Fitness costs of delayed pollination in a mixed-mating plant

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- **Background** To predict the evolutionary consequences of pollinator declines, we need to understand the evolution of delayed autonomous self-pollination, which is expected to evolve as a mechanism of reproductive assurance when cross-pollination becomes unreliable. This involves estimating the costs of increased levels of selfing as well as those associated with floral senescence.

- **Methods** We studied the mechanisms and costs of delayed self-pollination in the mixed-mating vine *Dalechampia scandens* (Euphorbiaceae) by first assessing among-population variation in herkogamy and dichogamy, which together determine the rate and timing of autonomous self-pollination. We then tested whether floral longevity responds plastically to delayed pollination. Finally, we assessed the costs of delayed self-pollination in terms of seed number and size, explicitly separating inbreeding depression from effects of floral senescence.

- **Key results** Herkogamy varied extensively, while variation in dichogamy was more limited. Unpollinated blossoms increased their longevity, but seed quantity and quality decreased with increasing delays in pollination, independently of inbreeding depression.

- **Conclusions** In *Dalechampia scandens*, earlier autonomous selfing is facilitated by reduced herkogamy rather than reduced protogyny, providing reproductive assurance while maintaining the possibility for outcrossing events. Effective early autonomous self-pollination may evolve under reduced cross-pollination reliability in response to costs associated with floral senescence.

Key words: *Dalechampia scandens*, delayed self-pollination, dichogamy, floral ontogeny, flower longevity, herkogamy, inbreeding depression, mating system, plant fitness, pollination reliability, pollinator decline

INTRODUCTION

Most flowering plants rely on animal pollinators for seed production (Ollerton *et al.*, 2011), and even self-compatible species often exhibit mixed mating systems, with at least some offspring produced by pollinator-mediated cross-fertilization (Goodwillie *et al.*, 2005; Moeller *et al.*, 2017; Whitehead *et al.*, 2018). It is thought that if cross-pollination becomes unreliable due to pollinator declines, self-compatible species will usually evolve greater rates of autonomous selfing as a mechanism of reproductive assurance (Kalisz & Vogler, 2003; Kalisz *et al.*, 2004; Eckert *et al.*, 2006; Opedal *et al.*, 2016a). A key step towards predicting the consequences of pollinator declines for plants is therefore to understand the mechanisms by which self-pollination evolves when pollination reliability declines.

Whether selfing is selectively favoured at a given level of pollination reliability depends on the fitness of selfed relative to outcrossed offspring, i.e. the severity of inbreeding depression (Lloyd, 1979, 1992; Lande & Schemske, 1985; Charlesworth & Charlesworth, 1987). Furthermore, it depends on the potential loss of gametes and resources that could otherwise have been used for outcrossing, i.e. gamete discounting (Lloyd, 1992; Herlihy & Eckert, 2002; Porcher & Lande, 2005). Delayed selfing, occurring after the opportunity for outcrossing has passed, is considered particularly beneficial because it promotes outcrossing yet ensures seed set in unreliable pollination environments (´best of both worlds´-hypothesis; Lloyd, 1979, 1992; Becerra & Lloyd, 1992; Fausto *et al.*, 2001; Goodwillie & Weber, 2018). Delayed selfing does not inflict pollen or seed discounting, and is expected to be selected whenever the relative fitness of inbred offspring is greater than zero (i.e. inbreeding depression < 1; Lloyd, 1992).

The influence of inbreeding depression on the evolution of selfing has been the subject of extensive theoretical and empirical work (Lande & Schemske, 1985; Charlesworth &

Charlesworth, 1987; Husband & Schemske, 1996; Byers & Waller, 1999; Winn *et al.*, 2011). In contrast, fitness costs of delayed selfing other than inbreeding depression have been less well studied (Goodwillie & Weber, 2018; but see e.g. Kalisz & Vogler, 2003). Plants capable of delayed self-pollination may benefit from increased flower longevity to maximize the probability of outcrossing (Ashman & Schoen, 1994; Arathi *et al.*, 2002; Sato, 2002), but extended flower longevity imposes the costs of floral maintenance (Primack, 1985; Ashman & Schoen, 1994). Furthermore, as senescence progresses, female fecundity may decrease due to reductions in stigma receptivity and/or ovule viability, resulting in lower seed quantity and/or quality (Levy, 1988; Petanidou *et al.*, 2001; Arathi *et al.*, 2002; Castro *et al.*, 2008). Therefore, offspring produced by delayed selfing might have lower fitness than those produced by early outcrossing, independently of genetic effects due to inbreeding.

Variation in pollination environments may affect the rate and timing of selfing both in the short term, by determining the amount of cross-pollen arriving onto stigmas, and in the long term, by selecting on the extent of autofertility (seed set in the absence of pollinators; Kalisz & Vogler, 2003; Opedal *et al.*, 2016a). Thus, variation in plant mating systems is often associated with variation in herkogamy and/or dichogamy (i.e. spatial and temporal separation of sex functions, respectively). In particular, herkogamy often affects the rate of autofertility, while dichogamy may affect the timing of autonomous selfing within the blossom life span (Opedal *et al.*, 2016b; Koski *et al.*, 2018). This leads to covariation of these traits with the long-term pollination environment of populations or species (Goodwillie *et al.*, 2005; Brys *et al.*, 2013; Opedal *et al.*, 2016a).

Studies of the *Dalechampia scandens* species complex have revealed extensive variation in pollination reliability, mating system, and floral traits among populations and species (e.g. Armbruster, 1985, 1988; Opedal *et al.*, 2016a). In this mixed-mating species complex, the spatial proximity and temporal overlap of female and male functions within the blossom allow for competing or delayed self-pollination. The *D. scandens* complex therefore

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provides an opportunity to understand better the mechanisms by which delayed selfing evolves. We performed a series of experiments on 14 populations of *D. scandens* raised in a common greenhouse environment. We first assessed patterns of covariation among floral traits mediating the rate and timing of selfing by measuring herkogamy and dichogamy in populations differing in their natural pollination environment. Then, we experimentally quantified the degree of plasticity in floral ontogeny and longevity in response to delayed pollination, specifically testing whether pollinated and unpollinated blossoms differ in their ontogeny and longevity. Finally, we studied the costs of delayed pollination in terms of seed quantity and quality by conducting an experiment on three focal populations. By delaying self- or cross-pollination throughout blossom development, we explicitly separated fitness costs due to inbreeding depression from those associated with floral senescence.

MATERIAL AND METHODS

Study species and populations

Dalechampia scandens L. (s.l.) (Euphorbiaceae) is a species complex of perennial, mixed-mating vines occurring over a large geographical range in the Neotropics (Armbruster, 1985). Blossoms (pseudanthia) of *D. scandens* are functionally bisexual, comprising up to ten male flowers and three female flowers forming a functional unit (Figs 1, 2). Each female flower contains three ovules, resulting in a maximum seed set of nine seeds per blossom. A resin-secreting gland located above the male flowers serves to attract bee pollinators that use the resin for nest construction (Armbruster, 1984). The flowers are subtended by a pair of involucral bracts that open and close daily. *Dalechampia scandens* is functionally protogynous, self-compatible, and therefore potentially capable of autonomous self-pollination during the bisexual phase (i.e. period of overlap of both sex functions within a blossom; Armbruster & Herzig, 1984; Opedal *et al.*, 2015). The rate of autofertility decreases with increasing herkogamy (Armbruster, 1988; Opedal *et al.*, 2015), whereas the timing of

selfing within the blossom life span may depend both on herkogamy and the duration of the female phase (i.e. delay of the bisexual phase; Opedal *et al.*, 2016b; see below for a detailed description of blossom morphology and ontogeny).

