INBREEDING REDUCES FLORAL LONGEVITY AND FLOWER SIZE IN THE MIXED-MATING BIENNIAL *SABATIA ANGULARIS*

Rachel B. Spigler[1,]* and Annmarie Charles*

*Department of Biology, Temple University, 1900 North 12th Street, Philadelphia, Pennsylvania 19122, USA

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Premise of research. Inbreeding depression is well known to contribute to the maintenance of outcrossing in flowering plants because of its direct negative impacts on germination, survival, and fertility. Yet inbreeding depression could also impact fitness indirectly by affecting traits that govern biotic interactions. Relatively little is known about inbreeding depression on traits related to pollination success. Although often associated with pollinator attraction or reward, pollination success is also determined by floral longevity. Inbreeding depression in floral longevity has not been studied but has the potential to compound pollination disadvantages confronting inbred plants.

Methodology. We investigated inbreeding depression for traits related to pollination success in the mixed-mating biennial *Sabatia angularis* L. (Pursh) (Gentianaceae). Under controlled, pollinator-free conditions, we performed crosses to create families of selfed and outcross seed and raised these plants to flower. We compared selfed and outcrossed individuals for the following traits: flower number, flower size, corolla longevity, stigma longevity, and plant height. We also considered whether inbreeding depression was influenced by flower number–longevity trade-offs or plant size.

Pivotal results. Selfed plants produced significantly smaller flowers that lived for a significantly shorter amount of time than flowers of outcrossed plants, based on corolla and stigma longevity. Consistent with prior work, we found trade-offs between floral longevity and flower number and positive relationships between longevity and plant height. However, we did not detect inbreeding depression for flower number or plant size.

Conclusions. We find novel evidence that inbreeding reduces both floral longevity and flower size. Although moderate, inbreeding depression for these traits could leave selfed plants at a disadvantage in pollen-limited environments, especially where rates of pollen export outpace deposition such that plants are forced to rely on pollinators for seed production. Our work contributes to our understanding of how inbreeding depression can alter plant-pollinator interaction outcomes and raises questions about its influence on reproductive allocation.

Keywords: inbreeding depression, flower life span, floral traits, mating system, selfing, trade-off.

Introduction

Inbreeding depression is perhaps the most important factor influencing the evolution of selfing from outcrossing. Measured as the relative reduction in fitness of selfed offspring compared with outcrossed offspring, it offsets the automatic transmission advantage of selfing (Lloyd 1979; Lande and Schemske 1985; Charlesworth and Charlesworth 1987). Inbreeding depression is thought to be primarily attributable to (at least partially) recessive deleterious alleles (Charlesworth and Charlesworth 1999; Charlesworth and Willis 2009; Brown and Kelly 2020). Although some of these alleles have large effects and can be lethal, many mildly deleterious alleles contribute to inbreeding depression. The difference is critical because selfing should readily purge inbreeding depression caused by the former as recessive alleles are exposed but will be less efficient in ridding populations of weakly deleterious alleles that are often partially recessive and/or may not segregate independently (Lande and Schemske 1985; Barrett and Charlesworth 1991; Wang et al. 1999; Waller 2021), helping to explain the maintenance of mixed mating (Goodwillie et al. 2005).

In plants, inbreeding depression has been documented extensively for vital rates that directly impact fitness: seed production after initial self- or cross-pollination, subsequent germination, survival, and, to a lesser extent, fertility of the resultant offspring (Husband and Schemske 1996; Goodwillie et al. 2005). But inbreeding depression can also have indirect negative fitness effects when expressed in traits that influence critical ecological interactions with antagonists and mutualists. For example, some studies have demonstrated that inbred plants are more susceptible

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[1] Author for correspondence; email: rachel.spigler@temple.edu.

