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Fitness costs of delayed (self-) pollination in a mixed-mating plant

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Abstract

Delayed selfing may provide reproductive assurance for plants facing unreliable pollination environments, but may negatively affect the reproductive performance due to inbreeding depression. Furthermore, floral senescence may impose an additional cost to delayed (self-) pollination, if late pollination yields lower reproductive performance. Floral traits mediating the rate and timing of delayed selfing are expected to covary with the mating system of a population.

Here, we first test whether herkogamy and dichogamy, together determining the rate and the timing of delayed autonomous selfing, covary across 14 populations of the mixed-mating vine *Dalechampia scandens*. We also test whether floral longevity responds plastically to delayed pollination. We then study the costs of delayed selfing on reproductive performance, explicitly separating inbreeding depression from senescence costs.

Herkogamy and dichogamy covaried to some extent across populations. Although dichogamy was generally less variable than herkogamy, we still observed genetic differences in blossom ontogeny and longevity between species, and blossom longevity responded plastically to delayed pollination. Reproductive performance in terms of seed quantity and quality decreased with an increased delay of (self-) pollination independently of inbreeding depression.

Overall, the delay of pollination affects reproductive fitness following delayed selfing negatively, beyond the often assumed effects of inbreeding depression. In unreliable pollination evironments, this may select for selfing to occur earlier in the blossom life span. However, if floral traits are selected to increase (delayed) selfing, the evolution of blossom ontogeny and therefore dichogamy may be constrained more strongly than morphological traits.

Keywords: *Dalechampia scandens*, autonomous selfing, mating system, inbreeding depression, floral ontogeny, flower longevity, pollination timing, pollinator environment

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Introduction

Most angiosperms rely on animal pollinators for outcrossed seed production (Ollerton *et al.* 2011), and even self-compatible species often maintain mixed mating systems with at least some offspring produced by pollinator-mediated cross-pollination (Goodwillie *et al.* 2005). In self-compatible species, autonomous selfing is thought to evolve as a mechanism of reproductive assurance if cross-pollination is unreliable (Kalisz and Vogler 2003; Kalisz *et al.* 2004). Delayed selfing is considered particularly beneficial, because it favors outcrossing yet ensures seed set in unreliable pollination environments (´best of both worlds´-hypothesis; Lloyd 1979, 1992; Becerra and Lloyd 1992; Fausto *et al.* 2001; Goodwillie and Weber 2018). The fitness advantage of (delayed) autonomous selfing (within-blossom selfing in the absence of pollinators) depends on the fitness of selfed relative to outcrossed offspring, i.e. on the level of inbreeding depression (Lloyd 1979, 1992; Lande and Schemske 1985; Charlesworth and Charlesworth 1987). It may further depend on the potential trade-off of resources that could otherwise have been used for later outcrossed offspring production, i.e. gamete discounting (Lloyd 1992; Herlihy and Eckert 2002). In predominantly outcrossing species, inbreeding depression is often expressed throughout the life cycle, and may select against selfing (Husband and Schemske 1996). In contrast, frequent inbreeding may increase homozygosity across the genome and favor the purging of deleterious recessive alleles, decreasing inbreeding depression in early-life fitness components such as seed set and seed quality (Charlesworth and Charlesworth 1987; Husband and Schemske 1996; Winn *et al.* 2011).

Variation in the pollination environment 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 autofertility (seed set in the absence of pollinators; Kalisz and Vogler 2003; Opedal, Albertsen, *et al.* 2016). In most species, autofertility rates are determined by herkogamy and/or dichogamy (i.e. spatial and temporal separation of sex functions, respectively), and these traits are therefore expected to covary across populations and species in response to the long-term pollination environment (Goodwillie *et al.* 2005; Brys *et al.* 2013; Opedal, Albertsen, *et al.* 2016).

The influence of inbreeding depression in the evolution of selfing has been the subject of extensive theoretical and empirical work (Lande and Schemske 1985; Charlesworth and Charlesworth 1987). If inbreeding depression exceeds 0.5, it may generally select against selfing, but delayed selfing may be favored at any level of inbreeding depression when pollinators are scarce (Lloyd 1979, 1992). However, whether delayed selfing imposes a fitness cost beyond inbreeding depression has rarely been studied (Goodwillie and Weber 2018; but see e.g. Kalisz and Vogler 2003). Plants capable of delayed self-pollination may benefit from an increase in flower longevity in order to maximize outcrossed offspring production (Ashman and Schoen 1994; Arathi *et al.* 2002; Sato 2002). However, extended floral longevity may come with the cost of floral maintenance (Primack 1985; Ashman and

Schoen 1994). Furthermore, as senescence progresses, female fecundity may decrease due to a loss of stigma receptivity and ovule viability, resulting in lower seed quantity and quality (Levy 1988; Petanidou *et al.* 2001; Arathi *et al.* 2002; Castro *et al.* 2008). Prolonged floral life spans may therefore exhibit plasticity in response to the pollination environment, and also coevolve with the mating system in the long term.

Pollinator abundances worldwide are currently in decline (Potts *et al.* 2010), leading to intensified outcross pollen limitation (Ashman *et al.* 2004). Under these circumstances, delayed selfing may provide reproductive assurance, but even in the absence of inbreeding depression, the quantity and quality of seeds produced may decrease with a delay of pollination, and it is therefore important to understand better the ontogenetic and genetic consequences of delayed selfing on plant fitness.

Here, we study first whether dichogamy and herkogamy, which largely determine the mating system of self-compatible plant populations, have coevolved across 14 populations of the mixed-mating vine *Dalechampia scandens*. We also study the plasticity of floral ontogeny and longevity in response to variation in the pollination environment by assessing the effect of pollination on the rate of floral development. Second, to study the costs of delayed pollination on seed quantity and quality, we conduct an experiment on three focal populations, where we explicitly separate inbreeding depression from senescence costs.

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 and three female flowers. Each female flower contains three ovules, resulting in a maximum seed set of nine seeds per blossom. A resin-producing gland located above the male flowers serves to attract bee pollinators that use the resin for nest construction (Armbruster 1984). The flowers are surrounded by a pair of involucral bracts that open and close daily. *Dalechampia scandens* is self-compatible, functionally protogynous, and capable of autonomous selfing during the bisexual phase (i.e. period of overlap of both sex functions within a blossom; Armbruster and 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 protogyny (i.e. delay of the bisexual phase; Opedal, Listemann, *et al.* 2016).

The *D. scandens* complex comprises at least two, currently undescribed, species (Falahati-Anbaran *et al.* 2017). The 'large-glanded' species has relatively large resin glands, large showy bracts,

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and pronounced herkogamy (anther-stigma separation), indicating a history of frequent cross-pollination mediated by animal pollinators (Opedal, Albertsen, *et al.* 2016). Large anther-stigma distances in this species may prevent autonomous selfing in the beginning of the bisexual phase, but 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, and appears to be highly selfing. Self-pollination is expected to occur almost immediately upon opening of the male flowers, as facilitated by low herkogamy. Populations of the large-glanded species differ in their mating system from moderate outcrossing to nearly complete selfing, and population-mean herkogamy is strongly correlated with outcrossing rates (Opedal, Albertsen, *et al.* 2016).