The *D. scandens* complex comprises at least two, currently undescribed, species (Bolstad *et al.*, 2014; Falahati-Anbaran *et al.*, 2017). The 'large-glanded' species has relatively large resin glands, large showy bracts, and relatively pronounced herkogamy (anther-stigma separation, Fig. 1), indicating a history of frequent cross-pollination mediated by animal pollinators (Bolstad *et al.*, 2014; Opedal *et al.*, 2016a). Populations of the large-glanded species differ in their mating system from moderate outcrossing to nearly complete selfing (outcrossing rate: 0.16 – 0.49, $n = 4$ populations), and population-mean herkogamy is strongly correlated with outcrossing rates (Opedal *et al.*, 2016a). Relatively large anther-stigma distances in this species may prevent autonomous selfing early in the bisexual phase, yet it may occur towards the end of the bisexual phase when the male flowers abscise and contact the stigmas as they fall. The 'small-glanded' species has smaller glands, bracts and herkogamy (Fig. 2), and appears to be highly selfing. Self-pollination occurs immediately or shortly after the opening of the first male flowers, facilitated by very low herkogamy (Opedal *et al.*, 2016b).

This study was conducted in the greenhouse of the Department of Biology at the Norwegian University of Science and Technology (NTNU, Trondheim, Norway) using plants from 14 populations of both species (large-glanded species: $n = 9$ populations, small-glanded species: $n = 5$ populations). These plants were derived from seeds collected in the field in Costa Rica, Mexico, the Lesser Antilles, and Venezuela (Table 1). Populations were propagated in the greenhouse for at least one generation by random within-population crossing. Therefore, phenotypic differences among populations are expected to reflect mostly genetic differences. Plants were grown in the greenhouse under standardized conditions (26°C day / 24°C night; 13h light / 11h dark).

Blossom morphology and ontogeny

To quantify average herkogamy in each population, we measured anther-stigma distances (specifically, the distance between the central male flower and the stigma of the middle female flower within a blossom; see Fig. 1b) of one blossom per individual using digital callipers (0.01 mm precision). Measurements were taken on the first day of the bisexual phase. We recorded daily the blossom development from bud to seed dispersal in both hand-pollinated and unpollinated blossoms (Table 1). Starting with the first day of blossom opening, the female phase (Fig. 1a) typically lasts two-three days, until the first male flower opens (Fig. 1b), and the blossom enters the bisexual phase. The male flowers open successively during the bisexual phase (Fig. 1c), normally starting with the central (terminal) flower, and the largest number of open male flowers on the third day of the bisexual phase (Supplementary data Fig. S1). During this stage, bracts are white or light green, depending on population (Fig. 1a, b), and fully open for several hours during the day. At this time the blossoms may be most conspicuous to pollinators. The bisexual phase ends when the last male flower abscises or the entire male cymule (cluster of male flowers, including the resin gland) is shed. Around this time, the bracts change colour to a less conspicuous darker green (Fig. 1c, d), and do not open fully any longer. The loss of the resin gland and the change in bract colour presumably render the blossoms less attractive to pollinators. Blossoms may then enter a second female phase (Fig. 1d), which we considered as concluded when the bracts closed permanently, or the blossom was shed. We determined total blossom life span as the time from first blossom opening until the end of the second female phase, which represents the duration of floral attraction (i.e. ecological longevity), rather than the physiological floral longevity. Unpollinated blossoms are generally shed shortly after abscission of the male cymule. Pollinated blossoms initiate fruit growth and maturation and seeds are dispersed by explosive dehiscence of capsules about four to nine weeks after pollination, depending on

population.

Effects of pollination on floral ontogeny

We studied the plasticity in the duration of each ontogenetic phase in response to the timing of hand-pollination (i.e. "pollination environment"). To assess the effect of pollination on blossom development, two blossoms per individual ($n \geq 10$ plants per population, except population S8, with $n = 4$) were randomly assigned to one of two treatments. On the first day of blossom opening, blossoms receiving the pollination treatment were hand-pollinated by saturating the three stigmas with pollen from a randomly chosen paternal individual from the same population. The second treatment consisted of preventing pollination in a second blossom. To do so, we cut off the tips of the three styles on the first day of blossom opening in order to minimize autonomous self-pollination by greatly reducing the receptive surface and the probability that anthers and stigmas touch. We verified that unpollinated blossoms did not set seed when we ended blossom observation. We monitored the blossoms daily to record blossom development.

Fitness consequences of inbreeding and delayed pollination

To test the effects of inbreeding and delayed pollination on seed production and seed mass, we performed hand-pollinations with self or outcross pollen at different ontogenetic stages during blossom development in three focal populations. The populations were chosen to represent blossom phenotypes associated with mating systems ranging from moderate outcrossing to nearly complete selfing, as inferred from population-mean herkogamy. We chose a moderately outcrossing phenotype characterised by greater herkogamy and low autofertility (Tulum population), a less outcrossing phenotype with reduced herkogamy and greater autofertility (La Mancha), and a selfing phenotype with small or no herkogamy and high autofertility (Valladolid; Table 1).

Designated female (pollen-receiving) blossoms were emasculated on the first day of blossom opening, before any anthers had dehisced, to prevent autonomous self-pollination. We then pollinated the blossoms at one of four ontogenetic stages (Fig. 1): 1) the first day of the female phase; 2) the first day of the bisexual phase; 3) the last day of the bisexual phase; or 4) two days after the end of the bisexual phase (i.e. during the second female phase). Because male flowers had been removed, we used our observations of population-mean blossom ontogeny (see above) to schedule the hand-pollinations (Table 1). We used pollen from male flowers either from the same individual (i.e. geitonogamous selfing) or from another individual within the population (i.e. outcrossing). We replicated the eight treatment combinations (four pollination timings, with two pollen types each) on 15 individuals per population. Each individual served as a maternal plant and received all treatments in randomized order. Effects of paternal identity on seed mass are very limited in *D. scandens* (Pélabon *et al.*, 2015, 2016), allowing us to select sires randomly within each population. We pollinated blossoms by brushing the male flowers across the stigmas, saturating them with pollen. If the blossom was closed permanently (or almost closed) on the scheduled day of pollination, we gently opened the bracts to perform the pollination ($n = 36$, largely in the latest pollinations in the Valladolid and La Mancha populations). To collect the seeds, we bagged the blossoms with tea bags prior to explosive dehiscence.

If a blossom was shed before the scheduled pollination day, aborted before seed initiation, or failed to set viable seeds, the cross was recorded as unsuccessful. Seed sets with at least one viable seed were recorded as successful. For each successful cross, we counted the number of viable seeds per blossom and weighed the seeds individually ($n = 2204$) on a precision balance (0.1 mg precision). We used seed mass as a measure of offspring quality because it affects later offspring performance positively in *D. scandens* (Opedal *et al.*, 2015; Pélabon *et al.*, 2016). Underdeveloped seeds (< 10 mg, $n = 28$ in 15 seed sets) were removed from all analyses. Previous studies of *D. scandens* showed that blossom size may affect seed

size (Pélabon *et al.*, 2015, 2016). We accounted for this effect by measuring the diameter of the blossom peduncle with digital callipers (0.01 mm precision) and using this as a proxy for blossom size in the analyses.

