Relatively weak inbreeding depression in selfing but also in outcrossing populations of North American *Arabidopsis lyrata*

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Abstract

Hermaphroditic plants can potentially self-fertilize, but most possess adaptations that promote outcrossing. However, evolutionary transitions to higher selfing rates are frequent. Selfing comes with a transmission advantage over outcrossing, but self-progeny may suffer from inbreeding depression, which forms the main barrier to the evolution of higher selfing rates. Here, we assessed inbreeding depression in the North American herb *Arabidopsis lyrata*, which is normally self-incompatible, with a low frequency of self-compatible plants. However, a few populations have become fixed for self-compatibility and have high selfing rates. Under greenhouse conditions, we estimated mean inbreeding depression per seed (based on cumulative vegetative performance calculated as the product of germination, survival and aboveground biomass) to be 0.34 for six outcrossing populations, and 0.26 for five selfing populations. Exposing plants to drought and inducing defences with jasmonic acid did not magnify these estimates. For outcrossing populations, however, inbreeding depression per seed may underestimate true levels of inbreeding depression, because self-incompatible plants showed strong reductions in seed set after (enforced) selfing. Inbreeding-depression estimates incorporating seed set averaged 0.63 for outcrossing populations (compared to 0.30 for selfing populations). However, this is likely an overestimate because exposing plants to 5% CO_2 to circumvent self-incompatibility to produce selfed seed might leave residual effects of self-incompatibility that contribute to reduced seed set. Nevertheless, our estimates of inbreeding depression were clearly lower than previous estimates based on the same performance traits in outcrossing European populations of *A. lyrata*, which may help explain why selfing could evolve in North American *A. lyrata*.

Introduction

Roughly half of all flowering plants are self-incompatible (Razanajatovo *et al.*, 2016), and self-incompatibility has been reported in over 100 plant families (Igic *et al.*, 2008). However, transitions from outcrossing to selfing are frequent (Barrett, 2002). Compared to their outcrossing relatives, selfing species are characterized by smaller flowers that facilitate self-pollination (Sicard

& Lenhard, 2011; Tedder *et al.*, 2015) and often have shorter lifecycles (Razanajatovo *et al.*, 2016). Genetically, selfers have lower heterozygosity and diversity (Wright *et al.*, 2013) and therefore reduced evolutionary potential (Stebbins, 1957). The latter likely explains why selfers tend to be more susceptible to generalist herbivores (Johnson *et al.*, 2009). These features likely provide an advantage for outcrossing on long evolutionary timescales (Wright *et al.*, 2013), but on shorter timescales cannot prevent the evolution of selfing.

On shorter timescales, the fate of selfing lineages depends on the balance between the transmission advantage of selfing and the cost of selfing in terms of inbreeding depression. On the one hand, selfers have a

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transmission advantage over outcrossers, because they transmit both the maternal and paternal gene set to the seeds, rather than only the maternal set (Fisher, 1941). On the other hand, genomes with an outcrossing history are expected to accumulate deleterious recessive mutations (genetic load). In cross-progeny, only few of these mutations will be expressed, owing to high levels of heterozygosity. In self-progeny, however, increased homozygosity will result in expression of more recessive deleterious mutations and cause inbreeding depression (Charlesworth & Willis, 2009). As inbreeding depression constitutes the prime barrier to the evolution of selfing in the short term (Kondrashov, 1985; Lande & Schemske, 1985; Porcher & Lande, 2005), its estimation is important for understanding the conditions under which selfing can evolve.

Inbreeding depression can be assessed as the reduction in performance of self-progeny relative to cross-progeny (Ågren & Schemske, 1993). Stressful environmental conditions (e.g. drought) and interactions with herbivores may increase the intensity of inbreeding depression (Armbruster & Reed, 2005), and assessing inbreeding depression under benign conditions may thus lead to underestimates of inbreeding depression (Reed et al., 2002; Fox & Reed, 2011; but see Sandner & Matthies, 2016). Inbreeding-depression estimates may also depend on the life-history stage on which they are based. Ideally, estimates should be based on multiple stages covering the complete lifecycle. As this is particularly challenging for self-incompatible perennials, only few studies report lifetime estimates of inbreeding depression for such species (reviewed in Sletvold et al., 2013; also see Bellanger et al., 2015), and few have done so in benign and more stressful environments.