For this study, we used 14 populations from both species (large-glanded species: *n* = 9 species, small-glanded species: *n* = 5 species), covering a large range of geographical origins and inferred mating-systems (Table 1). The study was conducted on greenhouse populations originally established from seeds collected in the field in Costa Rica, Mexico, the Lesser Antilles, and Venezuela. Populations were propagated in the greenhouse for at least one generation, by random within-population crossing, and relationships among experimental individuals were maximally as close as sibling status. Plants were held in the greenhouse under standardized conditions (26°C day/ 24°C night; 13h light/ 11h dark). Therefore, phenotypic differences among populations are expected to reflect largely genetic differences.

Blossom morphology and ontogeny
We recorded the blossom development from bud to seed dispersal daily in both pollinated and unpollinated blossoms (*n* = 285) in the 14 populations of *D. scandens* (Table 1). Starting with the day of first blossom opening, the female phase (i.e. protogyny) lasts generally two to three days (Fig. 1a), until the first male flower opens (Fig. 1b), and the blossom enters the bisexual phase. Depending on population, the bisexual phase lasts four to six days, with the male flowers opening successively, normally starting with the central (terminal) flower (Fig. 1c, S1). The bisexual phase ends when the last male flower abscises or the whole cymule is shed. 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. The second female phase lasts on average one to seven days. 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. Average blossom lifespan ranges from eight to 15 days. While unpollinated blossoms are generally shed thereafter, pollinated blossoms start maturing seeds, which are dispersed explosively about four to nine weeks after pollination, depending on population.

Effects of pollination on floral ontogeny

To assess the effect of pollination on blossom development, we recorded blossom ontogeny in pollinated (n = 142) and unpollinated (n = 143) blossoms in the 14 populations. Two blossoms per individual ($n \geq 10$ plants per population, except population S8, with n = 4) were randomly assigned to one of the treatments. On the first day of blossom opening, blossoms receiving the pollination treatment were hand-pollinated by saturating all three stigmas with pollen from a randomly chosen paternal individual within the same population. The second treatment consisted in leaving a second blossom unpollinated. To do so, we cut the stigmas on the first day of blossom opening to prevent uncontrolled self-pollination and verified that blossoms did not set seed when we ended blossom observation. Blossom development was recorded daily. Additionally, we quantified herkogamy on one blossom per individual by measuring, with digital callipers (0.01 mm precision), the anther-stigma distance on the first day of the bisexual phase (specifically, the distance between the central male flower and the middle stigma; see Fig. 1b).

Fitness consequences of delayed pollination

To test the effects of delayed pollination on seed production and seed mass, we performed hand-pollinations at different developmental stages during blossom ontogeny 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 by population-mean herkogamy. We used a moderately outcrossing phenotype defined by large herkogamy (Tulum), a less outcrossing phenotype (La Mancha), and a selfing phenotype with small or no herkogamy (Valladolid; Table 1).

Designated female (pollen-receiving) blossoms were emasculated on the first day of blossom opening, before any anthers had dehisced, to prevent uncontrolled autonomous selfing. 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 a flower from either 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 times, 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 use any individual as a father. We pollinated blossoms by brushing the male flowers across the stigmas, saturating them with pollen. If the blossoms had

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already closed permanently on the scheduled day of pollination, we gently opened the bracts to perform the pollination. We bagged the blossoms to collect the seeds after the explosive dehiscence of the seed capsules.

If blossoms were shed before the scheduled pollination date, were aborted before seed initiation, or failed to set viable seeds, we recorded the cross 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 (< 0.01 g, n = 28 in 15 seed sets) were removed from all analyses. Previous studies on *D. scandens* showed that blossom size may affect seed size (Pélabon *et al.* 2015, 2016). Following the method used in these studies, we accounted for this effect, by measuring the peduncle diameter of the female blossoms with digital callipers (0.01 mm precision) on the first day of blossom opening and used this as a proxy for blossom size.

Statistical analyses

All analyses were performed with R version 3.3.1 (R Core Team 2017). 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 seeds as the response variable, and timing of pollination (four levels), pollen type (self/ cross) and population, as well as their interactions as fixed factors, and maternal individual as a random factor. 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 by using the inverse-logit transformation of the parameter estimates. Similarly, 95% CIs were obtained by using the inverse-logit transformation of the upper and lower bounds of the confidence intervals.

We analyzed 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. To analyze variation in seed mass we used linear mixed-effect models with timing of pollination, pollen type and population, as well as their interactions as fixed factors. We included seed set and peduncle diameter as covariates to account for a possible trade-off between seed mass and seed number within seed set, as well as possible blossom-size effects, respectively. Blossom identity nested within maternal individual was added as a random effect. We selected models fitted with maximum likelihood (ML) by comparing their Akaike information criterion corrected for small sample sizes (AICc) .

We estimated overall reproductive performance at the blossom level as: the probability of

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setting seeds × number of seeds per seed set × 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 relative performance following selfing (w_s) and outcrossing (w_o) with $\delta = 1-w_s/w_o$, if $w_s \leq w_o$, and $\delta = w_o/w_s-1$, if $w_s > w_o$, weighing inbreeding and outbreeding depression equally. Hence, positive values represent better reproductive performance following outcrossing than selfing, and negative values represent better reproductive performance following selfing than outcrossing. Similarly, we estimated the cost of delayed selfing as the ´selfing depression´ by comparing the relative reproductive performance of blossoms outcrossed early in flower life (w_o at timing 1 and 2) with the performance following delayed selfing later in blossom life (w_s at timing 2 or 3). We obtained 95% CIs of the inbreeding depression and the ´selfing depression´ from 1000 non-parametric bootstrap estimates, resampled at the blossom level.

Results

Blossom morphology and ontogeny

Herkogamy, measured as the average anther-stigma distance was about three times larger in the large-glanded species than in the small-glanded species (Table 1). In contrast, most part in the variation in protogyny (i.e. duration of the female phase) occurred at the among-blossom level, including variation between pollinated and unpollinated blossoms on each individual (Table 2). Protogyny did not differ detectably between species (0% variation due to species), and little variation (14%) in this trait occurred among populations. The bisexual phase was on average about one day longer in the large-glanded species, and 19% of the variation in its duration occurred among populations due to differential rates of male flower opening (Fig. S1). The duration of the second female phase was similar between species (2% variation due to species), with moderate variation (22%) 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).

Herkogamy and protogyny tended to be positively correlated among populations of the large-glanded species (Pearson´s $r = 0.32$, $p = 0.40$), and the small-glanded species ($r = 0.80$, $p = 0.103$; Fig. 2), but with weak statistical support. Both traits tended to be positively correlated among individuals within each population, however, this was not supported statistically in most populations (results not shown).

Effects of pollination on floral ontogeny

Across all 14 populations, unpollinated blossoms prolonged the bisexual phase (paired t-test, $t = 4.10$, $p < 0.001$) and consequently increased their longevity by about one day ($t = 3.18$, $p = 0.002$; Fig. 3). In contrast, pollination did not detectably affect the duration of the female phase ($t = 1.43$, $p =$

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0.155), and although the second female phase was about half a day longer in unpollinated blossoms, this difference was only marginally significant (t = 1.18, p = 0.072). The effect of pollination was stronger in the small-glanded species than in the large-glanded species, as indicated by larger differences between pollinated and unpollinated blossoms (Fig. 3).

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. 4). This pattern and its magnitude was 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 (Table 4).

