

BRIEF COMMUNICATION

**FLORAL EMASCULATION REVEALS POLLEN QUALITY LIMITATION
OF SEED OUTPUT IN *BULBINE BULBOSA* (ASPHODELACEAE)¹**

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Pollen commonly limits seed output in natural plant populations, but the relative contributions of pollen quantity and quality to pollen limitation remain largely unexplored. Estimates of these contributions are needed to promote better understanding of the ecological and evolutionary consequences of pollen limitation. We assessed pollen limitation and distinguished how pollen quantity and quality contributed to seed output in the predominantly outcrossing herb *Bulbine bulbosa*. We compared seed output of emasculated and cross-pollinated plants that could not self-pollinate with that of intact plants. Seed output was similar in emasculated and cross-pollinated plants, but it was about 50% less in intact plants. These findings indicate that the quantity of cross-pollen deposited by pollinators did not limit seed output. However, pollinators also deposited self-pollen, which reduced seed output because selfing disabled ovules, rendering them unavailable for cross-fertilization. We conclude that pollen quality rather than quantity can limit seed output under natural conditions, which supports recent proposals for greater consideration of pollen quality in the study of pollen limitation.

Key words: Asphodelaceae; *Bulbine bulbosa*; floral emasculation; ovule discounting; pollen limitation; pollination quality; pollination quantity; self-pollen interference.

Seed production of flowering plants is often limited by inadequate pollination. A recent review by Knight et al. (2005) found that fruit set was pollen limited in 63% of 482 data records. Similar findings have been reported in previous reviews (Burd, 1994; Larson and Barrett, 2000; Ashman et al., 2004). Reduced seed output owing to pollen limitation can potentially affect plant demography and cause selection on floral traits and plant mating systems (Lloyd, 1992; Burd, 1995; Ashman and Morgan, 2004; Ashman et al., 2004; Morgan and Wilson, 2005; Eckert et al., 2006). However, pollen limitation can be caused by deposition of (1) an insufficient quantity of pollen or (2) low-quality pollen on stigmas, although the individual contributions made by each have rarely been assessed. Estimating these contributions will advance our understanding of the ecological and evolutionary consequences of pollen limitation (Ashman et al., 2004; Aizen and Harder, 2007).

Limitation of seed output by pollen quantity occurs when too few pollen grains are deposited on stigmas to fertilize all ovules. When pollen quantity limits seed output, selection is expected to favor traits that increase pollen receipt, which in turn necessitates increases in pollen export (Harder and Routley, 2006; Aizen and Harder, 2007). Alternatively, selection could promote autonomous selfing to provide reproductive assurance and, hence, reduce reliance on pollinators (Lloyd, 1992; Morgan and Wilson, 2005; Eckert et al., 2006). By contrast, limitation by pollen quality occurs when low-quality pollen is deposited on stigmas and usurps ovules that otherwise would have been fertilized by higher-quality pollen (i.e., ovule discounting). This assumes that both high- and low-quality pollen are deposited on stigmas but that seed output is less than if low-quality pollen were eliminated. Ovules could be discounted if

self-fertilized or otherwise inbred embryos are aborted owing to early-acting inbreeding depression (Wiens et al., 1987; Husband and Schemske, 1996) or if ovules are disabled owing to late-acting (ovarian) self-incompatibility (Sage et al., 1999, 2006). When pollen quality limits seed output, selection is expected to favor traits that reduce the deposition of low-quality pollen, particularly self-pollen, thus allowing higher-quality pollen to fertilize ovules.

Pollen limitation is usually examined by supplementing stigmas with cross-pollen and comparing the resulting seed output with that of naturally pollinated plants. Increased seed output of supplemented plants is taken to indicate that naturally pollinated plants are pollen limited (Ashman et al., 2004; Knight et al., 2006; Aizen and Harder, 2007). However, Aizen and Harder (2007) have argued that supplementation experiments may be flawed because the respective effects of pollen quantity and quality on seed output are confounded. Because supplemental pollination entails adding excess cross-pollen to stigmas, supplemented flowers receive not only more pollen but also higher-quality pollen, on average, than naturally pollinated flowers (Ashman, et al., 2004; Aizen and Harder, 2007). As a result, supplementation studies could falsely conclude that seed output is limited by pollen quantity, when in fact pollen quality is the limiting factor. Further, studies could underestimate the combined limitations of pollen quantity and quality if supplemental pollen is added near the end of floral life and low-quality pollen has previously discounted ovules, thus preventing any increase in seed output (Ramsey and Vaughton, 2000; Aizen and Harder, 2007). Despite the relevance of pollen quality to seed output (Ramsey, 1995; Ramsey and Vaughton, 2000; Finer and Morgan, 2003; Chacoff et al., 2008), the implications of pollen quality for pollen limitation have been studied infrequently (Aizen and Harder, 2007).

