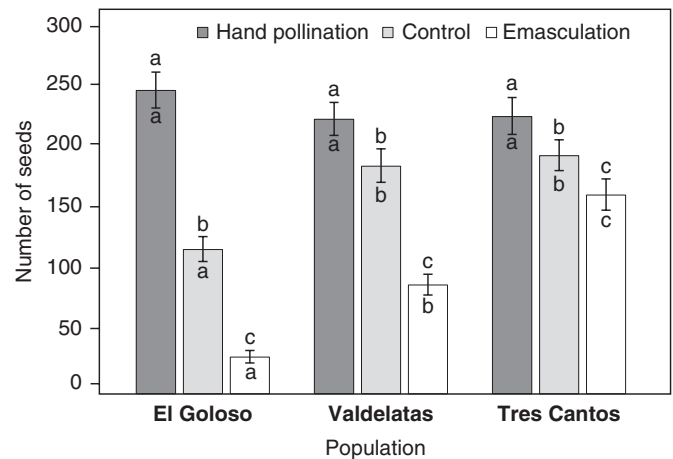


FIG. 2. Seed production per plant for control, hand-pollinated and emasculated flowers in the three study populations. Bars indicate back-transformed means (± 1 s.e.) for each population \times treatment combination. Different letters above each error bar indicate significant differences among treatments for each population, whereas different letters underneath each error bar indicate significant differences among populations for each treatment according to an *a posteriori* Tukey's pairwise test ($P < 0.05$). Populations were ordered according to increasing pollinator visitation.

results contribute to a body of knowledge supporting the hypothesis that delayed selfing guarantees seed production when pollinator visits are rare.

In addition to supporting the reproductive assurance hypothesis, our study provides further insights into the evolutionary consequences of pollen limitation for flower size in a mixed-mating system. Although we lack estimates for flower size heritability, there is reason to suspect that this trait exhibits high evolvability potential (Opedal, 2019). Flower size seems to be particularly important for pollinator attraction in nectarless species such as *T. guttata*. Muscoid flies, the prevalent pollinators in our study, are nectar-feeding pollinators that commonly visit open- and yellow-flowered species (Larson *et al.*, 2001; Lázaro *et al.*, 2008). Still, other attractive traits correlated to flower size differing among populations may be relevant to these pollinators. Similar visitation rates exhibited by control and emasculated flowers of *T. guttata* support the hypothesis that our study species is mainly pollinated by deceiving nectar-seeking insects (Thakar *et al.*, 2003), reinforcing the importance of floral traits, such as corolla size, that contribute to pollinator attraction and subsequent outcrossing. According to expectations, pollinator-mediated phenotypic selection favours larger flowers by increasing pollinator attraction and outcrossed pollen deposition. In particular, deposition of outcrossed pollen accelerated on large flower sizes in the two most pollen-limited populations, El Goloso and Valdelatas (Figs 1 and 5), stressing the relevance of floral traits promoting pollinator attraction for increasing outcrossing (Totland, 2001; Sahli and Conner, 2011; Trunschke *et al.*, 2017). However, reproductive assurance weakened selection for large flowers, especially when pollinators were scarce and pollen limitation was high (Fig. 5).

Selection for increased flower size through seed production is not uncommon in pollen-limited, self-incompatible xenogamous species (Totland, 2001; Hodgins and Barrett, 2008; Trunschke *et al.*, 2017). On the other hand, transitions