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to herbivory (Carr and Eubanks 2002; Ivey et al. 2004; Kariyat et al. 2013; Schrieber et al. 2019). Additionally, inbred plants may be less attractive to pollinators and thus could suffer lower pollination success on top of any losses in fertility (i.e., reduced pollen and ovule production or viability; e.g., Robertson et al. 1994; Carr and Dudash 1995; Jóhannsson et al. 1998). Although these impacts are less commonly studied, there is evidence that selfed individuals can have smaller floral displays and smaller flowers (Ouborg et al. 2000; Glaettli and Goudet 2006; Carr et al. 2014; Kariyat et al. 2021) that produce fewer volatiles and/or rewards (Ouborg et al. 2000; Ferrari et al. 2006; Delphia et al. 2009; Kariyat et al. 2021). Consistent with predictions that inbred plants are less attractive to pollinators, studies of *Mimulus guttatus* demonstrated that selfed plants had lower visitation rates—less than half that of outcrossed plants (Ivey and Carr 2005; Carr et al. 2014).

One floral trait key to pollination success that has not yet been examined in studies of inbreeding depression—and is often overlooked in studies of floral traits more generally—is floral longevity. Floral longevity refers to the amount of time a flower is open and functional. Consequently, it dictates the period of opportunity for pollen export (outcross siring success) and pollen deposition (seed set). When these opportunities are rare or unpredictable, longer floral life spans can buy time, compensating for low pollinator visitation rates (Arroyo et al. 2006). Studies have documented considerable variation in floral longevity among species (Primack 1985; Stratton 1989; Ashman and Schoen 1996; Song et al. 2022), and a relationship between floral longevity and pollen limitation across species (Ashman and Schoen 1996) reinforces the importance of this trait for pollination. Yet there is also some evidence of heritable variation in floral longevity within species (Krahl and Randle 1999; Spigler and Woodard 2019). In populations where intraspecific competition for limited pollinators occurs (Spigler and Chang 2009; Johnson et al. 2012; Ward et al. 2013), floral longevity will be particularly important for less attractive plants. If selfed plants are less attractive to pollinators, inbreeding depression in floral longevity could further compound their pollination disadvantages.

Here, we examine inbreeding depression for floral longevity in the mixed-mating biennial *Sabatia angularis* (L.) Pursh (Gentianaceae). *Sabatia angularis* is known to suffer from inbreeding depression across its life cycle (Dudash 1990; Spigler et al. 2017), but beyond flower number, we do not know whether there is inbreeding depression for traits related to pollination success. Although selfing in *S. angularis* can provide reproductive assurance under some ecological conditions (Spigler 2018), pollen limitation (Dudash 1993; Spigler and Chang 2009; Spigler 2018) and selection for traits related to pollinator attraction, such as flower size (Emel et al. 2017), still occur. Given that floral longevity may be another important avenue by which plants minimize pollen limitation in *S. angularis* when pollinator visitation rates are low, inbreeding depression in floral longevity could represent one mechanism contributing to lower seed production of selfed individuals in wild populations. We also evaluated inbreeding depression for flower size, total flower number, and plant height—traits potentially correlated with floral longevity (Spigler and Woodard 2019) and under selection in wild populations (Emel et al. 2017)—and considered whether any potential inbreeding depression for longevity remained when accounting for trade-offs and allometry.

Material and Methods

Study Species

Sabatia angularis is a biennial native to eastern North America. Wild populations grow in grasslands and other open-light environments, including roadsides. Plants flower July to August, producing pink, nectarless flowers that are visited by a mixture of generalist pollinators (mainly bees and flies). Individual flowers contain ~1000–1200 ovules, are protandrous (i.e., anthers dehisce before onset of stigma receptivity), and last an average of ~12 d when left unpollinated under benign greenhouse conditions (representing "potential" or "maximum" longevity) but can live as many as 20 d (Spigler 2017). Variation in floral longevity has a genetic basis but is also plastic in response to pollination and resource availability (Spigler 2017; Spigler and Woodard 2019). This variation is mainly due to female phase duration; the time between anthesis and onset of stigma opening (i.e., male phase) is not correlated with total floral longevity (Spigler and Woodard 2019). Populations can experience pollen limitation, the degree of which varies across populations and years (Dudash 1993; Spigler and Chang 2009; Spigler 2018). *Sabatia angularis* is self-compatible, and populations range from mixed mating to effectively complete outcrossing (range: multilocus outcrossing rate $t_m = 0.60–0.98$; mean: 0.78; SD: 0.12; Spigler et al. 2010). However, inbreeding depression for germination, growth, and flower production as well as seed production in the field can be high (Dudash 1990; Spigler et al. 2017).