Classical theory predicts that the inherent transmission advantage of selfers should drive the evolution of selfing when inbreeding depression is less than a 50% threshold (Lloyd, 1979; Lande & Schemske, 1985; Schemske & Lande, 1985). The assumptions of this basic model may not always apply as it does not allow for functional relationships among components of reproductive fitness (Johnston et al., 2009). The frequent occurrence of stable intermediate outcrossing rates shows that complete selfing does not always evolve automatically if inbreeding depression is low (e.g. Goodwillie et al., 2005; also see Cruzan & Barrett, 2016). Nevertheless, it is generally accepted that genetic load forms the prime barrier to the evolution of selfing after the breakdown of self-incompatibility. The increased expression of recessive deleterious alleles in selfed offspring may result in purging of the genetic load in self-compatible lineages (Crnokrak & Barrett, 2002). Of major interest for the breakdown of self-incompatibility, however, is the genetic load in the ancestral self-incompatible lineages. Few studies have quantified inbreeding depression in self-incompatible species, and even fewer have done so in species with both self-incompatible and self-compatible lineages (e.g. Busch, 2005). The latter are, however, of special interest for understanding the evolution of selfing.

Here, we test the prediction that genetic load forms a barrier to the evolution of selfing after the breakdown of self-incompatibility by making use of the mating-system variation in *Arabidopsis lyrata*. The species is normally self-incompatible and outcrossing, but in its North American range, a breakdown of self-incompatibility has led to several fully self-compatible populations. These populations have high selfing rates and occur in close geographic proximity to populations where plants are self-incompatible (Mable et al., 2005; Mable & Adam, 2007; Foxe et al., 2010; Griffin & Willi, 2014). Specifically, we (1) quantify inbreeding depression in six outcrossing populations and compare our estimates to previously published estimates for the European subspecies of *A. lyrata* and (2) test whether purging of genetic load has occurred in five predominantly selfing populations. We do this in a common greenhouse in a full factorial design with benign growth conditions crossed with drought stress and induced defence, so that we could also test (3) whether environmental stress magnifies inbreeding depression.

Materials and methods

Study species and seed material

Arabidopsis lyrata (Brassicaceae) is a 10- to 30-cm-tall perennial herb. It is native to Europe (*A. lyrata* ssp. *petraea*) and to North America (*A. lyrata* ssp. *lyrata*) and grows on rocky to sandy substrates in open woods or on river banks, at altitudes of up to 2200 m (Al-Shehbaz & O'Kane, 2002). It is normally self-incompatible, but in the North American subspecies, several populations have lost self-incompatibility and evolved high selfing rates (Foxe et al., 2010). To generate cross- and self-seeds from populations with contrasting mating systems, we sowed seeds that had originally been collected in the field from 11 North American *A. lyrata* populations (kindly provided by Barbara Mable, University of Glasgow). Five of these populations had previously been characterized as predominantly selfing and six as outcrossing (Foxe et al., 2010). In 2012 and 2013, we produced seeds by manually cross- and self-pollinating up to eight plants per population (Appendix S1). To produce seeds by outcrossing (cross type 'cross'), we emasculated a flower prior to anther dehiscence and rubbed over its stigma a freshly dehisced anther from a haphazardly chosen plant from the same population. To produce seeds by selfing (cross type 'self'), we self-pollinated several flowers and immediately placed the plants in an individual airtight container, which was then filled with a 5 % CO_2–air mixture from a pressurized gas cylinder. We kept self-pollinated plants in this

environment with elevated CO_2 concentration for 6–12 h at ambient temperature, which allows (partially) bypassing the self-incompatibility response (Nakanishi *et al.*, 1969; see Stift *et al.*, 2013 for details). We used this method to produce 'self-' seeds both for self-compatible and self-incompatible plants, but to test for potential side effects of the CO_2 treatment, we also produced 'self-' seeds under ambient greenhouse conditions without CO_2 enrichment for self-compatible plants. Although there was a minor side effect of CO_2 enrichment on seed set (selfings in 5% CO_2 yielded 5.9% fewer seeds than selfings in ambient conditions), there were no side effects for germination proportion, and any of the later plant performance traits (Appendix S2). Therefore, we did not distinguish between the two 'self' cross types in the analyses.