These arguments highlight the need for experimental protocols that separate the effects of pollen quantity and quality. One approach involves emasculating flowers, which renders intra-

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floral self-pollination impossible (Schoen and Lloyd, 1992; Eckert et al., 2006). Provided that emasculation does not affect visits by and behavior of pollinators, comparison of emasculated and intact flowers can be used in nonautonomous plants to assess quality limitation caused by pollinator-mediated selfing. Greater seed output by emasculated flowers compared with intact flowers and verification that only pollinators deposit self-pollen indicate that pollinator-mediated selfing contributes to pollen quality limitation (e.g., Ramsey et al., 2006). Pollen quantity limitation can be estimated by hand-pollinating flowers on other plants with abundant cross-pollen. If emasculated flowers have lower seed output than cross-pollinated flowers, this is evidence that cross-pollen is limiting. Further, the difference between intact and cross-pollinated flowers represents total pollen limitation owing to both pollen quantity and quality. We are not aware of previous studies that have undertaken these experimental comparisons and interpreted the results in the light of pollen limitation.

We used this approach to assess pollen limitation in the predominantly outcrossing herb *Bulbine bulbosa* (Asphodelaceae). Previously, we have shown that naturally pollinated plants are pollen limited and produce about 50% fewer seeds than plants that are experimentally cross-pollinated, despite large pollen loads on stigmas (Owen et al., 2007). Autonomous selfing does not occur, and pollinators are required for seed set. Experimental selfing reduces seed output by 75% compared with experimental outcrossing. When we pollinated flowers with an equal mixture of self- and cross-pollen, seed output was intermediate to that of self- or cross-pollination only, which indicates that pollen quality could cause pollen limitation because selfing discounts ovules. Finally, pollen quality also reduces progeny fitness. Although individual seed mass following selfing or outcrossing did not differ, cumulative inbreeding depression for seed germination and early seedling growth was 0.50 (Owen et al., 2007).

Here we assessed the extremes of pollen quality by comparing self-pollen and cross-pollen pooled from multiple donors, recognizing that “pollen quality” includes variation in pollen quality among outcross donors. We first established that emasculating flowers did not affect pollinator visits. We then compared seed output of plants with intact, emasculated, or cross-pollinated flowers in two populations of *B. bulbosa*. Our results reveal that both populations are pollen limited owing to low-quality self-pollen rather than to insufficient quantities of cross-pollen.

MATERIALS AND METHODS

Study species and sites—*Bulbine bulbosa* (R. Br.) (Haw) is a lilioid perennial herb that occurs in grassland, woodland, and open forest habitats across southeastern Australia. In late spring, plants produce a flowering scape with about 25–30 hermaphroditic flowers that open acropetally over several weeks. Most plants open one flower per day (mean \pm SE, = 1.2 ± 0.2 , range 1–3) and each flower lasts for about 8 h. Flowers are ca. 20 mm in diameter and have six yellow elliptic tepals. The stamens are clustered on one side of the flower, and clavate hairs attached to the apex of each filament surround each anther. The style is decumbent and bends away from the stamens. Anther-to-stigma distances are 3–4 mm. Flowers are fragrant but do not produce nectar. Fruits are capsules and contain a maximum of 12–13 seeds (Godden, 1993; Owen et al., 2007).

Our study was conducted at two sites near Armidale, New South Wales, Australia (30°29.8'S, 151°29.9'E, 960 m a.s.l.). Sites were located in grassy woodland and were about 1 km apart. Native bee and syrphid fly species and introduced honeybees were the major floral visitors at both sites. Each site had several hundred flowering plants.