Experimental Design

We created families of selfed and outcrossed seeds by hand-pollinating *S. angularis* plants ("maternal plants") grown in Temple University's Plant Growth Facility. Maternal plants were a product of outcrossing, derived from experimentally outcrossed fruits in a wild population in southeastern Pennsylvania (population UB2 in Spigler 2018). We removed anthers from all flowers on maternal plants before stigma receptivity to prevent incidental autonomous self-pollination and rubbed either self or outcross pollen on stigmas once receptive. Outcross pollen came from haphazardly selected donor individuals unrelated to the maternal plant; one donor was used per flower, but different donors were used to pollinate different flowers within a maternal plant. In total, we were able to produce families of selfed and outcrossed seeds from 19 maternal plants, which we raised to flower to estimate inbreeding depression.

We raised five selfed and five outcrossed plants per family to flower. We first germinated seeds in germination chambers (model GR-36VL, Percival Scientific, Perry, IA) under 14-h days at 25°C/15°C day/night. After ~2 mo of growth, we transplanted five rosettes of each cross type individually into pots (3.25 in. diameter) filled with a mix of 3:1 Premier Pro-Mix BX soil media (Premier Tech, Rivière-du-Loup, Quebec) to Turface (Profile Products, Buffalo Grove, IL) media. After ~2 more months of growth, we placed them in winter conditions (5°C/2°C day/night, 10-h days) for 12 wk and subsequently placed them in summer conditions to flower (25°C/15°C day/night, 14-h days). Not all of the plants flowered; we ultimately measured traits on a total of 143 plants.

Plants were checked three times per week to determine when the first flower opened. After at least one flower on a given plant was open, we tagged two or three flower buds and used these to measure corolla and stigma longevity. Once the tagged buds were large and pink (indicating that they will open within 1–2 d, based on Spigler 2017), we used forceps to gently open them just enough to access the undehisced anthers and remove them; anther removal prevents autonomous self-pollination and subsequent pollination-induced stigma wilting without damaging flowers (Spigler 2017). We checked tagged flower buds daily to record the date they opened and continued to check them daily to record dates of corolla and stigma wilting. Longevity of *S. angularis* corollas and stigmas within a flower often differ (Spigler 2017), which could reflect different functions. Whereas the former may indicate how long a flower is advertised to pollinators, the latter may indicate how long the flower can receive pollen. Stigmas were scored as wilted when at least the tips of both lobes of the bilobed stigma were brown (as in Spigler 2017; Spigler and Woodard 2019). We classified corollas as wilted when all five petals began to curl. Ultimately, we followed 319 flowers from anthesis to wilt. On two additional flowers per plant, we measured petal length and width on a haphazardly selected petal and estimated petal area as the product of these measurements. To standardize measurements (longevity, size) and account for any potential differences with inflorescence position, we sampled flowers located at terminal positions of the lateral branches (i.e., secondary positions). However, in a minority of cases, we needed to sample at tertiary positions (7% of flowers used for flower life span and 3% of flowers used to measure petal size); we averaged flower life span and flower size across measurements for each plant. Once plants ceased flowering, we measured plant height and counted the total number of flowers produced per plant.

To carry out data analyses, we used SAS software (ver. 9.3; SAS Institute, Cary, NC). We first calculated Pearson correlation coefficients representing phenotypic correlations among the following traits: total flower number, corolla longevity, stigma longevity, petal area, and plant height. To test for inbreeding depression in these traits, we used mixed general linear models. For each trait, we included cross (to indicate whether the plant was derived from selfing or outcrossing) as a fixed predictor and family (maternal plant identity) as a random variable. Given prior work demonstrating trade-offs between flower number and floral longevity and between flower number and size in *S. angularis* (Spigler and Woodard 2019) and to account for any influence of plant size, we repeated analyses for corolla longevity, stigma longevity, and petal size including flower number and plant height as covariates. For all models, we first tested for heterogeneity of variances between selfed and outcrossed plants; where significant, we accounted for heterogeneity in the model by including cross as a random group effect in proc glimmix. For traits with a significant cross effect, we calculated inbreeding depression as $(1 - w_s/w_o) \times 100$, where w_s represents the average measurement for selfed plants and w_o represents that for outcrossed plants.