Statistical analyses

All analyses were performed in R version 3.3.1 (R Core Team, 2016). To test the effects of delayed pollination with self- and cross-pollen on the probability of successfully setting seed, we fitted generalized linear mixed-effect models with the probability of setting seed as the response variable. The timing of pollination (four levels), pollen type (self/ cross) and population, as well as their interactions, were fixed factors, and maternal individual was a random factor accounting for unmeasured maternal effects on seed quantity and quality. The model was fitted with a binomial error distribution and logit link-function (lme4 R-package; Bates *et al*., 2015). We obtained the probability of successfully setting seed in each treatment through inverse-logit transformation of the parameter estimates. Similarly, 95% CIs were obtained through inverse-logit transformation of the upper and lower bounds of the confidence intervals.

We analysed seed set per blossom by fitting linear mixed-effect models with timing of pollination, pollen type and population, as well as their interactions as fixed factors and maternal individual as a random factor. Although seed number is a count variable, the distribution of residuals was close to normal, allowing for the use of a linear model. To analyse variation in seed mass we fitted linear mixed-effect models with timing of pollination, pollen type and population, as well as their interactions as fixed factors. We further included seed set and peduncle diameter as covariates to account for a possible trade-off between seed mass and seed number within seed set, and possible blossom-size effects, respectively. Blossom identity nested within maternal individual were included as random effects. We selected models fitted with maximum likelihood (ML) by comparing Akaike information

criterion values corrected for small sample sizes (AICc).

Inbreeding depression and cost of delayed pollination

We estimated overall reproductive performance at the blossom level as: reproductive performance = probability of setting seed × number of seeds per seed set × blossom average seed mass. Unsuccessful crosses were assigned a reproductive performance of zero. Following Ågren and Schemske (1993), inbreeding depression (δ) was estimated for reproductive performance as the performance after selfing (w_s) relative to the performance after outcrossing (w_o) with $\delta = 1-w_s/w_o$, if $w_s \leq w_o$, and $\delta = w_o/w_s-1$, if $w_s > w_o$, thus weighing inbreeding and outbreeding depression equally. Hence, positive values represent better reproductive performance after outcrossing than selfing, and negative values represent better reproductive performance after selfing than outcrossing. We estimated inbreeding depression for each population and each pollination time separately.

Finally, we obtained a measure of the 'delayed self-pollination cost' by comparing the relative reproductive performance of blossoms outcrossed early in flower life during the female phase (w_o at timing 1 and 2 combined) with the performance following delayed self-pollination later in blossom life (w_s at timing 2, 3, or 4). We obtained 95% CIs of the inbreeding depression and the delayed self-pollination cost from 1000 non-parametric bootstrap estimates, resampled at the blossom level.

RESULTS

Blossom morphology and ontogeny

Average herkogamy (anther-stigma distance) was about three times larger in the large-glanded species than in the small-glanded species (Table 1), and 73.2% of the variation in herkogamy was due to this between-species difference (Table 2). In contrast, most of the variation in dichogamy (duration of the female phase) occurred at the among-blossom level,

and no extra variation occurred between species (Table 2). On average, the bisexual phase was about one day longer in the large-glanded species (Table 1) representing 30.2% of the total variation (Table 2). The duration of the second female phase was similar across species (2.5% variation due to species differences), with moderate variation (22.2%) among populations (Table 2). Total blossom life span was about two days longer in the large-glanded species than in the small-glanded species (Table 1), but the largest part of the variation occurred at the blossom level (48.2%; Table 2).

Herkogamy and dichogamy tended to be correlated positively among populations, though with poor statistical support (large-glanded species: Pearson´s $r = 0.32$, $p = 0.40$, $n = 9$; small-glanded species: $r = 0.80$, $p = 0.10$, $n = 5$; Fig. 3). Likewise, the correlation among individuals within each population was generally not statistically different from zero (mean $r = 0.12$, with $p \geq 0.09$ in all populations), except in the highly selfing Valladolid population ($r = 0.86$, $p = 0.001$).

Plasticity of floral ontogeny in response to pollination

Across all 14 populations, unpollinated blossoms prolonged the bisexual phase by 0.31 days on average (paired t-test, $t = 4.10$, $p < 0.001$), and increased their total longevity by about one day ($t = 3.18$, $p = 0.002$; Fig. 4). In contrast, pollination did not detectably affect the duration of the female phase ($t = 1.43$, $p = 0.155$). Although the second female phase was about half a day longer in unpollinated blossoms, this difference was weakly supported statistically ($t = 1.81$, $p = 0.072$). The effect of pollination on floral ontogeny was stronger in the small-glanded species than in the large-glanded species, as indicated by greater differences between pollinated and unpollinated blossoms (Fig. 4).

Fitness consequences of delayed pollination

Blossoms pollinated on the first day of the bisexual phase nearly always set seeds,

while earlier and especially later pollinations tended to be less successful (Table 3, Fig. 5). These patterns and their magnitudes were population-specific, with the strongest decline in the probability of setting seed observed in the population with the selfing phenotype (Valladolid). Pollen type (self/cross) did not detectably affect the probability of setting seed in any population (Supplementary data Table S1).

The number of seeds produced in successful seed sets (291 out of 358 crosses) declined with the delay in pollination (Table 3, Fig. 5). The largest numbers of seeds resulted from pollination on the first day of the bisexual phase, while delayed pollination during the second female phase yielded only around two thirds of the maximum seed set. This pattern was consistent across pollen types and populations (Supplementary data Table S1). Of the unsuccessful crosses, 17% were due to blossoms being shed before the scheduled pollination on or after the last day of the bisexual phase. These were distributed equally across populations. The remaining unsuccessful crosses were due to blossom abortion before seed maturation or failure to mature fully the seeds. Underdeveloped seeds occurred very rarely (ca. 1% of all seeds) and were randomly distributed across treatments and populations. These seeds were removed from further analyses. In the highly selfing Valladolid population, about 27% of the blossoms that had closed permanently on the scheduled day of pollination, successfully set seed. In contrast, ca. 83% of the blossoms that were closed on the scheduled day of pollination successfully set seeds in the intermediately outcrossing La Mancha population.

Average seed mass varied among populations and decreased with the delay in pollination (Table 3, Fig. 5). The effects were population specific, but did not generally depend on pollen type (Supplementary data Table S1). We detected the most severe decline in seed mass in the highly selfing Valladolid population. In this population, seeds produced by self-pollination were lighter than those produced by cross-pollination, with self-seeds resulting from pollination during the second female phase being 18% lighter than the

population mean seed mass (Table 3). Peduncle diameter and seed set had weak, and generally statistically non-significant, effects on seed mass (Supplementary data Table S2).

Reproductive performance (estimated as the product of the probability of setting seed, seed number and seed mass), which combines the effects of delayed pollination on seed number and seed mass, declined strongly with the delay in pollination in all populations, with the strongest decline observed in the highly selfing Valladolid population (Fig. 5).