Does floral emasculation affect pollinator visitation?—Over 5 d at each site, we monitored pollinator visits to either emasculated or intact plants with one open flower (plants per treatment, site 1: $N = 17$; site 2: $N = 18$). Plants did not open flowers on all days, and different plants were used each day. We used jeweler's forceps to emasculate flowers by removing anthers before they dehisced, taking care to minimize damage to the clavate hairs, which surround and hide anthers and initially attract pollinators. Flowers on intact plants were left untouched. We monitored emasculated and intact plants simultaneously during four 15-min periods between 0900 and 1300 hours on each day and counted pollinator visits to flowers. To assess the effect of emasculation on pollinator visits, we used an analysis of variance (ANOVA) with site and emasculation as fixed factors and day as a random block. Similar visitation to emasculated and intact flowers indicates that pollinators do not discriminate between the treatments and validates the use of emasculation to test whether seed output is limited by pollen quality.

Is seed output limited by pollen quantity or pollen quality?—At each site, we haphazardly allocated plants with one scape that had just begun to flower to one of three treatments: intact, flowers untouched and naturally pollinated; emasculated, anthers removed from flowers and naturally pollinated; or cross-pollinated, anthers removed and flowers manually cross-pollinated ($N = 16$ –20 plants per treatment per site). Flowers were emasculated as just described. Cross-pollinated plants were bagged to exclude pollinators and received pollen from 3–4 donor plants located ca. 30 m distant to ensure maximum seed output by preventing possible biparental inbreeding. Outbreeding depression is unlikely; crossing distances of up to 1 km do not affect seed output (seed set: 1 km, $56 \pm 3\%$ [SE]; 10 m, $52 \pm 4\%$). Further, neither bagging nor emasculation affects seed output of cross-pollinated flowers (seed set: bagged + emasculated, $53 \pm 5\%$; not bagged + emasculated, $55 \pm 3\%$; not bagged + not emasculated, $57 \pm 3\%$; G. Vaughton and M. Ramsey, University of New England, unpublished data). We treated plants over 20 consecutive days, and all or most (>85%) flowers opened.

After 4 wk, we scored fruit set (fruits/flower) and counted numbers of ovules, filled seeds, and aborted seeds in four fruits per plant, or all available fruits. Filled seeds were firm. Aborted seeds were shriveled, smaller than filled seeds, and larger than unfertilized ovules. We probably underestimated abortions; zygotes that aborted early in development could not be detected and would have been scored as unfertilized ovules. We calculated seed set as filled seeds / (filled seeds + aborted seeds + unfertilized ovules) and seed abortion as aborted seeds / (intact seeds + aborted seeds). We estimated combined reproductive output as fruit set \times mean seed set. We then used these estimates to calculate (1) total pollen limitation as $1 - (\text{intact/cross-pollinated})$ and (2) cross-pollen limitation as $1 - (\text{emasculated/cross-pollinated})$. Values of both indices vary between 0 and 1. Values near zero indicate no pollen limitation, whereas larger values indicate increasing pollen limitation. Total pollen limitation reflects the combined limitation of pollen quantity and quality, and cross-pollen limitation reflects the limitation by pollen quantity only. Higher values of total pollen limitation than of cross-pollen limitation indicate that pollen quality limits seed output more than pollen quantity does.

We compared fruit set among treatments using analysis of deviance (using generalized linear models), with site and pollination treatment as factors and plants as replicates. For seed set and seed abortion, we used an ANOVA with site and pollination treatment as fixed factors, plant as a random factor nested within site and treatment, and fruits as replicates. Denominator degrees of freedom were calculated using Satterthwaite's approximation, owing to unequal nesting and unequal numbers of replicates per plant.

Statistical analyses—For analyses of deviance, we used GLMStat version 6.0 (Beath, 2004) with binomial error structures, logit link functions, and scaled deviances to remove overdispersion (Crawley, 1993). For ANOVAs, we used JMP version 5.0.1 (SAS Institute, Cary, North Carolina, USA). We transformed pollinator visits (square root) and seed set and seed abortion (arcsin square root) to ensure that assumptions of ANOVA were met. Means (\pm SE) are given.

RESULTS

Does floral emasculation affect pollinator visitation?—Pollinator visits to emasculated and intact flowers did not differ ($F_{1,62} = 0.16$, $P = 0.691$). On average, flowers received 4.6 visits per hour of observation, although the number of visits required

for full seed set was not determined (visits per hour, site 1: emasculated, 3.82 ± 0.56 ; intact, 3.91 ± 0.32 ; site 2: emasculated, 5.50 ± 0.42 ; intact, 5.33 ± 0.40). Visitation differed between sites and among days (sites: $F_{1,62} = 21.20$, $P < 0.0001$; days: $F_{4,62} = 6.07$, $P < 0.001$), but the treatment \times site interaction was not significant ($F_{1,62} = 0.53$, $P = 0.470$).