Results

We detected several correlations among the measured traits (fig. 1). Corolla longevity was significantly and positively correlated with stigma longevity and petal area. Stigma longevity was significantly and negatively correlated with flower number, indicating that plants with more flowers tend to have flowers with shorter-lived stigmas. We found a similar trend suggesting a trade-off between corolla longevity and flower number. Finally, plant height was significantly positively correlated with flower number and both corolla and stigma longevity.

Inbreeding depression was significant for corolla longevity, stigma longevity, and petal area (fig. 2). Maximum potential corolla longevity of selfed plants was ~1 d shorter, or 10.5% lower, than that of outcrossed plants ($F_{1,123} = 8.47$, $P = 0.004$). Results were similar for maximum potential stigma longevity; stigmas on selfed plants lasted ~0.8 d (8.3%) less than those on outcrossed plants ($F_{1,120} = 5.52$, $P = 0.02$). Selfed plants also produced petals that were 8% smaller than outcrossed flower petals ($F_{1,122} = 5.26$, $P = 0.02$). Interestingly, selfed plants tended to have more flowers than outcrossed plants, although the difference was not statistically significant ($F_{1,122} = 3.42$, $P = 0.07$). Height of selfed and outcrossed plants was similar ($F_{1,112} = 1.06$, $P = 0.30$). Accounting for flower number and plant height again revealed their negative and positive influences, respectively, on longevity (table 1). These effects did not change the results for inbreeding depression in corolla longevity but removed support for inbreeding depression in stigma longevity. Neither plant height nor flower number influenced petal area of our study plants (table 1).

Discussion

Inbreeding depression in floral traits could critically influence plant-pollinator interactions, potentially amplifying costs paid earlier in the life cycle and compounding inbreeding depression in fertility components. Studies have illustrated inbreeding depression in rewards and volatile compounds that attract pollinators or in visual cues, such as flower or display size (see citations in the introduction). We show novel evidence of inbreeding depression for another essential but understudied floral trait central to successful pollination: floral longevity. Our results reveal a combination of shorter floral life spans and smaller flowers in selfed *Sabatia angularis* plants, indicating that these plants could face disproportionately reduced pollination success under certain pollen-limited conditions.

Floral longevity could serve male and/or female function (Schoen and Ashman 1995), with evidence supporting both (Stratton 1989; Gao et al. 2015), but it is expected to primarily benefit female function in protandrous plants and/or plants with many ovules per flower. Consistent with this prediction, total floral longevity scales with the length of female phase, not male phase, in *S. angularis* (Spigler and Woodard 2019). The ability for selfing in *S. angularis* to provide reproductive assurance under certain ecological conditions notwithstanding (Spigler 2018), floral longevity can be key to reproductive success when pollen removal rates outstrip deposition rates, precluding autonomous selfing. Indeed, pollen is rapidly removed within 2 d of flower opening in *S. angularis* in some populations (Dudash 1991; R. B. Spigler and S. Ostrowski, unpublished data). Consequently, we would expect inbreeding depression for floral life span to disproportionately impact female fitness in *S. angularis*, with no to little impacts on outcross siring success, depending on the pollination environment.