The number of seeds produced in successful seed sets (291 out of 358 crosses) also declined with a delay of pollination (Table 3, Fig. 4). 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 (Table 4). Aborted seeds occurred very rarely (ca. 1% of all seeds), were randomly distributed across treatments and populations, and were not analyzed further.

Averaged over all successful seed sets, seed mass varied among populations, with the most outcrossing population (Tulum) having the heaviest seeds, and the highly selfing population (Valladolid) having the lightest seeds (Table 3). Generally, seed mass decreased with the delay of the pollination (Fig. 4). The effects were population specific, but did not generally depend on pollen type (Table 4). The decline in seed mass in the intermediate outcrossing population (La Mancha) was the least pronounced and most gradual. We detected the most severe decline in seed mass in the most selfing population (Valladolid). In this population, lighter seeds were produced following self-pollination than following cross-pollination, with seeds from selfing 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 not statistically significant effects on seed mass, differing in direction among populations (Table S1). Overall reproductive performance declined strongly with the delay of pollination in all populations, with the strongest decline in performance in the highly selfing population (Valladolid; Fig. 5).

Patterns of inbreeding depression with delayed pollination
Inbreeding depression (δ) in reproductive performance was weak in all populations (Table 3). In three quarters of the treatments, δ was not statistically significant as indicated by the 95% CIs overlapping zero (Fig. 6). The only exception was strong inbreeding depression of 37% following

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pollination on the last day of the bisexual phase in the intermediate outcrossing population (La Mancha; Table 3). We also detected cases of negative inbreeding depression (i.e. 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 population (Valladolid).

Our estimation of the ´selfing depression´ incorporated the timing of the pollination into the estimation of the relative reproductive performance following selfing and outcrossing. In the most outcrossing population (Tulum), reproductive performance from selfing on the first day of the bisexual phase was 10% (95% CI: -20 − -1%) higher compared to early outcrossing. However, a delay of selfing until the last day of the bisexual phase decreased reproductive performance by 58% (43 − 72%) compared to early outcrossing. In the intermediate outcrossing population (La Mancha), selfing on the first day of the bisexual phase yielded similar reproductive performance as early outcrossing (´selfing depression´ = 9%, 95% CI: -11 − 30%), but when selfing was delayed until the last day of the bisexual phase, reproductive performance decreased by 46% (29 − 66%) compared to early outcrossing. In the highly selfing population (Valladolid), reproductive performance was 26% (-38 − -15%) higher following selfing on the first day of bisexual phase compared to outcrossing at this stage. However, selfing on the last day of the bisexual phase decreased reproductive performance by 60% (41 − 79%) compared to early outcrossing.

Discussion

Variation and covariation in mating-system traits

Geographical variation in the pollination environment may exert differential selection pressures on blossom traits, mediating variation in plant mating systems (Armbruster 1985, 1988; Moeller 2006; Pérez-Barrales *et al.* 2007; Eckert *et al.* 2009; Opedal, Albertsen, *et al.* 2016). Genetically based variation in floral morphology and ontogeny among species and populations may therefore allow inferences about their mating-history (Ashman and Schoen 1994). As expected from previous work, we found blossom traits to be highly variable within the *Dalechampia scandens* species complex. In particular, herkogamy differed greatly between our study species. Herkogamy is commonly used as an indicator for selfing rates in a species or population, as it is negatively associated with autofertility rates, and presumably reflects adaptation to the pollination environment (Armbruster 1985, 1988; Moeller 2006; Opedal *et al.* 2015; Opedal, Albertsen, *et al.* 2016). In contrast, however, variation in floral development across populations has not been studied in *D. scandens*.

During blossom development, dichogamy should theoretically have the largest impact on the ratio of self- to outcross-pollen achieving fertilizations, as well as the timing of the pollination within the blossom life span. However, we did not find a difference in dichogamy (i.e. protogyny) between species, suggesting the trait to be conserved within *D. scandens*. Nevertheless, protogyny tended to

covary positively with herkogamy, both among populations within species (Fig. 2), as well as within population, as expected if these traits are either genetically correlated or under joint selection by the pollination environment (Armbruster and Schwaegerle 1996). However, covariation between these traits appears to be weak in *D. scandens*, and much less pronounced than observed in other species (e.g. Holtsford and Ellstrand 1992).

Populations of the large-glanded species, with more outcrossing phenotypes (greater herkogamy), had longer blossom life spans, consistent with maximizing pollinator-mediated pollen transfer (Primack 1985; Ashman and Schoen 1994; van Doorn 1997), while still allowing for some delayed selfing later in flower life to ensure reproduction. If populations consistently encounter low pollinator availability, selfing at an early time in blossom life may be favored, facilitated by low herkogamy, to ensure reproduction and reduce the costs of floral maintenance. Accordingly, floral longevity was shorter in the small-glanded species with the selfing phenotype (less herkogamy). Therefore the timing of delayed autonomous selfing appears to mediate on floral longevity, and may affect the cost of delayed selfing to the maternal plant.

Pollination and floral ontogeny

Optimal floral ontogeny and longevity is determined by the balance between maximizing reproductive output and minimizing resource costs to the maternal plant (Primack 1985; Ashman and Schoen 1994). We detected high variation in blossom ontogeny and longevity among and within individual plants. Delayed pollination led to prolonged blossom maintenance, whereas pollination shortened blossom longevity, indicating that individual blossoms plastically respond to their pollination environment to minimize the cost of floral maintenance (Fig. 3). Similar patterns of shortened blossom life spans, triggered by completion of female function, have been reported in numerous other species (Stead 1992; van Doorn 1997; Arathi *et al.* 2002; Sato 2002; Castro *et al.* 2008). However, many species, including *D. scandens*, exhibit a minimum blossom life span regardless of female success, presumably to increase the chances for pollen removal and maximize male fitness (Arathi *et al.* 2002; Castro *et al.* 2008). This may be most advantageous when pollinator visitation is reliable. All together, high individual plasticity in response to the pollination environment may allow for evolution of floral longevity in the long term. Extended floral longevity may however come with a fitness cost, as floral maintenance can be expensive to the maternal plant.

Fitness consequences of delayed pollination

Many mixed-mating plants are able to compensate insufficient cross-pollen receipt early in blossom life via delayed autonomous self-pollination (Kalisz and Vogler 2003; Goodwillie *et al.* 2005; Goodwillie and Weber 2018). Predominantly outcrossing populations may delay selfing to maximize

chances for cross-pollen receipt, but ensure reproduction late in the floral life span in the event of pollinator failure. We detected highest success in all reproductive fitness components following pollinations early in flower life. Female receptivity peaked on the first day of the bisexual phase, indicating that stigmas are presented already slightly prematurely and mature throughout the blossom life span, possibly as a mechanism to maximize the overall blossom life span and thereby chances for pollen receipt. However, a delay of pollination towards the end of the blossom life span decreased reproductive performance (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). However, we could not perform a number of the latest pollinations, because the blossoms were shed before the scheduled pollination date, or abscised before seed initiation, and part of the observed decrease in the probability of setting seed is therefore due to short floral life spans, rather than curtailed female fecundity. This phenomenon was expectedly most pronounced in the highly selfing population (Valladolid) and only rarely observed in the more outcrossing population (Tulum), partly explaining the strongest decline in reproductive performance with delayed pollination in the Valladolid population (Fig. 5).