Inbreeding depression

Inbreeding depression (δ) for reproductive performance was weak and generally not statistically different from zero (indicated by 95% CIs overlapping zero; Table 3, Fig. 6). We found no differences in inbreeding depression among populations (more outcrossing Tulum population: $\delta = 0.06$, 95% CIs = -0.15, 0.24; intermediately outcrossing La Mancha population: $\delta = 0.13$, 95% CIs = -0.06, 0.30; and highly selfing Valladolid population: $\delta = -0.19$, 95% CIs = -0.41, 0.06), or among treatments. The one exception was the strong inbreeding depression (37%) that followed pollination on the last day of the bisexual phase detected in the intermediately outcrossing La Mancha population. We also detected cases of significant outbreeding depression following pollination during the second female phase in the La Mancha population, and during the first day of bisexual phase in the highly selfing Valladolid population (Table 3, Fig. 6).

Combined fitness costs of inbreeding depression and delayed pollination

Combining the cost of delayed pollination (which presumably reflects floral senescence) with inbreeding depression provides an estimate of the cost of delayed self-pollination. This cost increased with increasing delay in pollination (Fig. 6). A delay of self-pollination until the last day of the bisexual phase decreased reproductive performance in the more outcrossing Tulum population by 58% (95% CI: 44% – 73%) relative to early

outcrossing, and, in the intermediately outcrossing La Mancha population, by 46% (29% –

63%). In contrast, in the highly selfing Valladolid population, reproductive performance was

26% (15% – 39%) higher following selfing on the first day of bisexual phase relative to

outcrossing at this stage, while selfing on the last day of the bisexual phase decreased

reproductive performance by 60% (40% – 79%) relative to early outcrossing.

DISCUSSION

Variation and covariation in mating-system traits

Geographical variation in pollination environments is often correlated with variation in plant

mating systems and associated floral traits (Armbruster, 1985, 1988; Moeller, 2006; Pérez-

Barrales *et al.*, 2007; Eckert *et al.*, 2009; Opedal *et al.*, 2016a). In self-compatible

populations, we expect reduced herkogamy to be associated with increased rates of

autofertility, and reduced dichogamy to be associated with selfing earlier in the floral lifespan.

As expected from previous work, we found herkogamy to vary extensively within the *D.

scandens* species complex. In contrast, we detected only limited variation in floral ontogeny

across populations. Although populations of the more outcrossing, large-glanded species had

longer blossom life spans, we found only minor differences in dichogamy across populations

and species. This confirms the results of an earlier study finding an association of pollinator

abundance with herkogamy but not protandry in *Clarkia xantiana*, even though both traits

affect reproductive success via autonomous selfing, and thus should be selected similarly by

the pollination environment (Moeller, 2006).

Herkogamy and dichogamy may be subject to similar selective pressures by the

pollination environment, thus generating a positive correlation between the two traits (e.g.

Holtsford & Ellstrand, 1992). In *D. scandens,* these traits appear to have evolved somewhat

independently, as indicated by the generally weak among- and within-population correlations

between the two traits. The lack of covariation between herkogamy and dichogamy within

populations (Opedal *et al.*, 2015; and see Results) suggests that any among-population covariation results from correlated selection rather than genetic covariance (Armbruster & Schwaegerle, 1996). The large difference in herkogamy between species also suggests that early selfing in the more selfing, small-glanded *D. scandens* species is facilitated primarily by reduced herkogamy. The duration of the first female phase appears to be a highly conserved trait within the *D. scandens* species complex, maintaining the possibility of outcrossing events, even in the most autogamous taxa. This is in contrast to studies in other species, for example in *Blackstonia perfoliata,* where dichogamy was shorter in populations from pollinator-poor environments, suggesting evolution towards earlier selfing (Brys *et al.*, 2013).

Consistent with the expectation of reduced investment in floral maintenance in selfing taxa, the total blossom life span was somewhat shorter in the small-glanded than in the large-glanded species of the *D. scandens* complex. Our pollination experiment indicated that individual blossoms were able to respond plastically to their pollination environment (Fig. 4). In the absence of pollination, blossoms were maintained for longer periods of time. In turn, pollination shortened the period of female receptivity and initiated earlier seed maturation. Similar patterns of shortened periods of blossom receptivity and floral attraction triggered by completion of the female function have been reported in numerous other species (Stead, 1992; van Doorn, 1997; Arathi *et al.*, 2002; Sato, 2002; Castro *et al.*, 2008). This might represent an adaptation to minimize the cost of floral maintenance, while maximizing the chances for late pollination in unpollinated blossoms (Ashman & Schoen, 1994; Sato, 2002).

The stigmas of blossoms left unpollinated were cut off to prevent autonomous self-pollination. Because the portion of the style removed represents only a small fraction of the female flower, we did not expect this manipulation to have reduced the maintenance cost of the female flowers, and thereby increased the longevity of the blossoms. If anything, the tissue damage inflicted may have reduced blossom life span, possibly masking the effect of the pollination treatment. Because unpollinated blossoms subjected to the stigma-removal

treatment lasted longer than pollinated blossoms, it is likely that the direct effect of cutting off the stigmas on blossom development was negligible and did not confound our results regarding the effect of pollination on blossom ontogeny or longevity.

Fitness consequences of delayed pollination

Reproductive performance of the blossoms peaked following pollination early in flower life and declined with later pollination (Fig. 5). As suggested for numerous other species, female fecundity may decrease with flower age due to physiological processes such as decreased stigma receptivity or ovule viability (Levy, 1988; Petanidou *et al.*, 2001; Arathi *et al.*, 2002; Castro *et al.*, 2008). Some of the latest pollinations could not be performed because the blossoms were shed before the scheduled pollination date or abscised before seed initiation. Thus, part of the observed decrease in the probability of setting seed was due to short floral life spans rather than curtailed female fecundity. As expected, this phenomenon was most pronounced in the highly selfing, small-glanded Valladolid population and was only rarely observed in the more outcrossing Tulum population. This partly explains the strongest decline in reproductive performance with delayed pollination observed in the highly selfing Valladolid population (Fig. 5).

When late pollination yielded seeds, these were fewer and smaller than those obtained by early pollination. In *D. scandens*, reduced seed production by old flowers often occurred through complete seed-set failure of individual flowers within a blossom, while the other flowers produced near-maximum seed set (L.S.H. *personal observation*). This may indicate differential rates of senescence among flowers within a blossom, or selective abortion of individual flowers. In either case, blossoms may remain open to promote cross-pollination as long as they contain fertile female or male flowers or to increase overall floral attraction of the plant individual (Primack, 1985; van Doorn, 1997). Seed mass, our measure of seed quality, also declined with the delay in pollination, suggesting that flower senescence not only

affects maternal performance, but also individual offspring quality. Reductions in seed number or quality with flower age have been reported in other species. For example, Arathi *et al.* (2002) found that pollination of *Mimulus guttatus* towards the end of the floral life span resulted in less than 20% of the number of seeds produced by early pollination. Likewise, in a study on *Polygala vayredae* by Castro *et al*. (2008), delayed pollination resulted in significant reduction in seed mass.