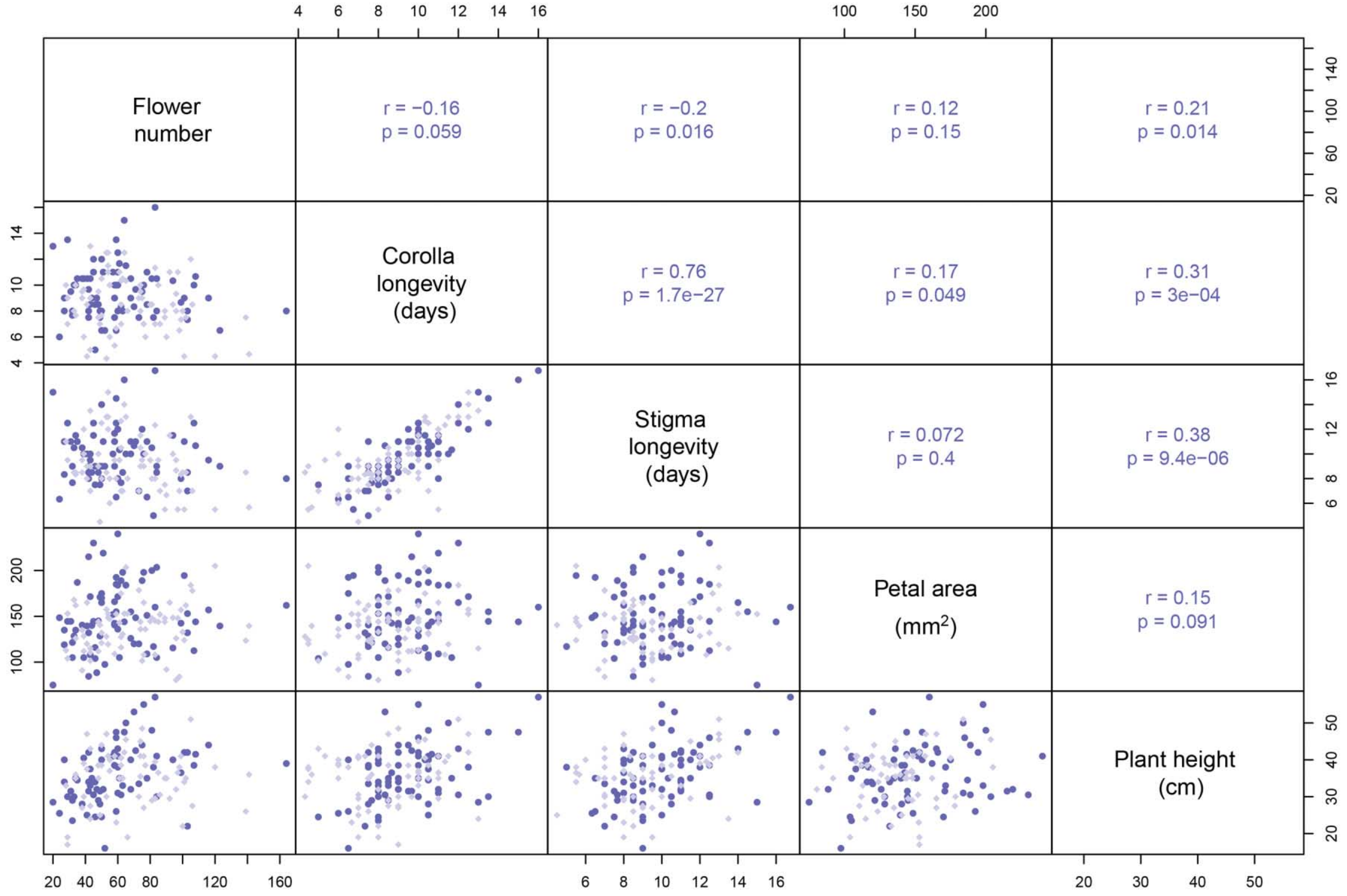

Fig. 1 Matrix illustrating phenotypic correlations among traits. Scatterplots are given below the diagonal. Dark purple circles represent data for outcrossed plants, and light purple diamonds represent data for selfed plants. Pearson correlation coefficients and corresponding *P* values are provided above the diagonal.