Is seed output limited by pollen quantity or quality?—Fruit set was similar in emasculated and cross-pollinated plants and was 15% greater in both than in intact plants. Fruit set differed significantly between sites, but the pollination \times site interaction was not significant (Fig. 1A, Table 1). Seed set and seed abortion of emasculated and cross-pollinated plants were also similar, and both produced 24% more seeds and had 22–27% fewer abortions than intact plants (Fig. 1B; seed abortion: intact, $53.7 \pm 1.8\%$; emasculated, $27.2 \pm 1.4\%$; cross-pollinated, $31.9 \pm 1.1\%$). Neither seed set nor seed abortion differed between sites, and the pollination \times site interactions were not significant, although plants varied significantly (Table 1). Combined reproductive output of intact plants was only half that of emasculated and cross-pollinated plants at both sites (Fig. 1C). Average total pollen limitation was 0.57 (site 1: 0.60; site 2: 0.54) and was substantially greater than average cross-pollen limitation, which approached zero (site 1: 0.03; site 2: 0.0).

DISCUSSION

Our study of *Bulbine bulbosa* was designed to test whether pollen quantity or pollen quality limited seed output, an issue that has been raised by recent reviews (Ashman et al., 2004; Aizen and Harder, 2007). Rather than using a standard pollen supplementation experiment, we compared seed output of emasculated and cross-pollinated plants that cannot self-pollinate with that of intact plants. Our results clearly demonstrate that under natural conditions, pollen quality but not quantity limited seed output. Two lines of evidence support this conclusion. First, similar seed output of emasculated plants and cross-pollinated plants indicates that the quantity of cross-pollen delivered by pollinators did not limit female fecundity. Second, greater seed output of emasculated plants compared with that of intact plants indicates that pollinators deposit self-pollen on stigmas as well as cross-pollen, which reduces the production of cross-fertilized seeds. From these data we estimated that total pollen limitation (0.57) greatly exceeded cross-pollen limitation (0.01). Thus, we conclude that pollinators deposit sufficient cross-pollen to effect full seed output but that seed output is reduced substantially because self-pollen is also deposited. However, because *B. bulbosa* is perennial, increases in seed output in one year may be offset by decreases in fitness in subsequent years (Dudash and Fenster, 1997). Despite this caveat, our study adds weight to proposals that pollen quality can limit seed output and that alternative methodologies to supplementation experiments may be required to characterize contributions made by pollen quality and quantity to total pollen limitation (Ashman et al., 2004; Aizen and Harder, 2007). Estimates of these individual contributions will promote better understanding of the ecological and evolutionary consequences of pollen limitation (Aizen and Harder, 2007).

A potential problem with emasculation studies is that pollinators may discriminate against emasculated flowers because floral signals or rewards have been altered (Schoen and Lloyd,

