Differences in dichogamy and herkogamy contribute to higher selfing in contrasting environments in the annual Blackstonia perfoliata (Gentianaceae)

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• Background and Aims The establishment of plant populations in novel environments may generate pronounced shifts in floral traits and plant mating systems, particularly when pollinators are scarce. In this study, floral morphology and mating system functioning are compared between recently established and older populations of the annual Blackstonia perfoliata that occur in different pollinator environments.
• Methods Hand-pollination and emasculation experiments were conducted to assess the extent of pollinator-mediated pollen deposition and pollen limitation, and the contribution of autonomous selfing to total seed production. Detailed measurements of key floral traits were performed to compare the flower morphology and mating system functioning between plants from both pollination environments.
• Key Results Pollinator-mediated pollen deposition was about twice as low in the recently colonized and pollinator-poor environment compared with the old and pollinator-rich sites, but total seed set was little affected by any type of pollen limitation. The contribution of autonomous selfing to total seed production was higher in the pollinator-poor sites than in the pollinator-rich sites (index of reproductive assurance = 0.56 and 0.17, respectively), and seed production was only poorly affected by selfing, whereas in the pollinator-rich populations selfing reduced total reproductive output by about 40% compared with outcross pollination. Plants originating from pollinator-poor environments produced smaller flowers that showed significantly lower levels of dichogamy (i.e. protogyny) and herkogamy. These reductions resulted in a 2-fold higher capacity for autonomous selfing under pollinator-free conditions (index of autonomous selfing = 0.81 and 0.41 in plants originating from the pollinator-poor and pollinator-rich environment, respectively).
• Conclusions The results illustrate that plant populations colonizing novel environments can differ markedly in floral morphology and mating system functioning. Due to a temporal shift in the male phase, the breeding system of B. perfoliata shifted from delayed selfing under pollinator-rich conditions towards competing selfing in recently established populations, providing greater reproductive assurance when pollinators and/or reproductive partners are limited.

Key words: Autogamy, competing selfing, delayed selfing, dichogamy, herkogamy, pollen limitation, pollen quality, reproductive assurance, Blackstonia perfoliata, Gentianaceae.

INTRODUCTION

The remarkable diversity of mating systems in flowering plants has fascinated biologists for more than a century [see, for example, Darwin’s (1876, 1877) monographs on plant sexual systems]. In particular, the evolutionary shift from outcrossing towards selfing has received much theoretical and empirical attention because of its profound biological importance for plant populations (e.g. Goodwillie et al., 2005). Increasing disturbance of plant–pollinator interactions and the resulting limitation in outcross pollen export and deposition can generate diverse selective forces on plant mating systems (e.g. Lloyd, 1992; Morgan and Wilson, 2005; Eckert et al., 2010), depending on the relative differences in male and female fitness resulting from self- and outcross-pollination (Charlesworth and Charlesworth, 1987). Severe pollen limitation can be particularly important during the early phases of colonization and population establishment (e.g. Baker, 1955; Levin, 2010; Cheptou, 2012), and is expected to cause selection for floral traits that facilitate autonomous self-fertilization (Cruden and Lyon, 1989; Lloyd, 1992). This may be particularly true in novel or disturbed environments that lack specialized pollinator communities (Aizen and Harder, 2007; Eckert et al., 2010).

Some of the most intriguing puzzles in plant mating system evolution concern the suite of changes in flower morphology and development, typically accompanying transitions from outcrossing to selfing, and their impact on mating system functioning itself (see Karron et al., 2012). In particular in self-compatible species bearing both male and female sex organs within individual flowers, subtle variation in traits that separate pollen presentation and pollen receipt are expected to cause significant differences in selfing probabilities and may be important targets of selection (reviewed in Goodwillie et al., 2005; Eckert et al., 2010). A higher capacity for autonomous selfing can generally be achieved through developmental
changes in two key floral traits that separate the sexual organs (anthers and stigma) either in space (herkogamy), time (dichogamy) or both (Schoen, 1982; Wyatt, 1986). In some species, decreased anther-stigma separation has been found to be one of the principal modifications providing a higher capacity for autonomous seed production when plants are exposed to outcross pollen limitation (e.g. Takebayashi and Delph, 2000; Moeller and Geber, 2005; Herlihy and Eckert, 2007; Brys and Jacquemyn, 2012).

Changes in the temporal separation of the male and female function, on the other hand, affect not only the capacity, but also the timing of autonomous selfing (Mallick, 2001). Dichogamy is presumed to function as a strategy to avoid intra-flower interference between pollen export and receipt (Bertin and Newman, 1993). When complete, self-pollination is totally precluded (Lloyd and Webb, 1986), but when some overlap in the presentation of both sexual functions occurs (i.e. partial dichogamy), opportunities for autonomous selfing may arise (Lloyd and Schoen, 1992). Even small changes in the level of dichogamy may exert large changes in the ability to self (Lloyd and Webb, 1986; Griffin et al., 2000; Barrett, 2003; Kalisz et al., 2012). Most self-compatible species that exhibit dichogamy are characterized by protogyny, implying that stigmas are receptive before pollen is dispersed (Bertin and Newman, 1993). In these species, a partial overlap in both sexual functions can result in delayed selfing. This mode of autonomous selfing is most beneficial when pollinators and/or potential mates are unpredictable in space and time, as it offers no pollen or seed discounting costs because opportunities for outcrossing have already passed (Lloyd, 1992; Morgan et al., 1997; Kalisz et al., 1999).