When late pollination yielded seeds, these were fewer and smaller than those obtained by early pollination. Reduced seed numbers from old flowers often occurred due to complete seed-set failure of single flowers within a blossom (*personal observation*), whereas other flowers produced nearly maximum seed set. This may indicate differential rates of senescence in flowers within a blossom, or selective abortion of individual flowers. In either case, blossoms may remain open 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, as our measure of seed quality, also declined with the delay of pollination, suggesting that flower senescence does not only affect maternal performance, but also individual offspring quality. Therefore, if populations encounter consistently unreliable pollinators, autonomous selfing may be selected to occur earlier in blossom life, and female investment after the early bisexual phase may be curtailed strongly in older blossoms.

Delayed pollination, inbreeding depression, and the evolution of selfing
Inbreeding depression is often assumed to antagonize the evolution of selfing (Lande and Schemske 1985; Charlesworth and Charlesworth 1987; Lloyd 1992). Furthermore, the effects of inbreeding on plant fitness are expected to vary among populations according to their mating history (Charlesworth and Charlesworth 1987; Husband and Schemske 1996; Winn *et al.* 2011). Even so, confirming previous studies in *D. scandens*, we failed to detect substantial inbreeding depression in early-life reproductive fitness components in all populations (Opedal *et al.* 2015). Moreover, inbreeding

depression did not increase systematically with the delay of pollination (Fig. 6). However, considerations of the cost of delayed selfing do commonly not incorporate the timing of self-pollination within the blossom life span. As reproductive success declines with flower age, the timing of self-pollination within the blossom life span may directly affect reproductive success, beyond the effects of inbreeding depression.

If autonomous selfing occurs in the more outcrossing populations of *D. scandens* (Tulum and La Mancha), it may normally be late in the bisexual phase, when the male flowers abscise and contact the stigmas as they fall. Most relevant in the estimation of the cost of delayed selfing may therefore be the comparison between the reproductive performance following outcrossing early in flower life to selfing in the end of the bisexual phase. A delay of selfing towards the end of the bisexual phase reduced blossom performance by around 58% (Tulum) and 46% (La Mancha) compared to early outcrossing, thereby decreasing reproductive performance to a larger extent, than inbreeding depression alone. In contrast, in the highly selfing population (Valladolid), autonomous selfing may occur in the beginning of the bisexual phase, facilitated by low herkogamy. Female performance following selfing at this stage was 26% higher compared to outcrossing, suggesting the greatest fitness benefit from delayed selfing, when it occurs early in blossom life. Selfing early in blossom life may therefore be favored in the long term, as it provides reproductive assurance, by simultaneously avoiding the cost of reduced female fecundity in old blossoms.

It should be noted however that inbreeding depression may be expressed at later life stages, not investigated in this study (Husband and Schemske 1996), and a previous study on *D. scandens* suggested pronounced inbreeding depression in natural populations (Opedal, Albertsen, *et al.* 2016). Furthermore, in sequentially flowering, perennial species like *D. scandens*, conclusions about the relative reproductive success following selfing or outcrossing should be considered carefully. Reproduction via selfing may draw resources from the maturation of outcrossed seeds (Herlihy and Eckert 2002), as well as for survival and reproductive fitness in the next season (Morgan *et al.* 1997). Therefore, resource trade-offs may further reduce the advantage of reproductive assurance via delayed selfing. However, despite all of these considerations, in the event of pollinator failure, delayed selfing should always be selected regardless of its costs, as long as it ensures reproduction (Lloyd 1992).

Conclusions: The evolution of delayed selfing under pollinator declines
Given the current decline in pollinator abundance, it is important to understand the effects of altered pollination reliability on plant reproductive success. Mixed-mating plants may ensure reproduction via delayed selfing. If floral traits are selected to increase (delayed) selfing, morphological traits, such as herkogamy may respond strongest to selection generated by unreliable pollination environments,

whereas dichogamy appears to evolve only to a small extent. Still, even if dichogamy was largely unaffected by delayed pollination, we observed genetic differences in blossom ontogeny and longevity between species, and blossom longevity responded plastically to delays in pollination. However, the greater the delay of pollination within the blossom life span, the lower was reproductive performance. Overall, delayed (self-) pollination may affect reproductive performance negatively, independently from the often assumed cost of inbreeding depression. If populations consistently encounter unreliable pollinators, autonomous selfing may hence be selected to occur earlier in blossom life. All together, further declines in pollinator abundances may affect plant performance via a variety of mechanisms acting simultaneously, and have severe negative effects, even in species capable of reproductive assurance via delayed selfing.

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Tables

Table 1: Population information and summary statistics for floral traits in 14 populations of *D. scandens*. All ontogenetic measurements are presented in days with their SD. Total blossom life span is the sum of the female phase (protogyny), bisexual phase, and second female phase. Populations used for 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)	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)	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)**	**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 diallel[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.* 2016, and [g] Bengtsson 2010.

Table 2: Variance component analysis for floral traits in 14 populations of *D. scandens*. Variance components were estimated as the random variance of mixed-effect models fitted with restricted maximum likelihood (REML). For each level of variation, we present the absolute variance and the percentage of the total variance in brackets.

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	Na	0.57 (60.66%)	0.46 (38.63%)	5.94 (50.31%)	6.39 (48.23%)
Total (CV^2)	5.85 (50.90%)	0.94 (12.89%)	1.18 (5.21%)	11.81 (89.63%)	13.25 (10.81%)

Table 3: Summary statistics for fitness components from self- or cross-pollination following four pollination timings in three populations of *D. scandens*. Inbreeding depression (δ) was estimated from the overall reproductive performance (see methods).

Population	Seed mass (g) ± SE	Timing	Probability of setting seed		Seed set (count)		Seed mass (g)		δ (95% CI)
			cross (95% CI)	self (95% CI)	cross (± SE)	self (± SE)	cross (± SE)	self (± SE)	
TUL	0.03486	1st day ♀	0.94 (0.65; 0.99)	0.87 (0.60; 0.97)	8.29 (0.11)	8.61 (0.07)	0.03740 (0.00033)	0.03594 (0.00044)	0.11 (-0.14; 0.35)
	(0.00019)	1st day ♀/♂	1	1	8.82 (0.03)	8.83 (0.04)	0.03645 (0.00038)	0.03686 (0.00038)	-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)	0.03212 (0.00047)	0.03178 (0.00059)	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)	0.03138 (0.00082)	0.03223 (0.00076)	0.20 (-0.24; 0.52)
LM	0.03046	1st day ♀	0.94 (0.65; 0.99)	0.87 (0.60; 0.97)	8.63 (0.09)	7.95 (0.11)	0.03246 (0.00030)	0.03188 (0.00036)	0.22 (-0.03; 0.44)
	(0.00016)	1st day ♀/♂	1	0.94 (0.65; 0.99)	8.73 (0.11)	8.72 (0.08)	0.03061 (0.00038)	0.03090 (0.00031)	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)	0.02876 (0.00044)	0.02982 (0.00051)	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)	0.02861 (0.00087)	0.02839 (0.00055)	-0.62 (-0.83; -0.30)
V	0.01833	1st day ♀	0.93 (0.63; 0.99)	1	8.46 (0.07)	8.66 (0.06)	0.01888 (0.00024)	0.01804 (0.00018)	-0.11 (-0.27; 0.03)
	(0.00009)	1st day ♀/♂	0.81 (0.53; 0.94)	1	8.66 (0.07)	8.69 (0.05)	0.01915 (0.00019)	0.01893 (0.00017)	-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)	0.01824 (0.00023)	0.01703 (0.00026)	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)	0.01702 (0.00034)	0.01507 (0.00045)	0.62 (-0.23; 0.97)