Seed set, germination and experimental set-up

To test for reductions in seed set associated with selfing (which may reflect inbreeding depression, but may also be due to residual self-incompatibility), we counted the mean number of seeds per silique produced by each plant after 'cross-' and 'self [CO_2]-' pollination treatments. We differentiated between normal seeds (regular, oval shape, golden brown seed coat) and abnormally developed seeds (irregular shape and/or black or greenish colour). Following Sletvold *et al.* (2013), we assumed that siliques without any seeds represented cases where self-incompatibility could not be bypassed with our CO_2 treatment, and we excluded these from seed counting.

To simultaneously assess the levels of inbreeding depression in outcrossing populations, and whether purging has led to reduced inbreeding depression in selfing populations, we performed a greenhouse experiment in the Botanical Garden of the University of Konstanz, Germany. To evaluate whether inbreeding estimates under (putatively benign) greenhouse conditions are representative for more stressful (and more realistic) environments, we also tested whether drought stress and induction of herbivore defence magnified inbreeding depression. In April 2014, we first selected mother plants for which sufficient 'cross-' and 'self [CO_2]-' seeds were available (at least 20 seeds per cross type). With two exceptions (populations RON and PTP, for which we had three and eight mothers, respectively), we could include five mothers per population (Appendix S1). As a procedural control, for all mothers from selfing populations, we also sowed 20 'self-' seeds that had been generated under ambient conditions [without CO_2 enrichment, hereafter referred to as self (ambient)]. For one mother (from population LPT), only 'self-' seeds formed without CO_2 were available. In total, our design included 'cross-' and 'self-' seeds of 30 mothers from the six outcrossing populations and 26 mothers from the five selfing populations (Appendix S1).

On the 16th of April 2014, we sowed 20 seeds per cross type per mother, in portions of five seeds per pot (7 × 7 × 6.5 cm square pots; Pöppelmann GmbH & Co. KG, Lohne, Germany) filled with potting soil (Einheitserde und Humuswerke Gebr.; Patzer GmbH & Co., Waldsiedlung, Germany). Pots were randomly assigned to positions in trays covered with transparent plastic lids in a growth chamber with 90 % relative humidity, 16 h light, at 21 °C and 8 h dark at 18 °C. On the 5th of May 2014, we transplanted eight randomly chosen seedlings per mother per cross type to individual pots (9 × 9 × 8 cm) with the same potting soil as used for germination. We then assigned four seedlings of each mother–cross-type combination to each of two adjacent greenhouse compartments (block I and block II). For three of the 56 mothers, fewer than eight (but at least four) seedlings were available for a certain cross type (see Appendix S1 for details), in which case we assigned four seedlings of that cross type to block I (discarding any remaining seedlings). For three of the 56 mothers, fewer than four seedlings were available for a certain cross type (Appendix S1), in which case we omitted the mother–cross-type combination altogether. Four seedlings (all in block I) did not survive the transplanting (Appendix S1). Thus, blocks I and II contained 528 and 508 pots, respectively. We randomized pot positions within each block. One week after transplanting, we switched on artificial illumination to extend the light period to 16 h. The temperature range was 20–23 °C during the light period and 15–18 °C during the dark period with a relative humidity of 70 %. Until the drought treatment, plants were watered *ad libitum* and fertilized with 0.1 % Scotts Universol® Blue (Everris International B.V., Waardenburg, Netherlands) once a week.