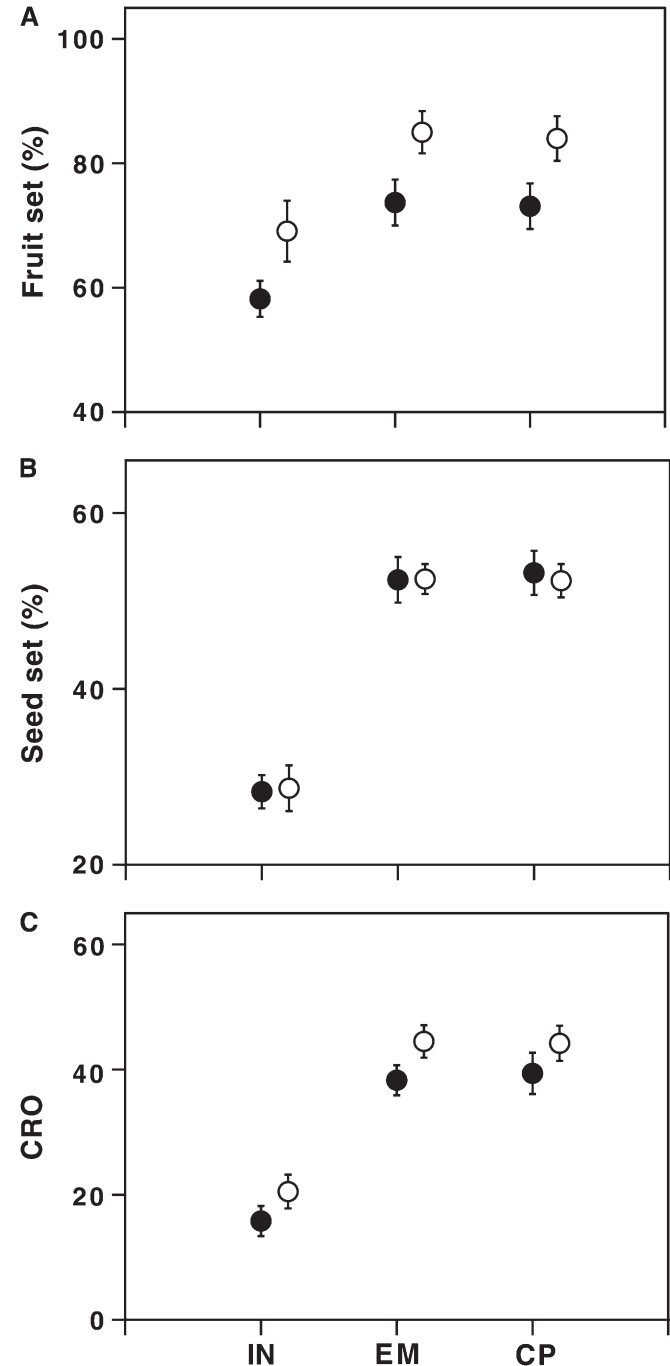


Fig. 1. Mean (\pm SE) percent (A) fruit set and (B) seed set, and (C) combined reproductive output (CRO \times 100) of intact (IN, natural self- and cross-pollination occurs), emasculated (EM, natural cross-pollination only), and cross-pollinated (CP, manual cross-pollination) plants in *Bulbine bulbosa*. Filled and open symbols represent sites 1 and 2, respectively. IN plants produced significantly fewer fruits and seeds than EM and CP plants ($P < 0.001$).

1992; Eckert et al., 2006). We found that pollinator visits to emasculated and intact *B. bulbosa* flowers did not differ, which is similar to our previous finding in *B. vagans* (Vaughton et al., 2008). In both species, clavate hairs that surround the anthers serve as an initial attractant to pollinators, and we were able to

TABLE 1. Results of an analysis of deviance (χ^2) and ANOVAs (F) examining the effects of site, pollination, the site \times pollination interaction, and plant nested within site and pollination (S, P) on fruit and seed set and seed abortion in *Bulbine bulbosa*. For ANOVAs, numerator and Satterthwaite denominator degrees of freedom are given. Data are given in Figure 1.

Source	Fruits			Seeds			Seed abortion		
	df	χ^2	P	df	F	P	df	F	P
Site	1	16.30	<0.001	1, 99.4	0.01	0.914	1, 99.7	0.03	0.874
Pollination	2	17.95	<0.001	2, 99.4	74.89	<0.001	2, 99.8	52.25	<0.001
Site \times pollination	2	0.113	0.945	2, 99.4	0.10	0.904	2, 99.8	0.308	0.736
Plant (S, P)	—	—	—	97, 301	1.89	<0.001	97, 301	1.66	<0.001

remove anthers while minimizing damage to the hairs (Vaughton et al., 2008). In *B. bulbosa*, foraging behavior of syrphid flies and native bees appeared not to be affected by emasculation, but honeybee visits were shorter in duration compared with visits to intact flowers. Nevertheless, emasculated flowers produced more seeds than intact flowers and produced as many seeds as flowers that were cross-pollinated, which is not expected if pollinators strongly discriminated against emasculated flowers.