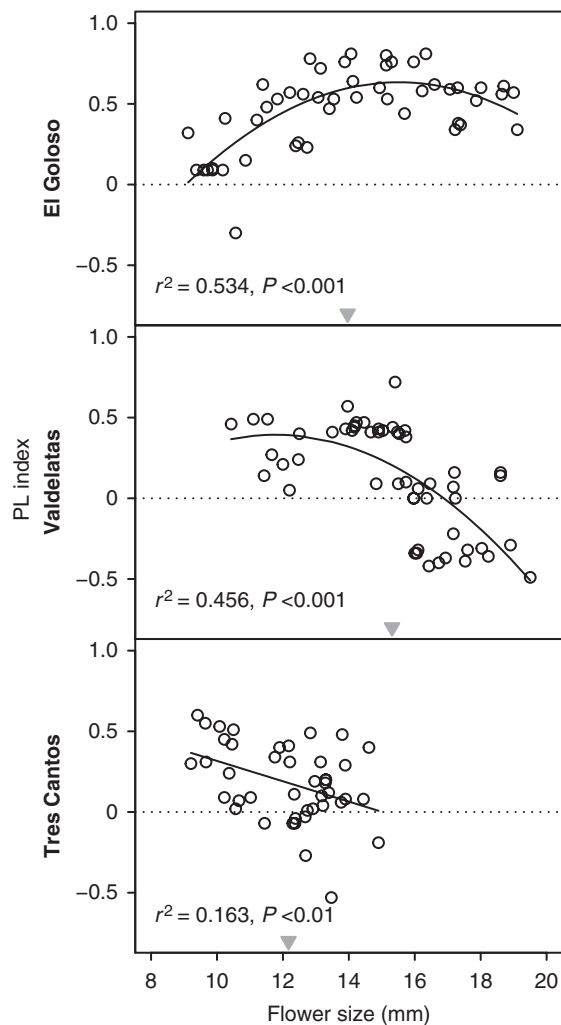


FIG. 3. Relationship of the pollination limitation (PL) index to mean individual flower size. For each population, the regression model (i.e. linear or quadratic) that best fits the data is depicted. The dotted line in each panel represents $RA = 0$ (the value at which benefits from delayed autonomous selfing equal benefits from pollinator-mediated cross pollination). Grey arrows above the x -axis indicate the mean flower size at each population. Populations were ordered according to increasing pollinator visitation.

in mating systems from outcrossing to selfing, as a strategy to provide reproductive assurance in plants subjected to severe pollen limitation, is often associated with reductions in flower size (Herlihy and Eckert, 2005; Goodwillie *et al.*, 2010; Carleial *et al.*, 2017). However, our study is the first to estimate to what extent phenotypic selection on flower size is decreased by reproductive assurance through delayed selfing. Similarly to outcrossing species, we identified that pollinator-mediated selection generally tends to favour larger flowers in a mixed-mating plant species, as evidenced by larger selection coefficients for control and emasculated flowers than for hand cross-pollinated flowers (i.e. not limited by either pollen quantity or quality) in at least two of the three populations (Fig. 5). Still, hand-pollinated flowers showed directional selection, albeit weak, towards larger flowers at El Goloso and Tres Cantos, possibly due to a positive correlation between ovule number and flower size in the study species (Herrera, 2004).

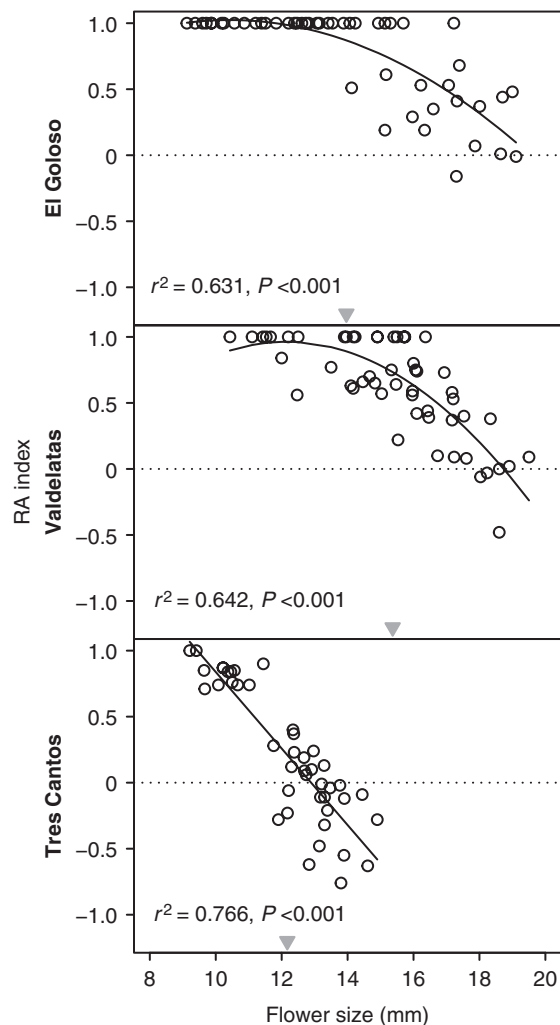


FIG. 4. Relationship of the reproductive assurance (RA) index to mean individual flower size. For each population, the regression model (i.e. linear or quadratic) that best fits the data is depicted. The dotted line in each panel represents $RA = 0$ (the value at which benefits from delayed autonomous selfing equal benefits from pollinator-mediated cross pollination). Grey arrows above the x -axis indicate the mean flower size at each population. Populations were ordered according to increasing pollinator visitation.