In cases of extreme outcross pollen limitation, further reduction in the level of dichogamy can be expected, leading to a system of competing or even prior selfing with little or no pollen and/or seed discounting (Morgan and Wilson, 2005). However, apart from effects on pollen and seed discounts, the ultimate outcome of these changes in floral mechanisms will also depend on the fitness of selfed seeds (Lloyd and Schoen, 1992). Husband and Schemske (1996), for instance, showed that self-pollination reduced seed production in a subset of 62 self-compatible species by an average of 20%, differences that could be mainly attributed to early inbreeding effects. Effects of purging, on the other hand, may diminish fitness differences over time (Waller, 1993), and are supposed to occur much more rapidly in small or recently established inbred populations (Wang et al., 1999).

In this study, we investigated floral morphology and mating system functioning in the annual, herkogamy-pollinated Blackstonia perfoliata occurring in two contrasting pollination environments. Two large populations were studied in a pollinator-rich coastal dune area where the species originally occurred and which is typically rich in insect pollinators. These populations were compared with two recently established B. perfoliata populations that were similar in size and density, and occurred in a newly created industrial area that is poor in pollinators and co-flowering plant species. Previous research on the related hoverfly-pollinated Centaureum erythraea in the same environments has indicated that short-lived species may be prone to rapid evolutionary or ecological shifts in floral traits and selfing capacity when populations establish in newly created anthropogenic sites (Brys and Jacquemyn, 2012). Based on these findings, we hypothesized that B. perfoliata is exposed to similar selective forces, favouring plants with a higher capacity for autonomous selfing that increases seed set in the face of outcross pollen limitation (i.e. reproductive assurance; Jain, 1976). To test this prediction, we investigated seed set and effects of selfing on female reproductive success, and assessed the contribution of pollinator-mediated outcross pollination and autonomous selfing to total seed set. In addition, detailed morphological measurements were conducted in combination with the determination of the timing of male and female function to elucidate the driving mechanisms explaining transitions in mating functioning.

MATERIALS AND METHODS

Study species and sites

Blackstonia perfoliata (L.) Huds. (yellow wort) is an annual, monocarpic herb belonging to the Gentianaceae. It is a widely distributed species, occurring throughout most of Western Europe and the Mediterranean (Tutin, 1972; Van der Sluis, 1985). Blackstonia perfoliata develops showy yellow, hermaphroditic and self-compatible flowers (Fig. 1A). Flowering of the species starts at the beginning of June and lasts until mid-July. Blackstonia perfoliata individuals produce on average 11.2 flowers per plant (range 2–65; n = 250). Within the same plant, flowers open gradually and anthesis of individual flowers generally takes 4 d (R. Brys, pers. obs.). During the night and at the end of a flower’s life span, flowers close again. Blackstonia perfoliata does not produce any nectar and is almost exclusively pollinated by pollen-gathering hoverflies (Diptera, Syrphidae) (Fig. 1B, C) and small flies (Empididae), but occasionally some bees (Hymenoptera, Apidae) may also visit the flowers (R. Brys and B. Geens, pers. obs.). In August, when fruits are ripe, plants produce tiny seeds (<0.01 mg) in large quantities (on average 702 ± 52 seeds per fruit).

In the summer of 2011, we studied four B. perfoliata populations in Belgium. Two of them [Dune population 1 (DP1, 51°5’10”N, 2°33’1”E) and Dune population 2 (DP2, 51°8’10”N, 2°41’51”E)] were located in an intact coastal dune area at the Western part of the Belgian coast, where several populations have been known for more than a century (Van Landuyt et al., 2006). In contrast, the other two populations were located within an industrial area (Waaslandhaven) close to the river Scheldt near Antwerp [Waaslandhaven 1 (WH1, 51°16’51”N, 4°18’31”E) and Waaslandhaven 2 (WH2, 51°14’48”N, 4°19’40”E)]. In this area, large patches of bare soil were created via sand supplementation projects between 1980 and 1990. Although B. perfoliata did not occur in this area before 1980, the first colonizing plants and establishing populations were reported from 1980 onwards (Van Landuyt et al., 2006, and pers. comm.). To date, several of these recently established populations have reached a population size of >1000 flowering individuals in this area. Each of the selected study populations consisted of a large number of individuals...
(n >1000) and showed comparable densities of flowering plants. Initial investigation of the pollinator community of *B. perfoliata* in each study population showed that it is almost exclusively visited and pollinated by hoverflies, with *Episyrphus balteatus* and *Eristalis tenax* making up to 80% of the total species composition (see Fig. 1B, C; Supplementary Data Table S1). Exploratory assessment of pollinator visitation rates and the pollinator assemblage in each of these populations revealed that plants were visited by the same pollinators and at similar frequencies in both environments, but that about five times more visits were observed in the coastal dune populations than in the populations occurring in the industrial area (see Supplementary Data Table S1 and Fig. S1). Due to the marked differences in pollinator availability between both areas, we further denote the dune populations as ‘pollinator-rich’, whereas the populations located in the industrial area are referred to as ‘pollinator-poor’ populations.