Table 4: Model selection for the effects of the timing of the pollination (timing), pollen type (self/cross) and population on the probability of setting seed, seed number per seed set, and seed mass in three populations of *D. scandens* (see methods). Maternal identity was included as a random effect in all models. Blossom identity nested within maternal identity was included as a random effect in analyses of seed mass. The highest ranked models (in bold) and alternative models, whose ΔAIC was less than two, are presented.

| Variable	Population	Model parameters	Parameters (K)	Log Likelihood	ΔAICc	AICc weight
Seed set probability	all	**timing + population + timing × population**	**13**	**-135.7**	**0**	**0.58**
	TUL	**timing**	**5**	**-38.82**	**0**	**0.63**
		timing + pollen type	6	-38.42	1.43	0.31
	LM	**timing**	**5**	**-43.81**	**0**	**0.52**
	V	**timing**	**5**	**-52.91**	**0**	**0.61**
Seed set	all	**timing**	**6**	**-584.74**	**0**	**0.60**
	TUL	**timing**	**6**	**-203.7**	**0**	**0.68**
		timing + pollen type	7	-203.36	1.62	0.30
	LM	**timing**	**6**	**-210.55**	**0**	**0.51**
		timing + pollen type	7	-209.62	0.44	0.41
	V	**timing**	**6**	**-163.6**	**0**	**0.47**
		timing + pollen type	7	-162.84	0.86	0.30
		timing + pollen type + timing × pollen type	10	-159.36	1.44	0.23
Seed mass	all	**timing + population + timing × population + seed set + peduncle**	**17**	**9608.82**	**0**	**0.72**
	TUL	**timing + seed set + peduncle**	**9**	**3313.87**	**0**	**0.70**
		timing + pollen type + seed set + peduncle	10	3313.94	1.93	0.27
	LM	**timing + seed set + peduncle**	**9**	**3270.67**	**0**	**0.71**
	V	**timing + pollen type + seed set + peduncle**	**10**	**3244.03**	**0**	**0.50**
		timing + seed set + peduncle	9	3242.35	1.28	0.26
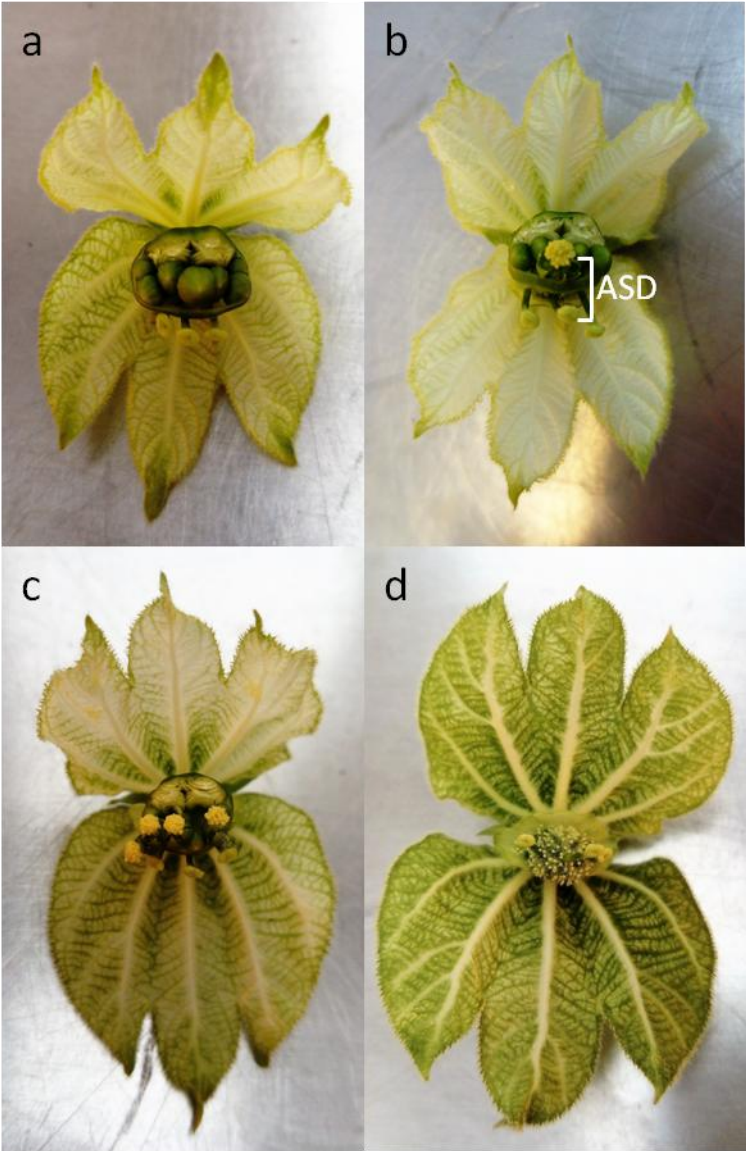
Figure 1: Blossoms of *D. scandens* throughout blossom ontogeny. Blossoms are shown in the female phase (a), on the first day of the bisexual phase (b), in the late bisexual phase (c), and during the second female phase (d). ASD: anther-stigma distance.