Inbreeding depression and the cost of delayed self-pollination

Inbreeding depression is the principal genetic factor selecting against selfing (Lande & Schemske, 1985; Charlesworth & Charlesworth, 1987; Lloyd, 1992). The effects of inbreeding on plant fitness are expected to covary with population mating histories due to differential purging of deleterious alleles (Charlesworth & Charlesworth, 1987; Husband & Schemske, 1996; Winn *et al.*, 2011). Inbreeding depression in plants is typically quantified by comparing the performance of selfed and outcrossed offspring resulting from pollination at a fixed developmental stage, e.g. during the female phase. Confirming previous studies of inbreeding depression in *D. scandens*, we failed to detect substantial inbreeding depression for early-life fitness components (Opedal *et al.*, 2015; Pélabon *et al.*, 2015). We also failed to detect a systematic increase of inbreeding depression when pollination was delayed (Fig. 6). However, inbreeding depression may be expressed at later life stages that were not investigated in this study (Husband & Schemske, 1996), and a previous study of *D. scandens* suggested substantial inbreeding depression in natural populations (Opedal *et al.*, 2016a). Furthermore, in sequentially flowering perennial species like *D. scandens*, the cost of selfing may be expressed in a variety of different ways not studied here. Reproduction via selfing may draw resources from maturing outcrossed seeds (Herlihy & Eckert, 2002) and affect survival and reproductive fitness in later seasons (Morgan *et al.*, 1997). Lastly, our pollination method of saturating the stigmas with pollen, and the resulting pollen competition, may have

mitigated inbreeding depression, rendering it undetectable (Armbruster & Rogers, 2004; Lankinen & Armbruster, 2007; but see Opedal *et al.* 2015).

Delayed selfing under natural conditions occurs, by definition, later than (potential) outcrossing (Lloyd, 1979, 1992; Goodwillie & Weber, 2018). In the more outcrossing populations of *D. scandens* (Tulum and La Mancha) autonomous self-pollination may normally occur late in the bisexual phase, when the male flowers abscise and contact the stigmas as they fall. Consequently, to estimate the cost of delayed selfing, an ecologically more relevant test consists in comparing reproductive performance following early outcrossing with the performance following selfing at the end of the bisexual phase. A delay of selfing towards the end of the bisexual phase reduced blossom performance by 58% in the more outcrossing Tulum population, and 46% in the intermediately outcrossing La Mancha population, compared to early outcrossing. In contrast, in the highly selfing Valladolid population, autonomous selfing typically occurs in the beginning of the bisexual phase, facilitated by low herkogamy. Reproductive performance following selfing on the first day of the bisexual phase was 26% higher than with early outcrossing, suggesting no fitness cost, and even some benefit, of selfing when it occurs early in blossom life. Due to logistic constraints, we did not replicate our experiment within mating-systems and observed differences in the consequences of delayed pollination could result from population characteristics other than the mating system. However, similar patterns were detected by Kalisz and Vogler (2003) in a study of *Collinsia verna*, where inbreeding depression in fruiting success was weak, but when selfing was delayed, reproductive success was lower than with outcrossing. As in our study, the effect in *C. verna* was inconsistent across populations and varied within the flowering season.

The contribution of floral senescence to realized inbreeding depression demonstrated in this study may have implications for the evolution of plant mating systems and the maintenance of outcrossing. If offspring produced by geitonogamous or pollinator-facilitated

within-blossom selfing early in blossom life have greater fitness than those produced by delayed autonomous selfing, this could lead to selection for greater pollinator attraction and hence greater arrival of both self and cross-pollen. Similarly, Holsinger and Thomson (1994) detected a positive relationship between self- and cross-pollen deposition in *Erythronium grandiflorum*, and they suggested this pattern as a mechanism contributing to the maintenance of outcrossing. Importantly, reduced fitness of offspring resulting from delayed pollination may be independent of offspring genotypes, and no purging of deleterious recessive alleles is therefore expected to result from delayed self-pollination costs as defined in this study.

CONCLUSIONS

The evolution of delayed self-pollination under pollinator declines

Our results add to the evidence suggesting that pollination ecology can strongly affect the evolution of plant mating system (Holsinger, 1991; Porcher & Lande, 2005; Devaux *et al.*, 2014). As long as some cross-pollination occurs, observations suggest that a mixed mating system is maintained in *D. scandens* (Opedal *et al.*, 2016a). Outcrossing could also be maintained by strong late-acting inbreeding depression (Opedal *et al.*, 2016a) or unknown trade-offs with paternal fitness through pollen export (Johnston *et al.*, 2009). Furthermore, costs of delayed selfing caused by floral senescence could lead to fitness differences between offspring resulting from early pollinator-mediated selfing compared to those resulting from late autonomous selfing. This process would not preclude the evolution towards earlier selfing under complete pollination failure but could contribute to the maintenance of a stable mixed mating system via the maintenance of protogyny. Furthermore, it could constrain the evolution towards competing selfing or complete autonomous selfing as long as some cross-pollination occurs.

Delayed selfing is nearly always advantageous in unreliable pollination environments (Lloyd, 1979, 1992), and, if pollination fails completely, selection is likely to favour

autonomous selfing early in the bisexual phase. In *D. scandens,* evolution of earlier selfing appears to occur primarily through the evolution of reduced herkogamy, while distinct protogyny is maintained perhaps as a mechanism of maintaining the opportunity for rare outcrossing events or as a result of some developmental constraint. Herkogamy appears to be a highly evolvable trait able to respond rapidly to selection imposed by declining pollination reliability (Bodbyl Roels & Kelly, 2011; Opedal *et al.*, 2017). This suggests that, in the context of declining pollinator populations, evolutionary shifts toward greater rates of autonomous self-pollination can be rapid.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: Model selection for the effects of the timing of the pollination, pollen type and population on the probability of setting seed, seed number per seed set, and seed mass in three populations of *Dalechampia scandens*. Table S2: Parameter estimates for the highest ranked models describing the effects of the timing of the pollination, pollen type and population on the probability of setting seed, seed number per seed set, and seed mass in three populations of *Dalechampia scandens*. Figure S1: Mean number of open male flowers on each day of the bisexual phase in 14 populations within two species of *Dalechampia scandens*.

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Figure legends

Figure 1: Blossom inflorescences of *D. scandens* (intermediately outcrossing La Mancha population of the large-glanded species) throughout blossom ontogeny. Blossoms are shown in the female phase (a), on the first day of the bisexual phase (b), late in the bisexual phase (c), and during the second female phase (d). Arrows indicate the first open male flower surrounded by closed male flower buds, and the stigmas of the three female flowers within a blossom. Average gland width in the large-glanded species is 7.28 ± 0.19 mm (Bolstad *et al.*, 2014). Photos by Per Harald Olsen.

Figure 2: Blossom inflorescences of *D. scandens* (small-glanded species) late in the bisexual phase. Arrows indicate the stigmas of the three female flowers within a blossom. Stigma tips are touching the pollen-loaded male flowers. Pollen visible on the stigmas was deposited by autonomous self-pollination. Average gland width in the small-glanded species is 5.15 ± 0.13 mm (Bolstad *et al.*, 2014). Photo by Per Harald Olsen.