**Seed production, pollen limitation and reproductive assurance in the field**

To investigate whether pollinator-mediated pollen deposition and reproductive assurance differed between pollination environments, an emasculation experiment was combined with supplemental hand pollinations in each of the study populations. Four pollination treatments were applied: (1) emasculation at the beginning of anthesis; (2–3) supplemental self- or outcross-pollination on the first day of flowering; and (4) flowers left intact and unmanipulated. This experiment also allowed us to assess whether plants experienced different levels of self- and/or outcross-pollen limitation. Within each study population, 80 similar sized flowering plants (20 plants per treatment) were selected representing the average size and floral display of that particular population. Because previous investigation showed that visitation rates did not differ

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**Fig. 1.** *Blackstonia perfoliata* flower (A) with floral measures reported in the study, and the two most abundant flower visitors (B) *Episyrphus balteatus* and (C) *Eristalis tenax*. 
significantly between emasculated and intact open-pollinated flowers \( (t_{1,50} = 0.888; \ P = 0.379; \) unpubl. results), we assumed that emasculation did not affect pollinator behaviour in this species. To minimize variation in pollen delivery in the supplemental hand pollination treatments, all flowers were pollinated by the same person using forceps to transfer pollen. Pollen for supplemental outcross pollinations were obtained from at least five donor plants in each population. On each of the selected plants, all flowers were exposed to the same pollination treatment. Once seeds were ripe, five fruits were harvested (if possible), and seed production was determined in the laboratory and averaged per plant. During the entire experiment, 29 plants were lost due to herbivory (14 in the pollinator-rich and 15 in the pollinator-poor environment), resulting in a total of 291 plants for which seed production was determined.

For each population, we calculated the mean pollinator failure index \( (P_{\text{pollinator}}) \) as \( [1 - \text{(mean seed production of plants with emasculated flowers/mean seed production of plants with supplemental outcross-pollinated flowers)}] \) and the index of reproductive assurance \( (RA) \) as \( [1 - \text{(mean seed production of plants with emasculated flowers/mean seed production of plants with intact flowers})] \) (see Lloyd and Schoen, 1992; Eckert et al., 2010). Mean levels of self- and outcross-pollen limitation \( (P_{\text{self}} \text{ and } P_{\text{outcross}} \text{, respectively}) \) were calculated as \( [1 - \text{(mean seed production of plants with intact flowers/mean seed production of plants with supplemental self-pollinated flowers)}] \) and \( [1 - \text{(mean seed production of plants with intact flowers/mean seed production of plants with supplemental outcross-pollinated flowers})] \) (see Eckert et al., 2010). For each index, we calculated 95 \% confidence intervals using the bootstrap procedure \( (n = 1000 	ext{ bootstraps}) \) implemented in PopTools (Hood, 2010).

**Floral measurements**

To investigate differences in floral traits between both pollination environments, in each population 40 flowering individuals were randomly selected across the entire population during peak flowering. For each plant, we measured plant height and counted the total number of flowers, and three freshly opened flowers were harvested. For each of these flowers, the degree of herkogamy was determined by measuring the minimum distance between stigmatic surface and anthers. At the same time, the corolla diameter was also measured and used as an estimate of flower size. All floral measurements were done with Image J (Rasband, 2011) using digital scans taken from a cross-section of the flowers.

To determine mean pollen and ovule production per flower, one extra flower was harvested on a subset of 15 plants per population. For this purpose, flowers were collected just before the onset of anthesis to ensure that pollen production could be accurately determined. In the laboratory, one anther was then excised per flower for further determination of pollen quantities using a ‘solution’ method and manual counting under a binocular microscope (for more details, see Brys and Jacquemyn, 2011). Total ovule production per flower was determined by dissecting the ovary, staining the ovules with a methylene blue solution (Tilton, 1980) and counting them under a binocular microscope. The pollen–ovule (P/O) ratio was then calculated for each flower.