Figure 2: Relationship between herkogamy and protogyny (i.e. dichogamy) in 14 populations within two species of *D. scandens*. Points represent mean trait values for each population. Error bars represent the ± SE of the mean. Lines indicate the correlation between herkogamy and protogyny within the large-glanded species (solid line) and small-glanded species (dashed line), respectively.
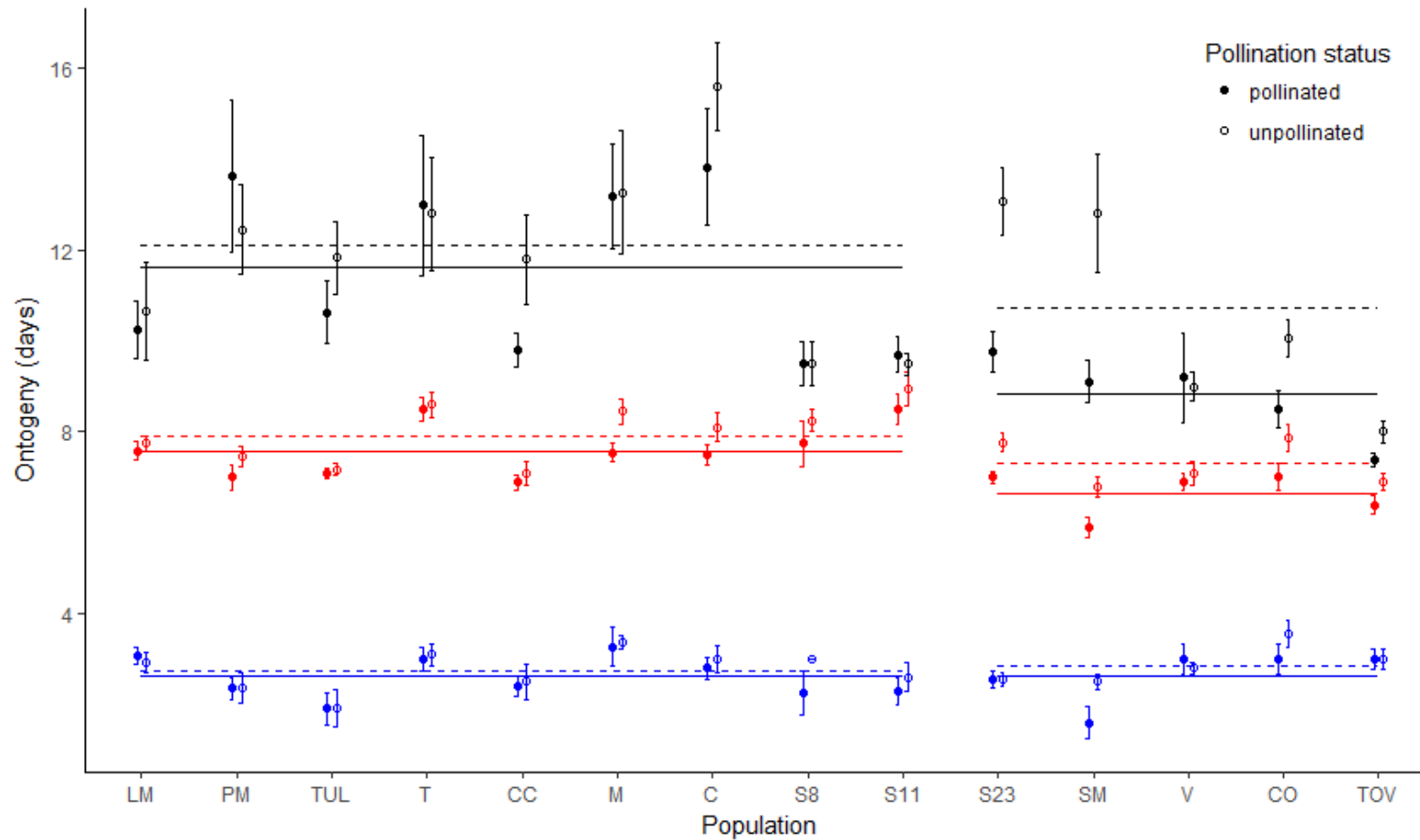
Figure 3: Pollination effect on the duration of each ontogenetic stage (in days after blossom opening) in 14 populations within two species of *D. scandens*. The x-axis represents the day of first blossom opening. Blue symbols: opening of male flowers (end of the female phase), red symbols: abscission of male cymule (end of bisexual phase), black symbols: blossom abscission or permanent closure (end of second female phase). Error bars represent the ± SE of the mean. 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), respectively.
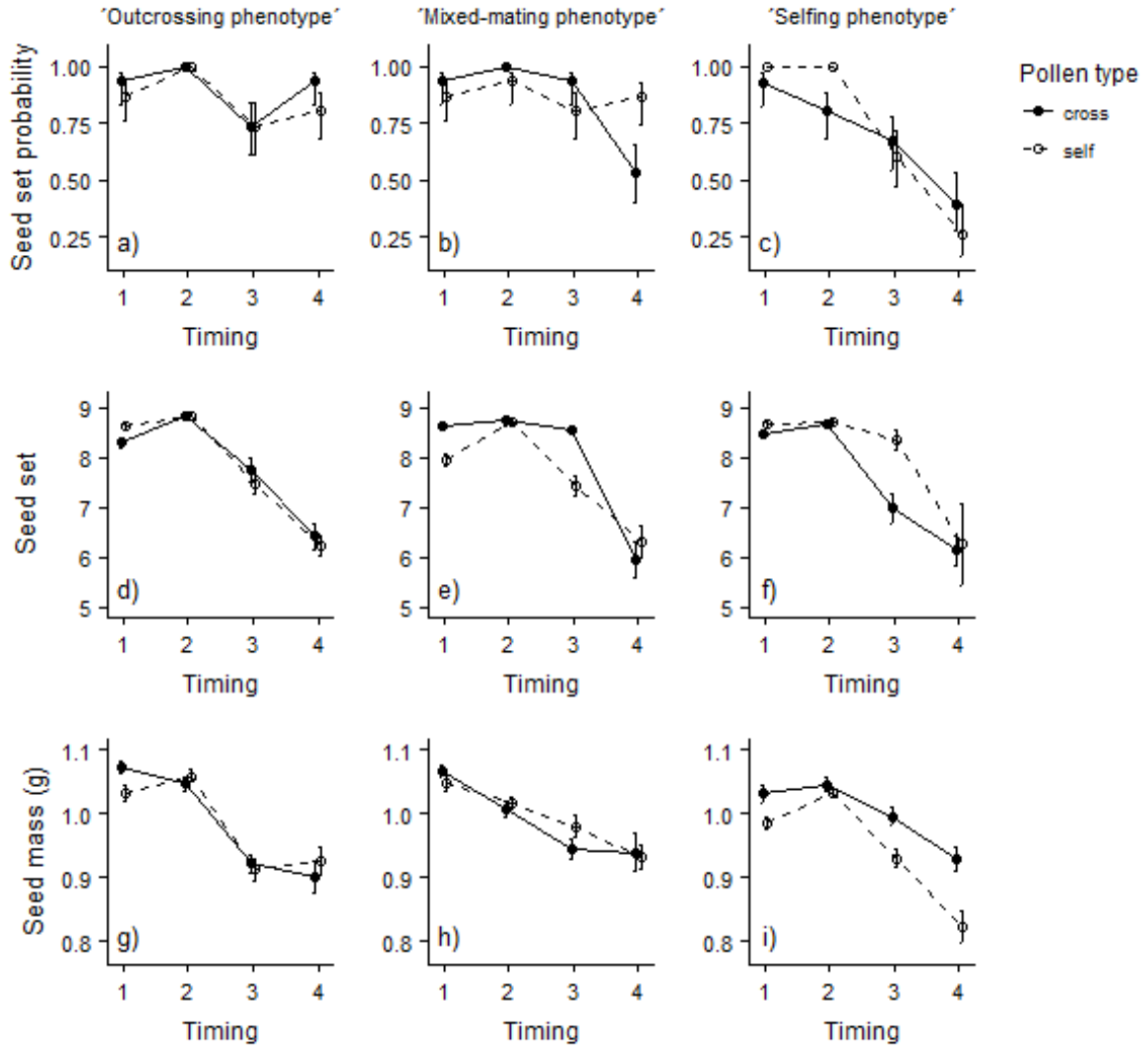
Figure 4: Effects of the timing of the pollination on the probability of setting seed (a - c), seed number per seed set (for successful seed sets; d - f) and seed mass (standardized by the population mean seed mass; g - i) in three populations of *D. scandens*: Tulum (a, d, g), La Mancha (b, e, h), Valladolid (c, f, i). Points represent the means for cross- (closed symbol, solid line) and self-pollen (open symbol, dashed line). 