Figure 3: Relationship between herkogamy and protogyny (i.e. dichogamy) in 14 populations within two species of the *D. scandens* complex. Points represent mean trait values for each population of the large-glanded species (closed circles) and the small-glanded species (open circles), respectively. Error bars represent ± SE. Lines indicate the correlation between herkogamy and protogyny within the large-glanded species (solid line; Pearson´s $r = 0.32$, $p = 0.40$) and small-glanded species (dashed line; $r = 0.80$, $p = 0.10$), respectively. Abbreviations next to the plotting symbols code for the population names (see Table 1).

Figure 4: Pollination effect on the duration of each ontogenetic stage (in days after blossom opening) in 14 populations within two species of the *D. scandens* complex. Points represent

the population-mean duration of each ontogenetic stage in pollinated (closed circles) and unpollinated blossoms (open circles), respectively. Error bars represent ± SE. The day of first blossom opening is at the y-axis origin. Blue symbols: opening of the first male flower (end of female phase), red symbols: abscission of male cymule (end of bisexual phase), black symbols: blossom abscission or permanent closure (end of second female phase). Horizontal lines indicate the mean duration of each ontogenetic stage in pollinated (solid line) and unpollinated (dashed line) blossoms in the large-glanded species (left group) and small-glanded species (right group). Populations are arranged according to the phylogeny estimated by Bolstad *et al*. (2014). Abbreviations code for the population names (see Table 1).

Figure 5: Effects of the timing of the pollination on the probability of setting seed (a - c), seed number per seed set (for successful seed sets; d - f), relative seed mass (standardized by population mean seed mass; g - i) and reproductive performance (standardized by population mean reproductive performance; j – l) in three populations of *D. scandens*: Tulum (more outcrossing phenotype), La Mancha (intermediately outcrossing phenotype), and Valladolid (selfing phenotype). Points represent the means for cross- (closed symbol, solid line) and self-pollen (open symbol, dashed line) at each of the four pollination timings. Error bars represent ± SE of the mean. Pollination timings were 1: first day of the female phase, 2: first day of the bisexual phase, 3: last day of the bisexual phase, and 4: during the second female phase.

Figure 6: Effects of the timing of the pollination on inbreeding depression (δ) for reproductive performance (a - c) and the delayed self-pollination cost (d - f; see methods) in three populations of *D. scandens*: Tulum (more outcrossing phenotype), La Mancha (intermediately outcrossing phenotype), and Valladolid (selfing phenotype). Points represent mean inbreeding depression and delayed self-pollination cost at each ontogenetic stage, obtained by non-parametric bootstrapping. Positive values indicate better performance of outbred blossoms

than inbred blossoms, and negative values indicate better performance of inbred blossoms than outbred blossoms. Error bars (95% CIs) overlapping zero indicate values not statistically different from zero. Pollination timings were 1: first day of the female phase, 2: first day of the bisexual phase, 3: last day of the bisexual phase, and 4: during the second female phase.

Tables

Table 1: Population information and summary statistics for floral traits in 14 populations belonging to two species of the *D. scandens* complex. Species means are presented separately for the large-glanded and the small-glanded species. Geographic information refers to the source populations of our experimental greenhouse populations. Herkogamy was measured as the average anther-stigma distance within each population. The autofertility estimates describe the percentage of seeds set after autonomous self-fertilization in the absence of pollinators. The duration of the female phase (protogyny), the bisexual phase, the second female phase, and the duration of seed maturation are given in days with standard deviations in parentheses. Total blossom life span is the sum of the female phase (protogyny), bisexual phase, and second female phase. Populations included in the second part of this study are in bold.

Population	Code	Country	Latitude	Longitude	Herkogamy (mm) ± SE	Autofertility (%)	Protogyny	Bisexual phase	2nd female phase	Seed maturation
Large-glanded species										
La Mancha[a]	**LM**	**Mexico**	**19°37'**	**96°28'**	**3.65 ± 0.29**	**68.5[e]**	**3.00 (0.72)**	**4.67 (0.64)**	**2.79 (3.00)**	**43.67 (2.27)**
Puerto Morelos[a]	PM	Mexico	20°51'	86°53'	5.21 ± 0.28	16.6[f]	2.36 (0.95)	4.86 (0.83)	5.82 (4.53)	36.73 (1.56)
Tulum[b]	**TUL**	**Mexico**	**20°13'**	**87°26'**	**3.81 ± 0.19**	**6.9[f]**	**1.91 (1.24)**	**5.22 (0.42)**	**4.13 (2.56)**	**39.30 (2.31)**
Tulum[a]	T	Mexico	20°12'	87°27'	4.84 ± 0.24	9.6[g]	3.05 (0.76)	5.50 (0.83)	4.35 (4.33)	35.60 (3.17)
Ciudad del Carmen[a]	CC	Mexico	18°56'	91°18'	4.30 ± 0.32	24.9[e]	2.45 (1.00)	4.55 (0.69)	3.80 (2.46)	37.30 (1.25)
Martinez de la Torre[a]	M	Mexico	20°05'	97°01'	4.96 ± 0.47		3.32 (1.04)	4.68 (0.89)	5.23 (4.10)	38.09 (1.87)
Comalcalco[a]	C	Mexico	18°21'	93°20'	4.86 ± 0.32		2.90 (0.85)	4.90 (0.85)	6.95 (3.39)[a]	36.40 (3.17)
Palo Verde A[c]	S8	Costa Rica	10°23'	85°19'	5.31 ± 0.08		2.63 (0.74)	5.38 (0.74)	1.50 (0.93)[c]	62.00 (19.10)
Punta Cacique[c]	S11	Costa Rica	10°34'	85°41'	4.25 ± 0.13		2.45 (0.94)	6.26 (1.05)	0.89 (1.05)	55.40 (4.58)
Species mean					4.48 ± 0.11		2.68 (1.02)	5.07 (0.92)	4.12 (3.68)	41.31 (8.65)
Small-glanded species										
Rincon de la Vieja[c]	S23	Costa Rica	10°46'	85°20'	0.59 ± 0.21		2.54 (0.58)	4.85 (0.73)	4.08 (2.52)	34.46 (1.20)
Martinique[d]	SM (1-6)	France	Martinique		0.84 ± 0.48		2.05 (0.94)	4.30 (0.66)	4.60 (3.33)	32.10 (1.66)
Valladolid[a]	**V**	**Mexico**	**20°42'**	**88°15'**	**1.21 ± 0.32**	**90[f]**	**2.90 (0.79)**	**4.10 (0.72)**	**2.10 (2.25)[a]**	**28.90 (1.45)**
Cozumel[a]	CO	Mexico	20°22'	86°59'	2.73 ± 0.45	52.1[f]	3.26 (1.00)	4.15 (0.93)	1.85 (1.31)	30.80 (2.04)
Tovar[b]	TOV	Venezuela	8°21'	71°46'	2.63 ± 0.46	65.6[g]	3.00 (0.65)	3.65 (0.67)	1.05 (0.60)	29.30 (0.82)
Species mean					1.50 ± 0.21		2.73 (0.88)	4.25 (0.84)	2.80 (2.58)	31.10 (2.46)

[a] Populations were sampled in 2007 and belong to the 2nd greenhouse generation.

[b] Populations were sampled in 1997 and belong to the 4th and 5th greenhouse generation.