**Timing of anther dehiscence, autonomous pollen deposition, stigma receptivity and autonomous selfing**

To investigate whether the timing of the male and female functions during floral ageing (also further denoted as flower or floral development), the capacity for autonomous pollen deposition and ultimately autonomous seed production differed between pollination environments, we transferred 30 *B. perfoliata* transplants per population into pots and brought them to a pollinator-free greenhouse. Because anthesis in *B. perfoliata* generally lasts for 4 d and mean floral longevity did not significantly differ between both environments (results not presented), we investigated the timing of anther dehiscence, autonomous self-pollen deposition, stigma receptivity and effective autonomous seed production during the following stages of flower development: (1) 24 h before anthesis; (2) first day of flower opening (0 h after anthesis); (3) second day after flower opening (24 h after anthesis); (4) third day after flower opening (48 h after anthesis); and (5) fourth day after flower opening (72 h after anthesis). All measurements were done between 1000 and 1200 h.

First, we recorded for each transplant the timing of anther dehiscence during each of the above-mentioned stages of flower development. Secondly, to determine the timing of autonomous self-pollen deposition during floral development and to investigate when autonomous self-fertilization takes place, we examined a subset of flowers on half of the transplants \( (n = 15 \text{ per population}) \). Per individual, five intact and unmanipulated flowers were marked, and their stigmas were harvested at each of the five afore-mentioned stages and stored in FAA (300 flowers in total). In the laboratory, stigmas were rinsed under distilled water and softened in 1 M NaOH for 24 h, before staining in a 0.005 \% aniline blue solution (phosphate buffer pH 8.5) for 1 h. To assess the number of autonomously deposited self-pollen grains, pollen loads were quantified under an UV epifluorescence microscope (see Supplementary Data Fig. S2). Once seeds were ripe, fruits were harvested and seed production was determined for each treatment.

Thirdly, stigma receptivity was investigated by means of pollen tube detection techniques on a subset of flowers on the same transplants used for the determination of autonomous pollen deposition \( (n = 15 \text{ per population}) \). Per individual, five flowers were emasculated 1 d before flower opening to avoid interference through autonomous self-pollen deposition. Flowers were then hand-pollinated with self-pollen at each of the five stages of flower development (300 flowers in total) and stigmas were harvested 8 h later by excising the pistil half-way, and immediately stored on FAA. In the laboratory, stigmas were then softened and stained using the same procedure as described above. The presence/absence of pollen tubes was again examined under a UV epifluorescence microscope (see Supplementary Data Fig. S2). When present in the stigmatic tissue, in all cases pollen tubes occurred in high abundances so that stigma receptivity could be determined unambiguously.
Finally, to provide insights into the total capacity for autonomous selfing, we used the other subset of transplants under pollinator-free conditions in the greenhouse (n = 15 per population). On these plants, three pollination treatments were applied: (1) supplemental self-pollination at the second day of flowering; (2) flowers left intact and unmanipulated; and (3) emasculation of flowers 1 d before flower opening with no further manipulation. For each plant, each pollination treatment was replicated twice, resulting in a total of six flowers that were treated per plant. Fruits were harvested once seeds were ripe, and seed production was determined manually. The capacity for autonomous selfing [i.e. autoreproductivity (AF)] was determined per plant as [(mean autonomous seed production per fruit)/(mean seed production of supplemental self-pollinated flowers)] (Lloyd and Schoen, 1992).

Impact of selfing and outcrossing on seed production

To investigate the impact of selfing on seed production, a subset of transplants (n = 15 per population) was exposed to supplemental hand pollinations under controlled pollinator-free conditions in the greenhouse. On each of these plants, five flowers were marked and supplementary pollinated with self-pollen, whereas another subset of five flowers was pollinated with outcross pollen. Once seeds were ripe, fruits of each of these differently treated flowers were harvested and seed production was determined. We calculated the relative difference in seed production following self- and outcross-pollination at plant level as \( w_s / w_o \), with \( w_s \) = total seed set per fruit following supplemental self-pollination and \( w_o \) = total seed set per fruit following supplemental outcross pollination.

Statistical analyses

To compare average seed production in the field between the different pollination treatments (i.e. open pollination, emasculation and supplemental self- and outcross-pollination) and both contrasting environments, a two-way analysis of variance (ANOVA; using PROC MIXED in SAS) was used, with pollination treatment, environment and their interaction as fixed factors. Population nested within environment was entered as a random factor in this model. A general linear mixed model (GLMM) was applied to test whether environment had a significant impact on plant height, total number of flowers per plant, corolla diameter and herkogamy, the number of pollen (P) and ovules (O) per flower, and the P/O ratio. To incorporate the nested structure of the design, population nested within environment was included as a random factor in the model to correct for random population effects.