Interestingly, reproductive assurance can lessen the selective pressure towards larger flowers by even one order of magnitude as observed in the most pollen-limited population. Therefore, although reproductive assurance in a mixed-mating species can greatly decrease selection for larger flowers, it does not completely negate the possibility of pollinator-mediated selection, particularly when there are outcrossing benefits.

A more in-depth inspection of our results indicates that phenotypic selection mediated by pollinators, pollen quantity and quality limitation, and reproductive assurance may also determine variation in flower size within and among populations. Specifically, evidence of stabilizing selection due to fitness deceleration in control flowers was found at Tres Cantos, the most pollinator-visited and the least pollen-limited population. This population also exhibited the smallest mean flower size and lowest variation in this trait. Several studies of female selection on flower size have found evidence for stabilizing selection,

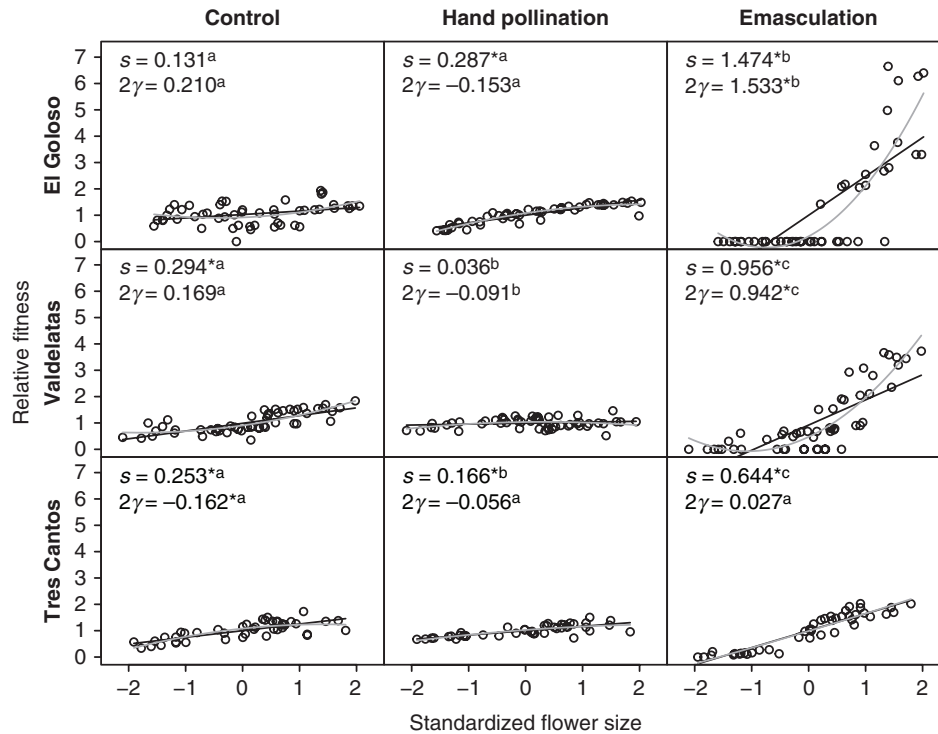


FIG. 5. Linear and quadratic phenotypic selection on mean individual flower size for control (left), hand-pollinated (middle) and emasculated (right) flowers through seed production in each of the three study populations. Significant linear or quadratic selection coefficients ($P < 0.05$) are marked with an asterisk. For each population, linear or quadratic selection coefficients with different lower case letters indicate statistically significant differences ($P < 0.05$) between treatments for linear and non-linear selection coefficients. Populations were ordered according to increasing pollinator visitation. Raw data are shown in [Supplementary Data Fig. S1](#).

but mostly due to an adaptive flower–pollinator morphological matching (Herrera, 1993; Wright and Meagher, 2004; Sahli and Conner, 2011) or due to the presence of antagonistic species (i.e. florivores, nectar robbers, etc.) that counteract the positive, directional selection exerted by pollinators (Strauss and Whittall, 2006). Pollinator-mediated selection at Tres Cantos was also the weakest, because relatively high pollinator visitation meant that even most of the smallest flowers received some outcross pollen, diminishing the strength of pollinator-mediated selection towards larger flowers. In agreement with this weak pollinator-mediated selection, the variance in relative fitness (i.e. opportunity for selection) from outcross pollination receipt (i.e. emasculated flowers) at Tres Cantos was also the lowest (Fig. 5). Therefore, stabilizing selection and average small flower size in this population could result from high rates of autonomous selfing and ensuing pollen quality limitation conferring low fitness in small-flowered individuals, and increasing costs of producing large flowers that are not outweighed by increasing outcrossing benefits in large-flowered individuals.