We employed a full factorial design in which plant defence was induced or not, and in which plants were exposed to drought or not. To induce defences, we sprayed plants with the phytohormone jasmonic acid (JA), instead of using real herbivores, as these tend to introduce noise due to variation in feeding intensity (Joschinski *et al.*, 2015). Jasmonic acid is a signalling hormone known to induce the plant–herbivore-defence pathway (Baldwin, 1998; Moore *et al.*, 2003). We applied JA on the 26th and 27th of May 2014, when plants had on average 10.4 ± 0.01 (mean ± standard error) leaves, by spraying plants until imminent run-off with a 1 mM solution of JA (Sigma-Aldrich Co., Saint Louis, MO, USA) and repeating the procedure 1 h later when droplets had evaporated. Control plants were treated in the same manner, but with water. In a side-experiment with two additional plants for each mother–cross-type combination (one treated with JA, one with water), we confirmed that JA induced a 36.9 % increase in peroxidase activity (Appendix S3), which is thought to toughen cell walls (Moore *et al.*, 2003). To impose drought, we stopped watering plants

until they visibly lost their leaf turgor (i.e. started to wilt), which we monitored daily. Wilted plants received 150 mL water to allow recovery. Plants in the drought treatment wilted 2–7 times during the 8 weeks until harvest. Control plants were watered *ad libitum* such that the substrate was constantly moist.

Measurements

To compare the performance of 'cross-' and 'self-' progeny (i.e. to assess inbreeding depression) in outcrossing and selfing populations under the given treatments, we scored performance traits representing the whole lifecycle. We scored days until germination for each seed individually until seedling transplanting (3 weeks after sowing). Before transplanting, we scored the proportion of seeds that had germinated per mother–cross-type combination (germination proportion). During the final harvest on the 28th and 29th of July 2014, 12 weeks after transplanting, we recorded individual plant survival. To estimate sexual performance of the surviving plants, we recorded whether individual plants had reached the reproductive stage (defined as bolting or flowering) or not, and harvested reproductive tissues (any part of the inflorescence, including the stalk, stalk leaves and flower parts). To estimate vegetative performance, we harvested the remaining aboveground tissue (i.e. the rosette leaves). To determine biomass of reproductive and vegetative tissues, we dried the plant tissues at 70 °C for about 72 h before weighing.

Statistical analyses

Our procedural control indicated that producing 'self-' seeds under CO_2-enriched conditions did not have side effects on offspring performance (Appendix S2). Therefore, we pooled 'self-' seeds produced under CO_2 with those produced under ambient conditions for further analyses. To assess whether there were differences between mating systems in performance and inbreeding depression, and to test whether these differences depended on drought and induced defence, we used general and generalized linear mixed-effects models implemented in the package *lme4* (Bates *et al.*, 2014) in R (R Core Team, 2016) run through RStudio 0.98.1103 (http://www.rstudio.org/). Only progeny from mother plants for which both 'cross-' and 'self-' progeny were available were included in analyses. For this reason, the progeny from one mother plant from the LPT population was excluded from analyses beyond the germination proportion, as insufficient 'cross-' seeds germinated. We analysed seed set after manual pollination (SS), germination proportion (G), survival (S) and aboveground vegetative biomass (B) separately, and combined in cumulative measures of vegetative performance per seed (G × S × B) and per pollination (SS × G × S × B). We also assessed flowering success and reproductive

biomass. For all traits, the models included the fixed effects mating system (selfing vs. outcrossing), cross type ('cross' vs. 'self') and their interaction, and the random-effects population (nested in mating system) and mother (nested in population). In addition, for traits assessed after application of the jasmonic acid (JA) and drought treatments (i.e. flowering, survival and biomass), the fixed part also included JA treatment (sprayed with JA vs. water control), drought treatment (drought vs. *ad libitum*) and their interaction.