To test whether the timing of anther dehiscence varied between pollination environments, a \( \chi^2 \)-test was applied on the pooled data from both populations per environment. Before pooling the data, we first tested whether the timing of anther dehiscence did not differ significantly among populations belonging to the same environment (\( \chi^2 = 0.513 \); d.f. = 4; \( P = 0.972 \) and \( \chi^2 = 0.513 \); d.f. = 4; \( P = 0.972 \) for the pollinator-poor and pollinator-rich populations, respectively). Based on these data, we calculated the time (i.e. floral age) at which 50 % of the flowers in the sample started to dehisce (AAD, average anther dehiscence) for each population. A logistic regression analysis, using PROC GLIMMIX in SAS, was used to test whether stigma receptivity was significantly related to floral development, and whether this relationship was affected by environment. Floral developmental stage (continuous variable), environment (fixed factor) and their interaction were entered as the explanatory variables in this model, whereas stigma receptivity was entered as a binary response variable, using the binomial distribution and a logit link function. Plants were nested within populations, whereas population was nested within environment. Per population, we also calculated average stigma receptivity (ASR, the time at which 50 % of the stigmas in the sample were receptive) as \(-\mu/\alpha\), where \( \mu \) and \( \alpha \) are the intercept and slope, respectively, of the fitted regression curve (Armbruster et al., 2002). To obtain an estimate of mean dichogamy per population, we calculated the time interval between the time at which 50 % of the stigmas in the sample were receptive during floral development and the moment at which 50 % of the flowers started dehiscing their anthers (i.e. AAD – ASR).

A two-way ANOVA was used to investigate whether both autonomous self-pollen deposition and seed production under pollinator-free conditions changed during floral development and differed between both environments from which the investigated plants originated. Again flower age, environment and their interaction were included as explanatory variables, with two levels of nesting included to correct for random effects. In addition, a GLMM was again used to test whether the capacity (i.e. seed production) and index of autonomous selfing differed significantly between both pollination environments. Finally, a two-way ANOVA was used to test whether supplemental outcross- and self-pollination resulted in different seed production per fruit and whether this was affected by environment. In this model, pollination treatment, environment and their interaction were included as explanatory variables. Pollination treatment was nested in plant and population was nested in environment, and both factors were incorporated as random factors in the model. All statistical analyses were conducted using SAS version 9.2 (SAS Institute Inc.©).

RESULTS

Seed production, pollen limitation and reproductive assurance in the field

Under natural field conditions, average seed production was significantly affected by pollination treatment (\( F = 21.31; P < 0.0001 \)) and the interaction between environment and pollination treatment (\( F = 9.42; P < 0.0001 \)), whereas no significant main effect of pollinator environment was found (\( F = 5.20; P = 0.1495 \)). In the pollinator-rich environment, supplemental outcross-pollination resulted in a 25.7 and 39.1% increase in seed production compared with that of open-pollinated and emasculated flowers, respectively. In the pollinator-poor environment, supplemental hand pollination increased seed production by 0.1 and 55.6 % compared with that of open-pollinated and emasculated flowers (Table 1). Seed production of supplemental outcross-pollinated plants was 38.7 % greater in the pollinator-poor than in the pollinator-rich populations.
Emasculating flowers reduced seed production by an average 18.3 and 55.3% compared with open-pollinated control plants in the pollinator-poor and pollinator-rich environment, respectively (Table 1).

As a result, the pollinator failure index was significantly larger in the pollinator-poor than in the pollinator-rich environment (mean $PL_{\text{pollinator}} = 0.67 \pm 0.04$ and 0.31 $\pm 0.12$, respectively; Fig. 2A). The contribution of autonomous selfing to total seed set was >3-fold higher in the pollinator-poor than in the pollinator-rich environments, resulting in significantly larger indices of reproductive assurance (mean $RA = 0.56 \pm 0.11$ and 0.17 $\pm 0.13$, respectively; Fig. 2A). Although the indices of outcross-pollen limitation suggested that female reproductive success was more strongly reduced in the pollinator-rich than in the pollinator-poor sites, they did not differ significantly from zero (mean $PL_{\text{outcross}} = 0.19 \pm 0.31$ and 0.05 $\pm 0.24$, respectively; Fig. 2B). In addition, seed set was not restricted by a shortage of self-pollen deposition in either environment, as indicated by the negative values of self-pollen limitation and the 95% confidence intervals overlapping zero (mean $PL_{\text{self}} = -0.05 \pm 0.25$ and $-0.02 \pm 0.33$ in the pollinator-poor and pollinator-rich environments, respectively; Fig. 2B).

**Floral traits**

Plants in the pollinator-poor environment were significantly ($P < 0.001$) larger and produced more flowers per plant than plants growing in the pollinator-rich environment (Table 2). Flowers were significantly smaller and were characterized by a significantly lower degree of herkogamy and lower P/O ratios in the pollinator-poor than in the pollinator-rich environments (Table 2). Ovule production per flower was significantly larger in the pollinator-poor than in the pollinator-rich environment, but pollen production, on the other hand, did not differ significantly between both environments, indicating that the observed difference in the P/O ratio was mainly due to differences in the number of ovules produced per flower (Table 2).