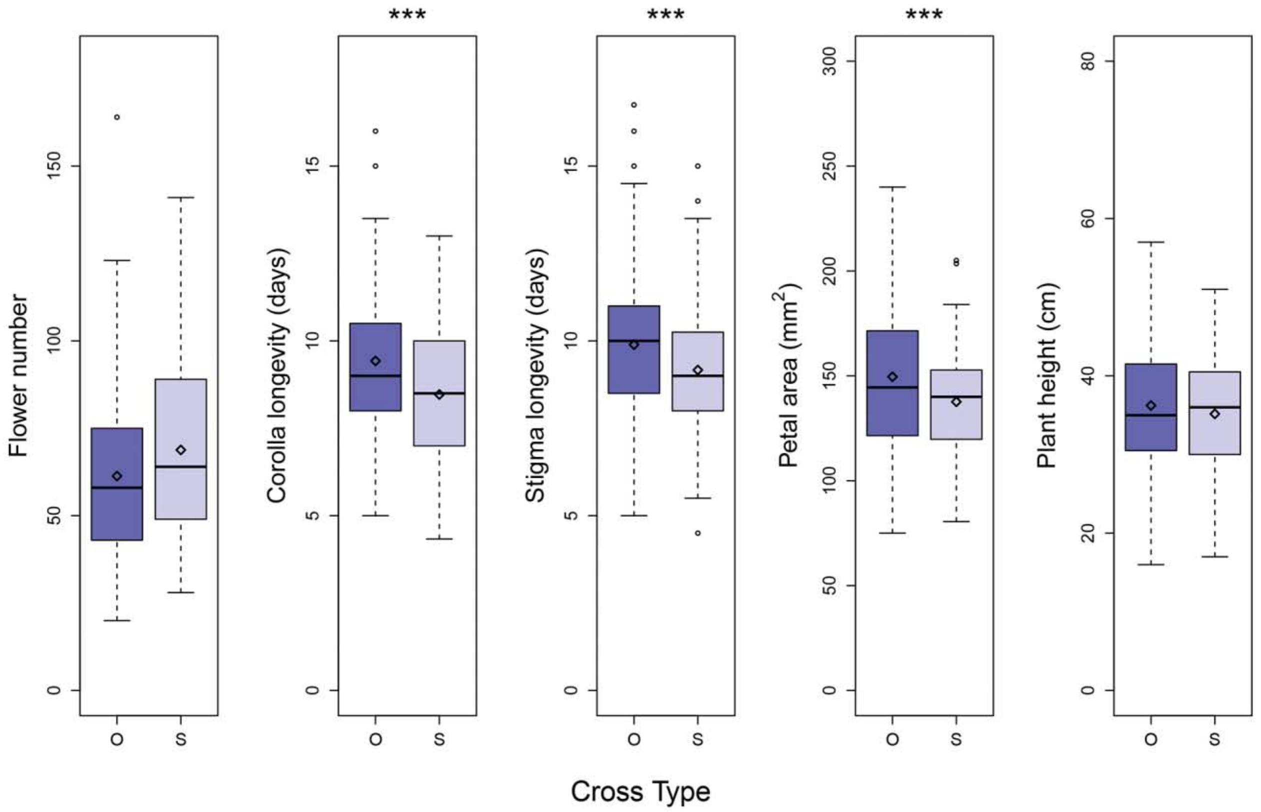

Fig. 2 Boxplots illustrating variation within and differences between selfed and outcrossed *Sabatia angularis* plants for (left to right) flower number per plant, mean corolla longevity, mean stigma longevity, mean petal area, and plant height. Raw data are plotted; significant differences based on the generalized linear mixed models are indicated by asterisks above the corresponding panel. *F* and *P* values for each comparison are given in the text. O = outcrossed plants; S = selfed plants.

Yet it is reasonable to question how much the modest ~1-d difference in longevity shown here may even matter for successful outcross pollen receipt in the field. After all, we found maximum floral longevity of selfed plants, based on corolla longevity, was on average 8.6 d, which is still longer than the realized longevity of flowers (~4–6 d) seen in two wild populations of *S. angularis*. However, shorter realized longevity in the field for this and many other species is largely due to plastic truncation of floral life span upon pollination (e.g., van Doorn 1997; Weber and Goodwillie 2007; Castro et al. 2008; Spigler 2017); flowers will live longer when seed production is pollen limited. For example, mean longevity of open-pollinated *Polygala vayredae* flowers in the field varied across years from ~8 d to ~14 d (Castro et al. 2008). Flowers of *S. angularis* plants in the field will live up to ~12 d when pollinators are excluded (R. B. Spigler, unpublished data), suggesting that if pollen deposition rates are low enough, the ability to live one more day could mean the difference between receiving too little or enough pollen to fertilize all ovules per flower. Furthermore, corolla longevity might impact fitness more via its influence on overall floral display. Harder and Johnson (2005) demonstrated this link, showing how plastic increases in floral display and flowering duration due to longer life span of individual flowers led to greater rates of pollinator visitation and success. Any increases in visitation from larger displays could be particularly important to selfed *S. angularis* plants, given that we also show here