Interestingly, we detected no significant directional selection on flower size in control flowers at El Goloso, the most pollen-limited population and the one showing the highest variation in this trait, indicating similar fitness benefits for flowers of any size (Fig. 5). Despite high PL (Table 1), this was the only population where seed production of the smallest-flowered plants was not penalized by pollen quality limitation (Fig. 3) associated with high RA rates (Fig. 4), perhaps because these individuals were already highly inbred (Barrett and Charlesworth, 1991). At the other extreme, we recorded a reduction in PL in

the largest-flowered plants of this population, perhaps because these individuals were the only ones benefiting from pollinator visitation (Figs 1–4). Therefore, this lack of directional selection and wide variation in flower size may be explained by a combination of high rates of RA in small-flowered individuals that were not handicapped by early-acting inbreeding depression, and large-flowered plants benefiting from pollinator-mediated outcrossing (see also Runquist *et al.*, 2017). Agreeing with this interpretation, El Goloso was the population in which we found the largest (albeit non-significant) positive quadratic selection coefficient, which might indicate an incipient trend towards disruptive selection. Finally, there was evidence of positive directional selection on flower size at Valdelatas, the population with the largest flowers and intermediate pollinator visitation levels. Seed production in the smallest-flowered individuals at Valdelatas was also guaranteed by high rates of RA but, unlike El Goloso, it was penalized by early-acting inbreeding depression resulting in quality PL, whereas individuals producing larger flowers were increasingly pollinator visited and thus increasingly benefited from cross pollination (Figs 3 and 4). Therefore, the interplay between pollinator availability, pollen limitation, reproductive assurance and inbreeding depression could be key factors in understanding variation in flower size within and among populations by determining the shape and strength of phenotypic selection.

Lastly, our results also illustrate that under conditions of weak to moderate pollen limitation, a mixed-mating system, even one involving delayed selfing, can represent an ‘evolutionary trap’ in the sense of representing a maladaptive species

characteristic (Vaughton and Ramsey, 2010a). Evidence in support of this view is provided by the results from Tres Cantos, where a large proportion of flowers in this population, particularly those with large corollas, exhibited a reduction in seed production as a consequence of selfing (Fig. 4). Although some negative values in RA could be the result of a sampling error, even differences as small as 5 % in the number of seeds produced by intact and emasculated flowers are highly unlikely to have occurred by chance when considering, as a reference value, a maximum of about 100 seeds that can be produced by a *T. guttata* flower. Being conservative and assuming a threshold difference of 10 %, still about one-third of the sampled individuals at Tres Cantos showed negative values exceeding this threshold, thus confirming that selfing can have an extensive cost in this population. As pointed out above, within-flower self-pollen deposition may not only occur autonomously, but may also be mediated by pollinators, especially in large-flowered individuals. In any event, this second form of self-pollen transfer is an unavoidable consequence of a mixed-mating system that can have negative fitness costs (Harder and Routley, 2006). In the context of a stable and adequate pollinator supply over time, this scenario could favour the evolution of self-incompatibility or a polymorphic sexual system that increases outcrossing (Barrett, 2002).

Conclusion

This study confirms previous findings showing that outcrossed seed production increases in the presence of pollinators, but the benefits of delayed selfing guarantee reproduction and reduce pollen limitation when pollinators are scarce. The incidence of outcrossing and delayed selfing not only varied across populations, but also varied among individuals within populations based on differences in visitation rates related to flower size. Most interestingly and as a novel finding, the intensity of both quantity and quality pollen limitation, reproductive assurance and pollinator-mediated outcrossing rates determined the strength and shape of selection on flower size. Pollinator-mediated selection maintains larger flowers by increasing the production of outcrossed seeds, but the benefits of reproductive assurance greatly weaken the strength of selection for outcrossing, particularly in the most pollen-limited population. However, in this latter population, pollinator-mediated outcrossing could still favour the evolution of large flowers in comparison with less pollen-limited populations. Our results also reveal that a mixed-mating system, even one that involves delayed selfing, can be maladaptive under an adequate pollinator supply because of early-acting inbreeding depression associated with pollinator-mediated selfing. Together, our findings support the view that the balance between reproductive assurance and pollinator-mediated outcrossing can be important forces in the evolution of flower size and in maintaining variation in this trait in species with mixed-mating systems.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of Figure S1: raw data of the number

of seeds vs. flower size for each population and treatment used for the phenotypic selection analysis.

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