**Mating system functioning and capacity for autonomous selfing**

The timing of anther dehiscence differed significantly between plants from both environments ($\chi^2 = 14.76$; d.f. = 4; $P = 0.005$). In individuals from the pollinator-poor populations, anthers started dehiscing sooner than in plants from the pollinator-rich sites (on average 50% of the plants in the sample started anther dehiscence 5.4 and 35.0 h after anthesis in the pollinator-poor and pollinator-rich populations, respectively; see Fig. 3A). Similarly, the timing of autonomous self-pollen deposition differed significantly between plants from both environments (Table 3; Fig. 3B). In individuals from the pollinator-poor environment, not only was pollen deposited earlier on the stigmatic surface, but also significantly more pollen grains were autonomously deposited at the end of a flower’s lifetime (on average 1166 $\pm 278$ and 736 $\pm 101$ pollen grains were deposited per stigma in individuals from the pollinator-poor and pollinator-rich environment, respectively) (Table 3). In plants from the pollinator-poor environment, >80% of the total pollen load was autonomously deposited at the second day of anthesis, whereas on average 32 and 75% of the total pollen load was deposited at the second and third day of anthesis in individuals from the pollinator-rich populations (Fig. 3B).

Stigma receptivity increased significantly with flower age (Wald $\chi^2 = 48.43$; $P < 0.001$) (see Fig. 3C), but neither environment (Wald $\chi^2 = 0.04$; $P = 0.84$) nor the interaction between environment and flower age (Wald $\chi^2 = 0.26$; $P = 0.61$) affected stigma receptivity significantly. The time at which 50% of the stigmas in the sample were receptive (ASR) was 9.0 and 5.9 h before the moment of anthesis in individuals from the pollinator-poor and pollinator-rich environment, respectively (Fig. 3C). The mean temporal separation between stigma receptivity and anther dehiscence (ADD – ASR) was on average 40.9 h in plants from the pollinator-rich populations and only 14.4 h in plants from the pollinator-poor populations.

Finally, the amount of seeds produced via autonomous selfing under pollinator-free conditions increased significantly with flower age, and the rate of increase was significantly higher in plants from the pollinator-poor environment than in individuals from the pollinator-rich environment (see Table 3; Fig. 3D). About one-third of the total autonomously produced seed set in plants from the pollinator-poor environment occurred on the second day of flowering, and increased to nearly 70% on the third day of flowering. In plants from the pollinator-poor environment, this increase was much slower, only 5 and 20% on the second and third day relative to flower opening, respectively (Fig. 3D). Finally, total autonomous seed production differed significantly ($F_{1,77} = 45.48$; $P < 0.0001$) between both environments (mean seed production: 184.0 $\pm$ 35.5 in the pollinator-rich environment and 700.5 $\pm$ 55.4 seeds per fruit in the pollinator-poor environment), resulting in significantly higher ($F_{1,77} = 48.37$; $P < 0.0001$) indices of autonomous selfing in the pollinator-poor (AF: 0.81 $\pm$ 0.50) than in the pollinator-rich environment (AF: 0.41 $\pm$ 0.06). Flowers that were emasculated prior to

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**Table 1. Mean seed production rates per fruit (± s.e.) of *Blackstonia perfoliata* occurring in both contrasting pollination environments (i.e. ‘pollinator-poor’ vs. ‘pollinator-rich’), following different pollination treatments in four natural populations**

<table>
<thead>
<tr>
<th>Population/pollination treatment ($n = 20$)</th>
<th>Pollinator-poor</th>
<th>Pollinator-rich</th>
</tr>
</thead>
<tbody>
<tr>
<td>WH1</td>
<td>766.6 ± 58.8</td>
<td>478.8 ± 78.6</td>
</tr>
<tr>
<td>WH2</td>
<td>1196.1 ± 105.3</td>
<td>410.3 ± 86.6</td>
</tr>
<tr>
<td>Emasculation</td>
<td>320.4 ± 31.5</td>
<td>344.2 ± 63.3</td>
</tr>
<tr>
<td>Supplemental cross-pollination</td>
<td>8050.0 ± 88.1</td>
<td>5971.1 ± 64.3</td>
</tr>
<tr>
<td>Supplemental self-pollination</td>
<td>727.3 ± 68.6</td>
<td>388.3 ± 69.4</td>
</tr>
</tbody>
</table>

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**References**

Brys et al. — Shifts in floral traits contribute to higher selfing.
flower opening did not produce any seeds, indicating that *B. perfoliata* is not able to develop any seeds through apomixis.

Impact of selfing and outcrossing on seed production

Supplemental self-pollination under controlled conditions in the greenhouse significantly lowered seed set per fruit.
compared with outcross-pollination ($F = 13.59; P = 0.0004$), and this effect was dependent on the environment from which the plants originated ($F = 3.94; P = 0.0409$). In plants obtained from the pollinator-rich populations, selfing reduced seed set by on average 40% compared with outcrossing, whereas in plants from the pollinator-poor sites, both pollination treatments resulted in a similar seed set (mean increase of 0.8% in seed set per fruit following selfing in comparison with outcrossing). Pollination environment, on the other hand, did not significantly affect seed production ($F = 3.94; P = 0.1268$). Finally, the relative index of female fitness following self- and outcross-pollination was significantly related to the capacity for autonomous selfing (Fig. 4).