[c] Populations were sampled in 2014 and belong to the 2nd greenhouse generation.

[d] Populations were sampled in 2016 and belong to the 2nd greenhouse generation.

Autofertility estimates are from [e] Opedal *et al.* (2015), [f] Opedal *et al.* (2016a), and [g] Bengtsson (2010).

Table 2: Variance component analysis for floral traits in 14 populations within two species of the *D. scandens* complex. Variance components were estimated as the random-effect variance of mixed-effect models fitted with restricted maximum likelihood (REML). For each level of variation, the absolute variance (σ^2 in squared units) and the percentage of the total variance (in parentheses) are presented. Levels of variation were between species ($n = 2$; small-glanded vs. large-glanded), among populations ($n = 14$; nested within species), among individuals ($n = 143$; nested within populations and species), and among blossoms (for ontogenetic traits: $n = 2$ per individual, measured in a pollinated and an unpollinated blossom; nested within populations and species). The CV^2 was computed as $\sigma^2/\mu^2 \times 100$, where σ^2 is the total variance and μ is the trait mean. Herkogamy was measured as the anther-stigma distance on one blossom per individual.

Level of variation	Herkogamy (mm^2)	Protogyny (days^2)	Bisexual phase (days^2)	2nd female phase (days^2)	Total life span (days^2)
Between species	4.28 (73.16%)	0 (0%)	0.36 (30.25%)	0.29 (2.47%)	1.76 (13.31%)
Among population	0.44 (7.58%)	0.13 (14.23%)	0.22 (18.67%)	2.63 (22.24%)	2.32 (17.49%)
Among individual	1.13 (19.26%)	0.24 (25.11%)	0.15 (12.46%)	2.95 (24.97%)	2.78 (20.96%)
Among blossom		0.57 (60.66%)	0.46 (38.63%)	5.94 (50.31%)	6.39 (48.23%)
Total	5.85	0.94	1.18	11.81	13.25
CV^2 (%)	50.90	12.89	5.21	89.63	10.81

Table 3: Summary statistics for fitness components from self- or cross-pollination at each of the four pollination timings in three populations of *D. scandens*: Tulum (more outcrossing phenotype), La Mancha (intermediately outcrossing phenotype), and Valladolid (selfing phenotype). Pollination timings were 1: first day of the female phase, 2: first day of the bisexual phase, 3: last day of the bisexual phase, and 4: during the second female phase. Inbreeding depression (δ) was estimated from the overall reproductive performance (see Methods), and highlighted in bold when statistically different from zero.

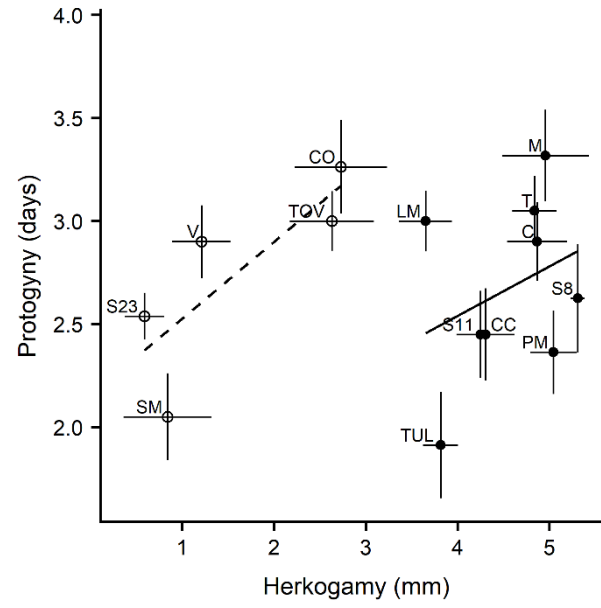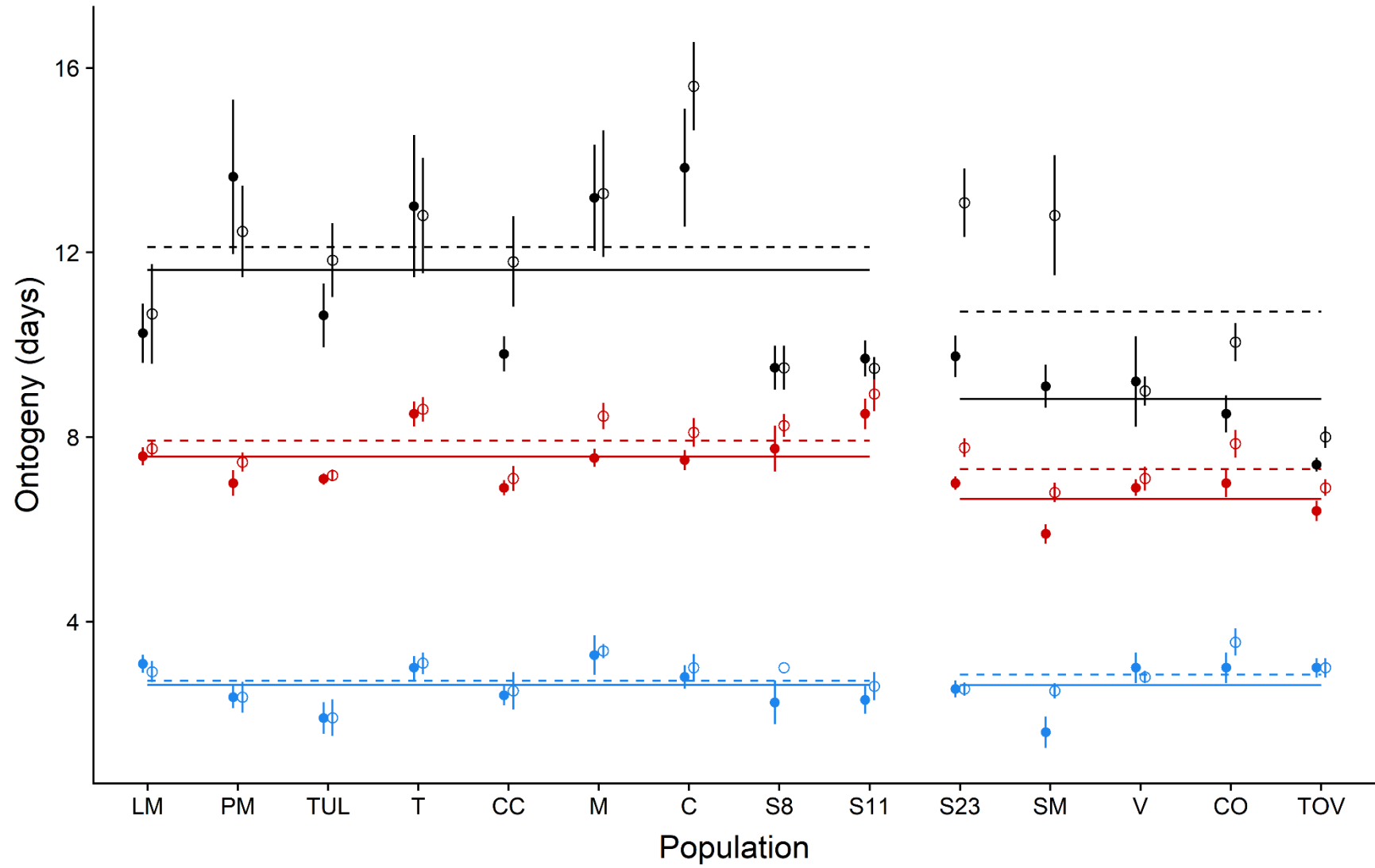| Population	Seed mass (mg) ± SE	Timing	Probability of setting seed		Seed number per seed set (count)		Seed mass (mg)		δ (95% CI)
			Cross pollen (95% CI)	Self pollen (95% CI)	Cross pollen ± SE	Self pollen ± SE	Cross pollen ± SE	Self pollen ± SE	
Tulum	34.86	1st day ♀	0.94 (0.65, 0.99)	0.87 (0.60, 0.97)	8.29 ± 0.11	8.61 ± 0.07	37.40 ± 0.33	35.94 ± 0.44	0.11 (-0.14, 0.35)
	± 0.19	1st day ♀/♂	1	1	8.82 ± 0.03	8.83 ± 0.04	36.45 ± 0.38	36.86 ± 0.38	-0.01 (-0.09, 0.07)
		Last day ♀/♂	0.74 (0.47, 0.90)	0.74 (0.47, 0.90)	7.74 ± 0.24	7.48 ± 0.21	32.12 ± 0.47	31.78 ± 0.59	0.01 (-0.48, 0.70)
		2nd ♀	0.94 (0.65, 0.99)	0.81 (0.53, 0.94)	6.41 ± 0.26	6.22 ± 0.21	31.38 ± 0.82	32.23 ± 0.76	0.20 (-0.24, 0.52)
La Mancha	30.46	1st day ♀	0.94 (0.65, 0.99)	0.87 (0.60, 0.97)	8.63 ± 0.09	7.95 ± 0.11	32.46 ± 0.30	31.88 ± 0.36	0.22 (-0.03, 0.44)
	± 0.16	1st day ♀/♂	1	0.94 (0.65, 0.99)	8.73 ± 0.11	8.72 ± 0.08	30.61 ± 0.38	30.90 ± 0.31	0.12 (-0.10, 0.34)
		Last day ♀/♂	0.94 (0.65, 0.99)	0.81 (0.53, 0.94)	8.55 ± 0.08	7.41 ± 0.20	28.76 ± 0.44	29.82 ± 0.51	**0.37 (0.13, 0.58)**
		2nd ♀	0.53 (0.29, 0.76)	0.86 (0.58, 0.97)	5.95 ± 0.37	6.30 ± 0.31	28.61 ± 0.87	28.39 ± 0.55	**-0.62 (-0.83, -0.30)**
Valladolid	18.33	1st day ♀	0.93 (0.63, 0.99)	1	8.46 ± 0.07	8.66 ± 0.06	18.88 ± 0.24	18.04 ± 0.18	-0.11 (-0.27, 0.03)
	± 0.09	1st day ♀/♂	0.81 (0.53, 0.94)	1	8.66 ± 0.07	8.69 ± 0.05	19.15 ± 0.19	18.93 ± 0.17	**-0.35 (-0.52, -0.20)**
		Last day ♀/♂	0.67 (0.41, 0.86)	0.60 (0.35, 0.81)	6.98 ± 0.30	8.34 ± 0.19	18.24 ± 0.23	17.03 ± 0.26	0.03 (-0.52, 0.94)
		2nd ♀	0.40 (0.19, 0.65)	0.26 (0.10, 0.53)	6.13 ± 0.31	6.25 ± 0.81	17.02 ± 0.34	15.07 ± 0.45	0.62 (-0.23, 0.97)