We used the *glmer* function to fit models for binomial traits (germination proportion and flowering success), employing the *bobyqa* optimizer with a maximum of 100 000 iterations. We used the *lmer* function to fit models for continuous traits (reproductive and vegetative biomass, cumulative vegetative performance), which were square root-transformed to improve residual normality and homogeneity of variance. Furthermore, we included greenhouse compartment as random effect. For estimating the coefficients of the fixed and the variance of the random terms, we fitted the models using restricted maximum likelihood (REML; Zuur *et al.*, 2013). Then, for assessing significances of the fixed terms, we fitted the models using maximum likelihood (ML), and compared models with and without each fixed term using likelihood-ratio tests (LRTs; Zuur *et al.*, 2013). Finally, to compare plant performance between outcrossing and selfing populations and among the different treatments, we calculated the inbreeding-depression index (δ), for each population with the formula: $\delta = (w_o - w_s)/\max(w_o, w_s)$, where w_o is the performance of 'cross-' progeny, and w_s is the performance of 'self-' progeny (Ågren & Schemske, 1993).

Results

Seed set, germination and survival

Self-pollination yielded significantly fewer normally developed seeds than cross-pollination, but this effect was much stronger in outcrossing (22.3 to 12.2 seeds per fruit) than in selfing populations (27.6 to 25.7 seeds per fruit; significant mating-system vs. cross-type interaction in Table 1; Table 2). Overall, 78.9 % of the seeds used in the experiment germinated (Fig. 1). Although there was no difference in weight between 'cross-' and 'self-' seeds (Appendix S4), there was significant inbreeding depression for germination proportion (significant effect of cross type in Table 1; Table 2). However, there were no differences between outcrossing and selfing populations in average germination proportion (no significant effect of mating system, Table 1) and inbreeding depression (no significant mating-system × cross-type interaction, Table 1). As almost all germinated plants (1009 of 1028; 98.2 %) survived until the end of the experiment, we did not analyse survival separately.

Table 1 Likelihood-ratio tests (model comparisons) for the effect of mating system and cross type on seed set and seed germination proportion of *Arabidopsis lyrata*.

Fixed effect	Seed set (poisson)*		Proportion of normal seeds per fruit (binomial)*		Germination proportion (binomial)*	
	χ^2	P	χ^2	P	χ^2	P
Mating system (MS)	**15.5**	**<0.001**	0.48	0.49	0.55	0.460
Cross type (CT)	**195.3**	**<0.001**	**10.9**	**<0.001**	**55.6**	**<0.001**
MS: CT	**183.3**	**<0.001**	**38.5**	**<0.001**	1.59	0.210
Random effect	St. dev.		St. dev.		St. dev.	
Mother	0.17		0.96		0.96	
Population	0.093		0.63		0.63	

Chi-square test statistics (χ^2) and corresponding *P*-values (bold if <0.05) are shown for fixed effects and interactions. All tests had d.f. = 1 (as all fixed effects had two levels). The mixed models included the random-effects *mother* and *population*, for which the standard deviations (St. dev.) they explained are indicated.
*Data were analysed with generalized linear mixed-effects models (*glmer*) with the indicated distribution.

Vegetative biomass

Among the 1009 plants that survived until harvest, there was significant inbreeding depression for vegetative biomass (significant effect of cross type, Table 3). However, there were no differences between outcrossing and selfing populations in vegetative biomass (no effect of mating system, Table 3) and inbreeding depression (no significant mating-system × cross-type interaction, Table 3; Appendix S5). Spraying with jasmonic acid (JA; i.e. inducing defences) significantly reduced vegetative biomass (Fig. 2, Table 3), but exposing plants to drought did not (Table 3), and there was no significant interaction between the stress treatments (Table 3). Neither JA nor drought (nor the combination of both) affected the magnitude of inbreeding depression for vegetative biomass (no significant interactions of JA and/or drought with cross type, Table 3; Fig. 2; Appendix S5).

Flowering and reproductive biomass

About half of the experimental plants flowered, that is were at least bolting (512 of the 1017 plants that survived). The proportion of plants flowering was significantly lower among JA-induced plants than among noninduced ones (Fig. 2; Table 3), but none of the other factors had a significant effect (Table 3). Among the 512 plants that flowered, there was significant inbreeding depression for reproductive biomass (Fig. 2; significant effect of cross type, Table 3; Appendix S5). However, there were no differences between outcrossing

and selfing populations in reproductive biomass (no significant effect of mating system, Table 3) and inbreeding depression (no significant mating-system × cross-type interaction, Table 3). Both drought and JA significantly reduced reproductive biomass (Fig. 2; Table 3), but only drought magnified the effect of inbreeding depression (significant drought × cross-type interaction, Table 3).