**DISCUSSION**

This work showed that the self-compatible, hoverfly-pollinated *B. perfoliata* experienced stronger failure of pollinator-mediated seed production in recently established populations. However, total seed production in these populations was not restricted by pollen limitation, and indices of reproductive assurance indicated that autonomous selfing significantly enhanced total seed set in these populations. Detailed investigation of flower morphology and mating system functioning further showed that plants from the pollinator-poor sites had a 2-fold higher capacity for autonomous selfing in the absence of any pollinator compared with plants from the pollinator-rich sites, indicating that the contribution of autonomous selfing to total seed set in the pollinator-poor populations was significantly related to the capacity for autonomous selfing (Fig. 4).

**Fig. 3.** (A) Mean proportional anther dehiscence, (B) mean autonomous pollen deposition proportional to the total amount of pollen that is autonomously deposited at the end of a flower’s lifetime ($\pm$ s.e.), (C) mean stigma receptivity, and (D) mean autonomous seed production per fruit proportional to the total numbers of seeds that are produced after supplemental self-pollination ($\pm$ s.e.) in relation to floral development in each of the studied *Blackstonia perfoliata* populations occurring under two contrasting environments. Times are in hours, where 0 indicates the beginning of anthesis. The arrows indicate the average developmental floral stage at which 50% of the flowers in the sample showed (A) anther dehiscence or (C) stigma receptivity.

**Table 3.** Results of a two-way ANOVA for the effect of pollinator environment (i.e. ‘pollinator-poor’ vs. ‘pollinator-rich’) and floral development (–24, 0, 24, 48 and 72 h relative to flower opening) in a pollinator-free environment, on autonomous self-pollen deposition and autonomous seed production rates proportional to the maximum number of autonomous deposited pollen grains and the maximum number of seeds that are produced via this mode of selfing, respectively, in four *Blackstonia perfoliata* populations occurring under two contrasting environments.

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autonomous self-pollen deposition ($n = 60$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pollinator environment</td>
<td>1, 2</td>
<td>76.55</td>
<td>0.0128</td>
</tr>
<tr>
<td>Floral development</td>
<td>4, 288</td>
<td>108.22</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Floral development $\times$ pollen environment</td>
<td>4, 288</td>
<td>8.06</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Autonomous seed production ($n = 80$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pollinator environment</td>
<td>1, 2</td>
<td>41.81</td>
<td>0.0208</td>
</tr>
<tr>
<td>Floral development</td>
<td>4, 59</td>
<td>103.82</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Floral development $\times$ pollen environment</td>
<td>4, 235</td>
<td>21.72</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
environment was much higher. Although the pollinator-rich populations showed lower levels of pollinator failure than the pollinator-poor populations, seed production at these sites was more severely limited by pollen quality (outcross-pollen deposition) than by pollen quantity (e.g. Vaughton and Ramsey, 2010). This can, at least partly, be attributed to the fact that self-pollination resulted in a 40% lower seed production compared with outcross-pollination at these sites, most probably due to early inbreeding effects (e.g. Husband and Schemske, 1996). The occurrence of certain levels of autonomous selfing, even in the pollinator-rich sites, may thus explain the higher indices of outcross-pollen limitation in *B. perfoliata* growing at these sites. The higher capacity for autonomous selfing was associated with a significant reduction in the level of herkogamy (70%), indicating that reduced spatial segregation of anthers and stigma is, at least partly, responsible for the observed increase in autofertility. This observation is in agreement with a number of studies that showed that variation in herkogamy significantly affected the extent of autonomous selfing (e.g. Rick et al., 1977; Ennos, 1981; Ritland and Ritland, 1989; Takebayashi and Delph, 2000; Moeller and Geber, 2005; Moeller, 2006; Herlihy and Eckert, 2007; Brys and Jacquemyn, 2011, 2012).

Whether a reduction in herkogamy increases the capacity for autonomous selfing significantly depends on the temporal separation of the male and female function within a flower (Lloyd and Webb, 1986). Here, we found that flowers in general exhibited protogyny, implying that stigma receptivity preceded pollen release. However, pronounced differences in dichogamy were observed between individuals from the two pollination environments. In individuals from pollinator-poor populations, anthers dehisced significantly earlier during flower development, resulting in a reduction of 65% in the level of dichogamy compared with plants originating from the pollinator-rich populations (from a temporal separation of on average 40.9 h between the female and the male phase in the pollinator-rich populations towards a temporal separation of 14.4 h in the pollinator-poor populations). This shift caused a similar deviation in the timing of autonomous pollen deposition and effective fertilization, and changed the breeding system from delayed selfing in the pollinator-rich environment towards competing selfing in the pollinator-impoveryished sites. Since we do not have any indication that pollinators show different preferences for flowers of a certain age, it can be expected that outcross pollen grains will have a higher chance of competing with autogamously deposited self-pollen in selfing than in outcrossing variants.