Similar seed output of emasculated and cross-pollinated plants in our study demonstrates that the quantity of cross-pollen deposited on stigmas was not limiting under natural conditions. Indeed, both flower types received about 4.6 visits per hour of observation, and we observed pollinators regularly moving between individual plants of *B. bulbosa* during foraging bouts. Flowers are open for only 8 h, and the high seed output of emasculated flowers attests to the efficiency of pollinators in transferring pollen between plants under the conditions of our study. However, we conducted our experiment during peak flowering when flowering densities were high, and levels of cross-pollination may be lower earlier or later in the season or in lower-density populations (Duncan et al., 2004b; Jakobsson et al., 2009). Interestingly, our results for *B. bulbosa* contrast with those for *B. vagans*, which often occurs in small, isolated populations. In *B. vagans*, emasculated flowers have reduced seed output compared with intact and cross-pollinated flowers, which indicates that cross-pollination is insufficient to maximize seed output (Vaughton et al., 2008; Vaughton and Ramsey, in press).

Intact and emasculated flowers were both exposed to pollinators, but self-pollination could occur only on intact flowers. The lower fruit and seed set and greater seed abortion on intact compared with emasculated flowers indicate that pollinators deposit a mixture of self- and cross-pollen on stigmas and that self-pollen interferes with the production of cross-fertilized seeds. We have previously argued that interference by self-pollen probably occurs as a result of self-pollen preempting ovules, rendering them unavailable for cross-fertilization (Owen et al., 2007). In that study, when we pollinated flowers with a mixture of self- and cross-pollen, seed output was intermediate to that following either pure self- or cross-pollination, despite apparently similar ovule fertilization in all treatments. Such reductions in seed output could increase the vulnerability of hermaphroditic plant populations to quality limitation (Aizen and Harder, 2007). Reductions in seed output after selfing occur in other lilioid species, and quality limitation may be common in natural populations of these species (Ramsey, 1995; Sage et al., 1999; Ramsey and Vaughton, 2000; Rosquist, 2001; Duncan et al., 2004a; Ramsey et al., 2006).

Flowers of *B. bulbosa* do not autonomously self-pollinate, and, thus, the amount of self-pollination and subsequent seed output will depend on interactions between insect pollinators

and floral biology (Owen et al., 2007). Self-pollination probably occurs predominantly via pollinator-mediated autogamy rather than geitonogamy because most plants have only one flower open per day. Notably, flowers are not dichogamous, and there are probably few opportunities for pollinators to deposit cross-pollen without concurrently depositing self-pollen (Owen et al., 2007). Although flowers are herkogamous, we observed pollinators contacting the stigma after they had been in contact with the anthers when foraging at flowers. As Lloyd (1992) noted, facilitated autogamy is probably an inevitable consequence of pollinators visiting flowers and is likely to vary greatly, depending on the foraging behaviors of visitors at flowers, the time they spend on each flower, and the positions of the anthers and stigmas. Floral visitors to flowers of *B. bulbosa* include pollen-collecting native bees and syrphid flies and introduced honeybees. Honeybees are expected to be less effective than native insect pollinators, but empirical data are scarce (Butz Huryn, 1997; Knight et al., 2005). In our study, honeybees comprised a relatively small proportion of the floral visitors (<20%), and we are currently examining their effectiveness as pollinators.

In *B. bulbosa*, selfing reduces seed output by 75% compared with outcrossing, and inbreeding depression over the life cycle is probably high (seed germination and early seedling growth, $\delta = 0.50$). As a consequence, few selfed progeny are likely to reproduce (Owen et al., 2007). If inbreeding depression causes selfed zygotes to abort, as we have previously proposed (Owen et al., 2007), then opportunities for purging of recessive lethal alleles would be reduced and high genetic loads could be maintained in populations (Lande et al., 1994). Here, we found that pollinators deposited sufficient cross-pollen to effect full seed set but that they also deposited self-pollen, causing pollen quality limitation. Consequently, we would expect that selection should favor traits that improve the quality of pollination and promote outcrossing. However, conflicting selection may constrain the evolutionary change that can occur. For example, increased herkogamy could reduce facilitated autogamy but also reduce cross-pollination if floral visitors fail to contact stigmas (Armbruster et al., 2009). Similarly, the evolution of dichogamy is probably constrained because flowers are short-lived, and if floral longevity were increased to more than a day, then geitonogamy could increase (Barrett, 2002; Finer and Morgan, 2003). Selection to promote outcrossing could also favor increased ovule production, allowing plants to compensate for the effects of embryonic lethal alleles (Porcher and Lande, 2005; see also Burd, 1995). However, given the apparently substantial genetic load in populations of *B. bulbosa*, ovule production would have to increase substantially for effective compensation to occur. Whether genetic variation for floral traits that promote outcrossing is present within populations remains to be determined.

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