Cumulative vegetative performance per seed and inbreeding depression

We found significant inbreeding depression for cumulative vegetative performance per seed (Fig. 2; significant effect of cross type, Table 3). There were no differences between outcrossing and selfing populations in cumulative vegetative performance per seed (no effect of mating system, Table 3) and inbreeding depression for this trait (no significant mating-system × cross-type interaction, Table 3; Appendix S5). JA reduced cumulative vegetative performance (significant effect of JA, Table 3), but neither JA nor drought (nor the combination of both treatments) affected the magnitude of inbreeding depression for cumulative vegetative performance (no significant interactions of JA and/or drought with cross type, Table 3; Appendix S5).

Cumulative vegetative performance per pollination and inbreeding depression

We found significant inbreeding depression for cumulative vegetative performance per pollination (significant effect of cross type, Appendix S6). Because self-pollination caused a significant reduction in seed set in outcrossing populations and plants from selfing populations produced more seeds (Table 1), cumulative vegetative performance per pollination was overall lower for outcrossing populations (Appendix S6), and self-pollination had a stronger negative effect (Appendix S6).

Discussion

Inbreeding depression in outcrossing populations of North American *Arabidopsis lyrata*

Inbreeding depression is the main barrier to the evolution of selfing after the breakdown of self-incompatibility (Kondrashov, 1985; Lande & Schemske, 1985; Porcher & Lande, 2005). We found that, under common greenhouse conditions, predominantly outcrossing populations of North American *Arabidopsis lyrata* (ssp. *lyrata*) had a mean cumulative inbreeding depression of $\delta_{per\ seed} = 0.34$. This estimate was based on cumulative vegetative performance calculated as the product of germination, survival and vegetative biomass, thus reflecting performance per seed. As we also found significant reductions in seed set after self- vs. cross-pollination for

Table 2 Mean seed set and germination rate after cross- and self-pollination for all six outcrossing and five selfing populations. Statistical analyses of the effects of mating system and cross type can be found in Table 1.

Mating system	Population	N*	Cross type	Number of normally developed seeds per fruit†		Proportion of normally developed seeds per fruit		Proportion of normally developed seeds that germinated	
				Cross-type means	Reduction due to selfing‡	Cross-type means	Reduction due to selfing‡	Cross-type means	Inbreeding depression (δ)
Outcrossing	IND	5	Cross	27.6	0.61	0.99	0.069	0.73	0.063
			Self	10.9		0.93		0.68	
	MAN	5	Cross	15.9	0.24	0.95	0.066	0.73	−0.014
			Self	12.1		0.88		0.74	
	PCR	5	Cross	23.0	0.54	0.97	0.022	0.88	0.14
			Self	10.5		0.95		0.76	
	PIN	5	Cross	20.8	0.34	0.99	0.018	0.93	0.24
			Self	13.8		0.97		0.71	
	SBD	5	Cross	25.0	0.43	0.98	0.048	0.86	0.22
			Self	14.3		0.93		0.67	
	TSS	4	Cross	25.8	0.55	0.92	0.040	0.84	0.048
			Self	11.8		0.88		0.80	
				Mean	**0.45 (0.058)**	**Mean**	**0.044 (0.0088)**	**Mean**	**0.12 (0.041)**
Selfing	LPT	5	Cross	23.1	0.018	0.92	−0.031	0.60	0.14
			Self	22.7		0.95		0.52	
	PTP	3	Cross	28.1	0.067	0.99	−0.0062	0.95	0.079
			Self	26.2		1.00		0.88	
	RON	8	Cross	30.2	0.072	0.98	0.0020	0.92	0.12
			Self	28.0		0.98		0.81	
	TCC	5	Cross	32.7	0.12	0.97	−0.0083	1.00	0.070
			Self	28.9		0.98		0.93	
	TSSA	5	Cross	22.3	0.040	0.88	−0.018	0.87	0.29
			Self	21.4		0.89		0.62	
				Mean	**0.063 (0.017)**	**Mean**	**−0.012 (0.0056)**	**Mean**	**0.14 (0.040)**