Figure 1
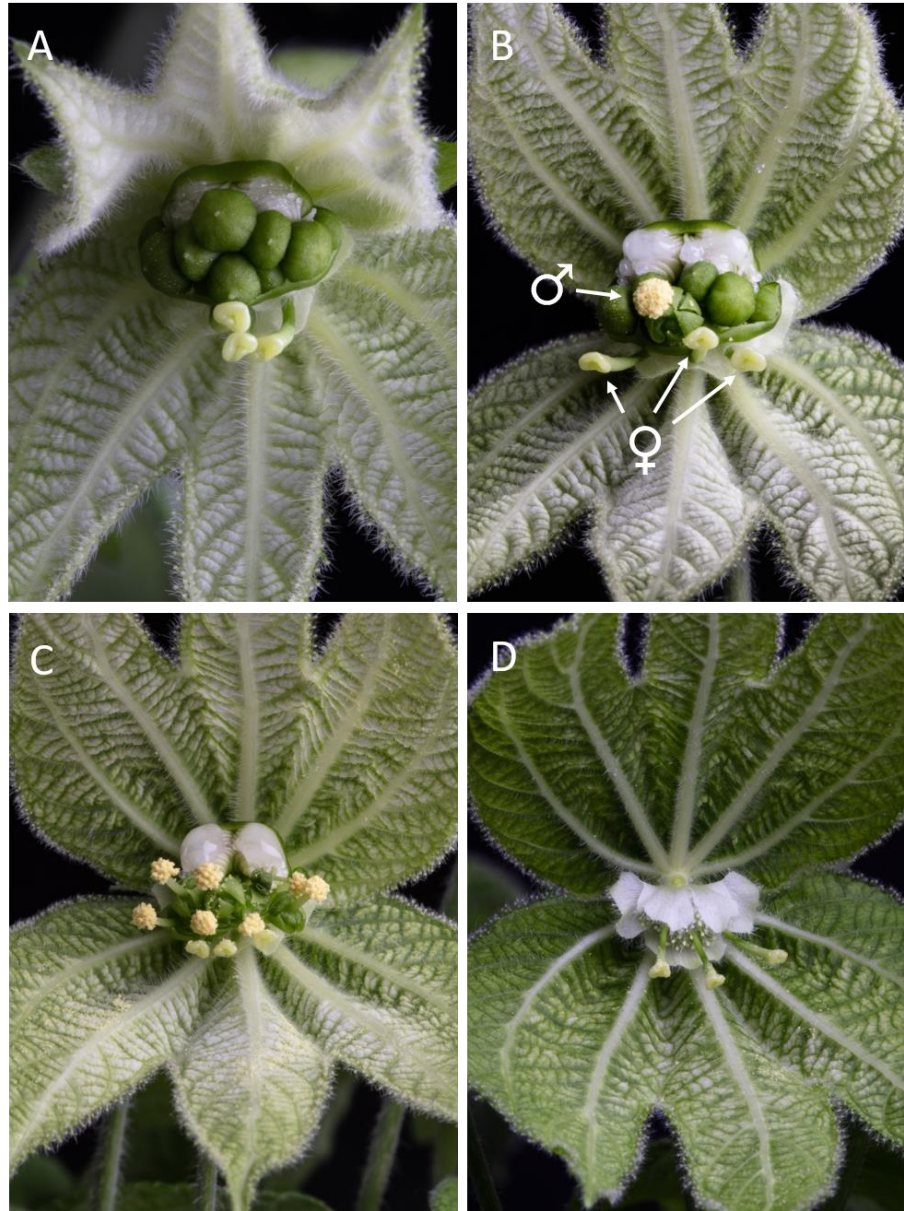
Figure 2

Figure 3

Figure 4

Figure 5

Figure 6