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.
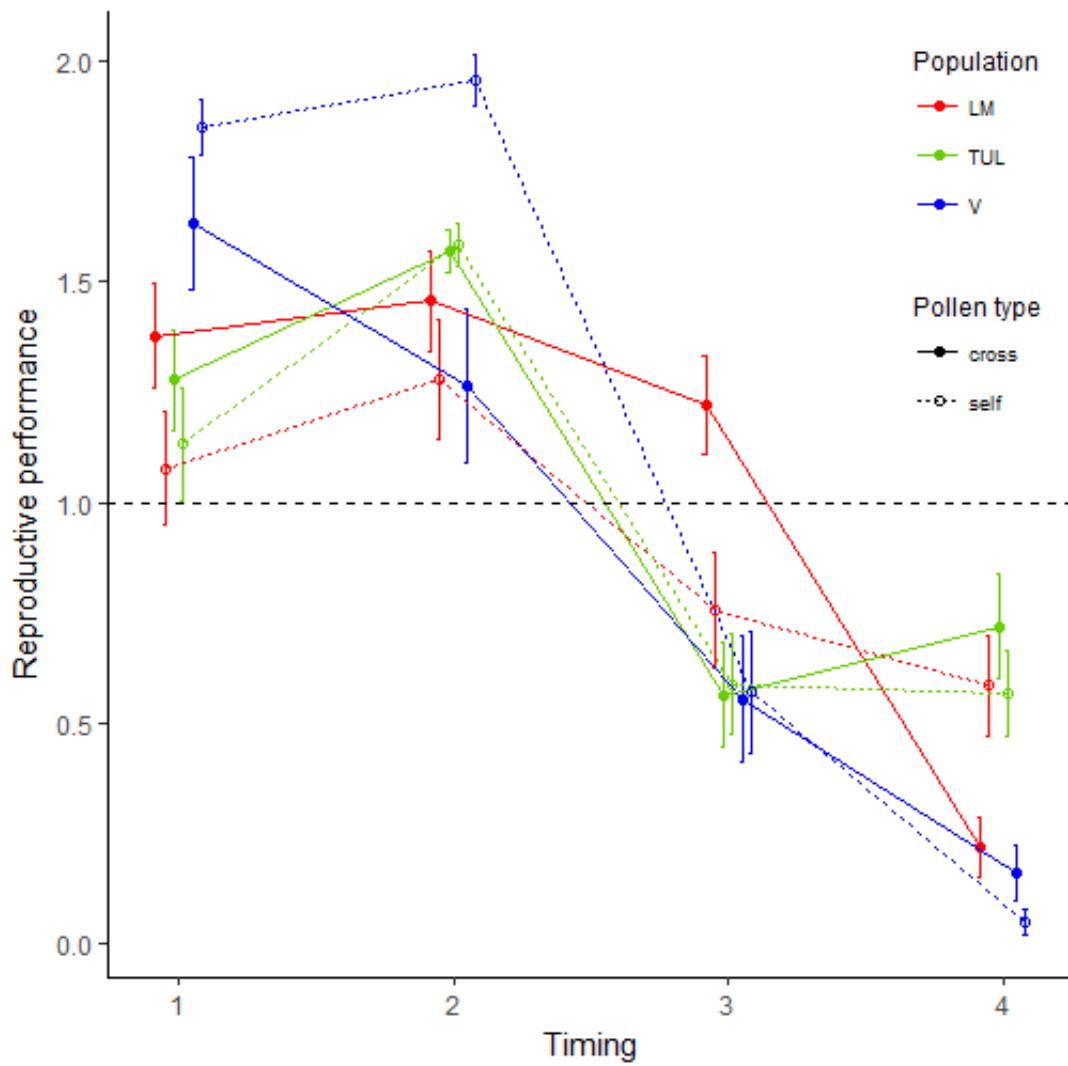
Figure 5: Effects of the timing of the pollination on reproductive performance (standardized by the population mean performance, indicated by the horizontal dashed line) in three populations of *D. scandens*: Tulum (green), La Mancha (red), and Valladolid (blue). Points represent the means for cross- (closed symbol, solid line) and self-pollen (open symbol, dashed line). 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.
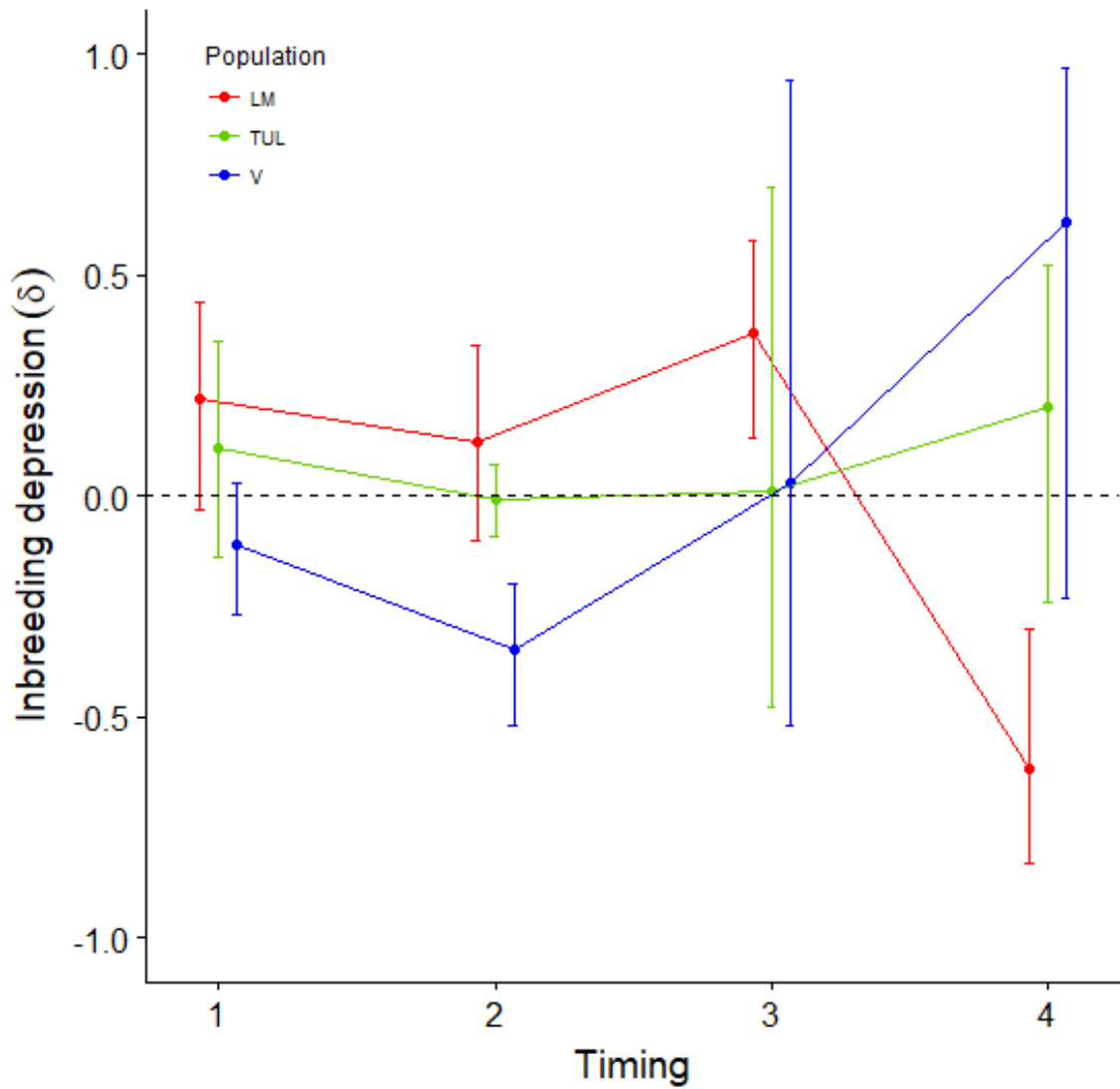
Figure 6: Inbreeding depression (δ) in reproductive performance with delayed pollination in three populations of *D. scandens*: Tulum (green), La Mancha (red), and Valladolid (blue). Positive values indicate inbreeding depression (performance of inbred blossoms < performance outbred blossoms) and negative values indicate outbreeding depression (performance of inbred blossoms > performance outbred blossoms). Error bars (95% CIs) overlapping zero indicate a δ not statistically significantly 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.