Investigation of anther development further showed that besides earlier dehiscence in the pollinator-poor environment, anthers also had a tendency to rotate more strongly during ripening in order to ease pollen release (see Fig. 5A–C). This, in combination with the close juxtaposition of anthers and stigma, may explain the higher capacity for autonomous selfing in these populations. A similar system in which anther rotation guarantees autonomous pollen deposition, thereby offering reproductive assurance in the absence of pollinators, has been reported in related *Centaurium* species (Brys and Jacquemyn, 2011; Brys et al., 2011). These results indicate that the observed reduction in herkogamy and dichogamy in combination with increasing anther rotation during ripening elevates the capacity for autonomous selfing, but, on the other hand, may diminish the chance for outcross pollen due to both shielding of anthers and clogging of the stigma with autonomously deposited self-pollen.

When a species or population has recently experienced an evolution towards autonomous selfing, co-evolution of other floral traits can occur in a sequential fashion, with changes in some traits preceding others (Vallejo-Marín and Barrett, 2009; Bodily-Roels and Kelly, 2011). In the case of the studied *B. perfoliata* populations, it can be assumed that recently established populations were exposed to severe outcross-pollen limitation, most probably due to the combined effects of founder events and pollinator limitation (e.g. Levin, 2010; Cheptou, 2012). Under these conditions, the first step may be the establishment of traits that are involved with reproductive assurance itself, such as the observed reductions in herkogamy and dichogamy, whereas others (reductions in the P/O ratio, flower size, floral lifetime, etc.) may follow as a secondary evolutionary response. In the pollinator-poor *B. perfoliata* populations, significantly lower P/O ratios were indeed observed compared with plants from the pollinator richer areas. This observation is consistent with the prediction that there may be less investment in the male function proportional to the female function and that reduced pollen production is more efficient in self-pollinating variants than in outcrossing variants (Lloyd, 1987; Sicard and Lenhard, 2011). In addition, *B. perfoliata* plants also produced significantly smaller flowers (i.e. smaller corolla size) in the pollinator-impoveryished populations, but the total number of flowers produced per plant was significantly larger than in the pollinator-rich populations. Given that plants were also larger in the pollinator-poor sites, this may explain the

![Figure 4](https://example.com/fig4.jpg)

**Fig. 4.** The association between the index of autonomous selfing (AF) and the relative fitness between seed set following supplemental self- (w_s) and cross-pollination (w_o) in each of the studied *Blackstonia perfoliata* populations occurring under both contrasting environments (mean ± s.e.).
higher capacity for flower production and also the higher ovule production on a per flower basis.

Although flower longevity is another feature that is frequently documented to shorten in more selfing taxa as a strategy to reduce unnecessary costs of resource investment (Lloyd, 1979; Arathi et al., 2002), especially in species or populations that are evolving towards competing or even prior selfing (Elle et al., 2010), such a shift was not detected in the studied populations. This poses the question of what the fitness advantage of accelerating the male phase might be if flowers do not show a noticeable reduction in floral lifetime and thus do not decrease the costs of floral maintenance (Schoen and Ashman, 1995). It might be plausible that when successful pollinator-mediated outcross-pollination remains restricted over time, this and other floral traits might evolve in the upcoming generations, and that the breeding system itself may shift towards prior selfing. On the other hand, it is also plausible that the observed differences in floral morphology and mating system functioning are caused by founder events during the first stages of population development, and that, in the most extreme scenario, the colonists already possessed a breeding system similar to the observed breeding system in current populations (e.g. Cheptou, 2012).

Overall, our findings illustrate that plants in recently established populations can show significant and rapid differentiation in floral morphology and mating system functioning, especially when pollinators are scarce. Although an increased capacity for autonomous selfing can be seen as an advantageous strategy for reproductive assurance and recruitment in the short term, these shifts may be detrimental in the longer term through important implications of inbreeding depression (Winn et al., 2011), distribution of genetic variation within populations, gene flow, capacity for adaptation, etc. (Lande and Schemske, 1985; Charlesworth, 2003). For a better understanding of the evolutionary and ecological consequences of such shifts in plant breeding systems, additional studies are therefore needed that focus more particularly on the longer term consequences, such as total lifetime fitness or population dynamics, than on the level of seed production alone (e.g. Aizen and Harder, 2007; Igic et al., 2008; Jacquemyn et al., 2012).

**SUPPLEMENTARY DATA**

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: insect species observed on flowers of *Blackstonia perfoliata* during observation intervals in the ‘pollinator-rich’ and ‘pollinator-poor’ environment in 2011. Figures S1: differences in pollinator abundance determined by pan-trapping in each of the study populations occurring under contrasting pollinator environments. Figure S2: pollen tube growth in stigma lobe of *Blackstonia perfoliata* following supplemental self-pollination at the first day of anthesis.

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**LITERATURE CITED**


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