Table 1

Results for Fixed Effects in Mixed General Linear Models Testing Inbreeding Depression in Floral Longevity and Flower Size, Accounting for Variation in Flower Number and Plant Height

	Corolla longevity			Stigma longevity			Petal area		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Cross	1, 110	5.91	.017	1, 107	2.27	.135	1, 109	5.48	.021
Flower number	1, 110	4.49	.036	1, 107	7.28	.008	1, 109	2.32	.130
Plant height	1, 110	15.90	.0001	1, 107	20.95	<.0001	1, 109	1.63	.204

Note. Degrees of freedom (df: numerator, denominator), *F* values, and *P* values for fixed effects are provided. See text for details.

that they have smaller, (presumably) less attractive flowers. Indeed, prior work found stronger selection for flower size and flowering duration in *S. angularis* populations with weaker plant-pollinator interactions (Emel et al. 2017).

Notably, we did not find inbreeding depression for flower number, in contrast to previous studies in *S. angularis* (Dudash 1990; Spigler et al. 2017). Several possibilities could account for this discrepancy. First, inbreeding depression for flower number may have been alleviated under our fairly benign study conditions, consistent with general patterns of environment-dependent inbreeding depression (Cheptou and Donohue 2011). However, given that we were able to detect relatively mild inbreeding depression for other traits, this seems unlikely. Second, it could be a peculiarity of our study population; inbreeding depression in *S. angularis* can vary markedly across populations (Spigler et al. 2017). A third and more interesting explanation is related to the trade-off between maximum floral longevity and flower number shown previously (Spigler and Woodard 2019) and replicated in this study. This trade-off represents a fundamental assumption of resource allocation models for the evolution of floral longevity (Schoen and Ashman, 1995). If we consider that all plants were provided with the same fixed amount of resources in our study and account for slightly more flowers per plant for selfed individuals, then it is possible that inbreeding depression in floral longevity and flower size represents different allocation patterns between outcrossed and selfed individuals. On the other hand, if we accept that the differences in flower number is not statistically significant, then instead there may be inbreeding depression in resource acquisition, forcing selfed individuals to forgo larger, longer-lived flowers to produce the same number of flowers as outcrossed plants. Further experiments can shed light on these possibilities, but both imply that reproductive trade-offs could have effectively eliminated inbreeding depression for flower number under our study conditions, akin to how a seed size–number trade-off mitigated inbreeding depression for germination in the mixed-mating shrub *Myrtus communis* (González-Varo and Traveset 2010). In contrast, prior studies demonstrating inbreeding depression for flower number in *S. angularis* were based on open-pollinated field plants, where there is greater opportunity for variation in resource acquisition and where pollination-induced truncation of floral life span can mean that maximum longevity is not realized, effectively erasing signatures and impacts of a flower number–floral longevity trade-off.

Conclusions

We show evidence for inbreeding depression in floral longevity and flower size. Because this inbreeding depression is mild and expressed late in the life cycle, the underlying deleterious alleles may be difficult to purge. In addition, mild inbreeding depression at any given stage does not mean that it is of no consequence. Indeed, because fitness across the life cycle is multiplicative (Arnold and Wade 1984), even small differences in the fitness of selfed versus outcrossed offspring can become magnified. Inbreeding depression in floral longevity and size can leave selfed individuals with fewer offspring by altering plant-pollinator interactions and reducing the opportunity to capture scarce or unpredictable pollinator visits (Rathcke 2003; Arroyo et al. 2006). Future work is needed to evaluate interactions between pollinator availability and inbreeding depression for floral longevity and attractive traits and their impacts on plant fitness.

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