Supplemental information

Table S1: Model estimates of the highest ranked models (see Table 4) of the effects of the timing of the pollination (timing) and pollen type (self/ cross) on the probability of setting seed, seed set, and seed mass in three populations of *D. scandens*. The intercept represents pollination at timing 1 (except for the effects on seed mass in the Valladolid (V) population, where the intercept represents pollination with cross-pollen at timing 1). Maternal identity was included as a random effect in all models. Blossom identity nested within maternal identity was included as a random effect in analyses of seed mass. 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. Statistically significant results are in bold.

| Variable	Population	Intercept (± SE)	Timing 2 (± SE)	Timing 3 (± SE)	Timing 4 (± SE)	Self-pollen (± SE)	Seed set (± SE)	Peduncle (± SE)
Seed set probability	TUL	**2.30 (0.64)**	32.46 (6572000)	-1.23 (0.76)	-0.33 (0.84)			
	LM	**2.27 (0.65)**	1.18 (1.19)	-0.33 (0.82)	-1.43 (0.74)			
	V	**3.33 (1.02)**	-1.14 (1.19)	**-2.79 (1.09)**	**-4.03 (1.09)**			
Seed set	TUL	**8.26 (0.33)**	0.54 (0.46)	**-1.44 (0.49)**	**-2.80 (0.47)**			
	LM	**8.02 (0.41)**	0.22 (0.49)	-0.40 (0.51)	**-3.31 (0.55)**			
	V	**8.50 (0.33)**	0.13 (0.47)	**-1.82 (0.52)**	**-3.90 (0.64)**			
Seed mass	TUL	**0.03788** (0.00351)	0.00002 (0.00101)	**-0.00473** (0.00120)	**-0.00520** (0.00129)		-0.00005 (0.00025)	-0.00098 (0.00243)
	LM	**0.02900** (0.00208)	-0.00131 (0.00074)	**-0.00306** (0.00074)	**-0.00269** (0.00100)		-0.00003 (0.00018)	**0.00418** (0.00144)
	V	**0.01747** (0.00141)	0.00069 (0.00045)	-0.00070 (0.00054)	-0.00120 (0.00077)	-0.00066 (0.00037)	0.00013 (0.00013)	0.00025 (0.00151)
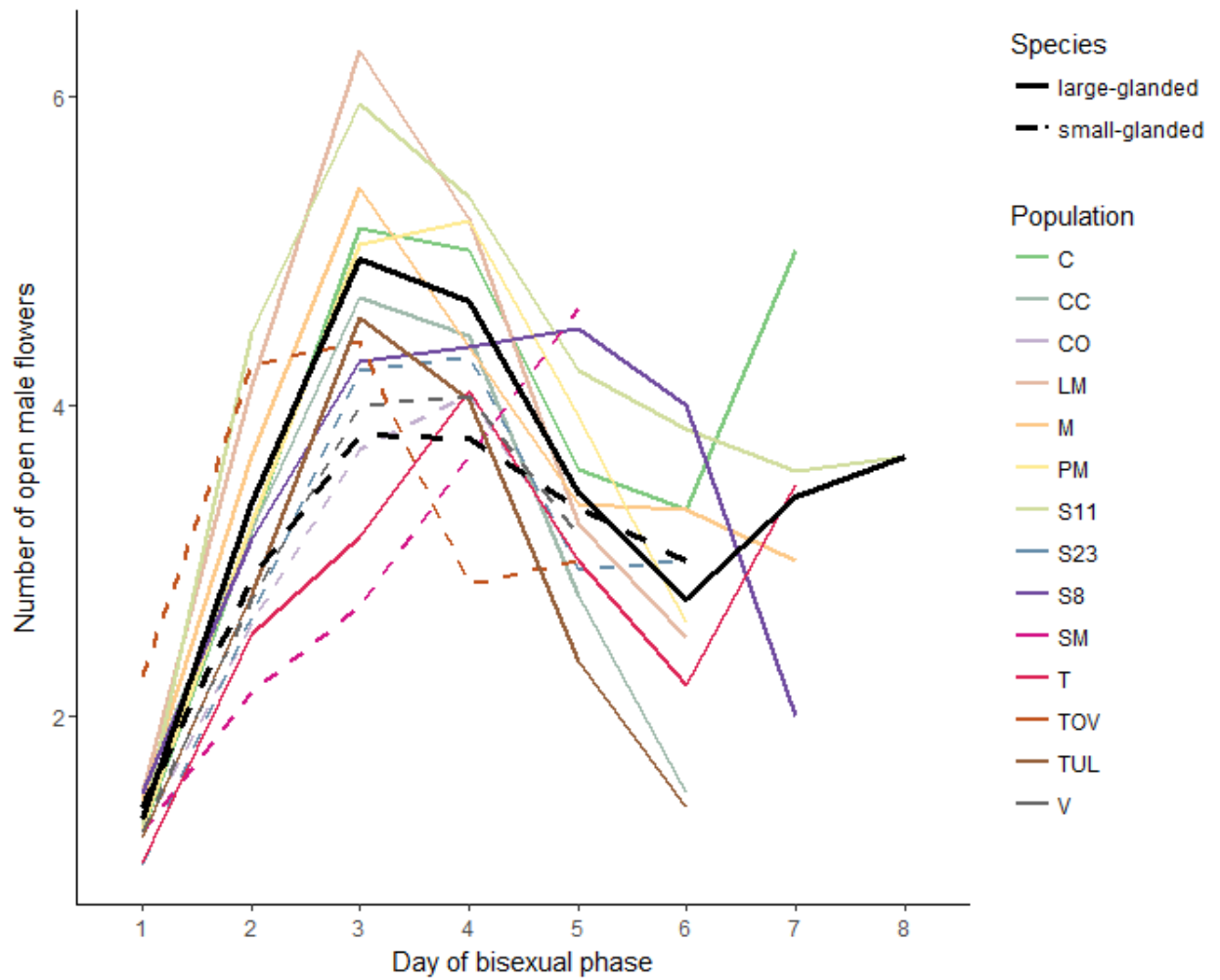
Figure S1: Number of open male flowers on each day of the bisexual phase in 14 populations within two species of *D. scandens*. Colored lines represent the average number of open male flowers per day per population within the large-glanded (solid lines) and small-glanded (dashed lines) species, respectively. The black bold lines indicate the average number of open male flowers per day in each species.