*N indicates the number of independent replicates (i.e. mother plants) per population.
†During seed counting, normally developed seeds (brown seed coat, regular shape, developed endosperm) were distinguished from abnormal seeds (green or black seed coat and/or irregular shape and/or no developed endosperm).
‡This reduction was calculated in the same way as one would calculate the inbreeding-depression coefficient δ. However, we prefer not to present it as inbreeding depression (δ), because for self-incompatible plants, a reduction in seed set due to selfing can both be due to incomplete bypassing of the self-incompatibility reaction, and inbreeding depression. For self-compatible plants, the estimates can be interpreted as inbreeding depression without reservation.

plants from outcrossing populations, this may underestimate the true level of inbreeding depression, and we therefore consider $\delta_{\text{per seed}} = 0.34$ to represent a lower bound for the true cumulative inbreeding depression. As an estimate of the upper bound, we calculated inbreeding depression based on cumulative performance per pollination (the product of seed set and the aforementioned cumulative performance per seed), which amounted to 'δ'$_{\text{per pollination}} = 0.63$ (Appendix S5). As this upper bound hinges on the assumption that reductions in seed set are solely due to inbreeding depression, and not due to residual effects of self-incompatibility (see 'Early-acting inbreeding depression (during the seed stage)' below), it should be interpreted with care.

Environmental stress (i.e. a period of drought, inducing defences and a combination of these) did not affect either of the estimates of inbreeding depression

($0.30 < \delta_{\text{per seed}} < 0.35$; $0.60 < \delta_{\text{per pollination}} < 0.65$, Appendix S5). In European populations of *A. lyrata* (ssp. *petraea*), which are all self-incompatible and obligately outcrossing, inbreeding depression based on the same cumulative performance traits averaged $\delta_{\text{per seed}} = 0.51$ and $\delta_{\text{per pollination}} = 0.81$ (Sletvold *et al.*, 2013). Our results therefore suggest that the barrier to the evolution of higher selfing rates is lower in North American than in European outcrossing populations. As bottlenecks can play a role in purging of the genetic load (Pujol *et al.*, 2009), our findings are not unexpected given that North American *A. lyrata* populations have gone through a bottleneck (Ross-Ibarra *et al.*, 2008; Mable *et al.*, 2017). This bottleneck and the associated purging of genetic load may thus have partly facilitated the evolution of selfing in North American *A. lyrata*.
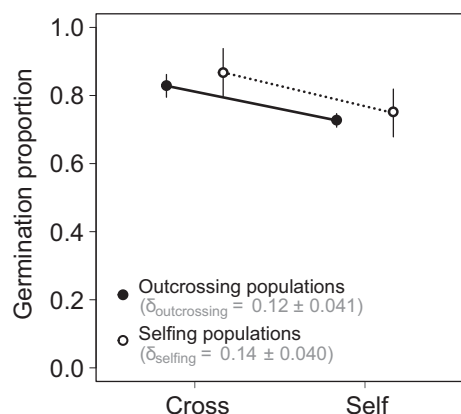
Fig. 1 Effect of cross type (cross vs. self) on germination proportion, for outcrossing (black circles) and selfing (open circles) populations of North American *Arabidopsis lyrata*. Vertical lines indicate standard errors (SE) of the means of population trait means. The legend shows the mean ± SE of the population inbreeding-depression estimates (δ) for both mating systems (Table 2).

Caveats for interpreting estimates of inbreeding depression

Inbreeding-depression estimates may depend on the life-stage for which performance is assessed (Husband & Schemske, 1996). In this study, we calculated a cumulative measure of vegetative performance as the product of germination proportion, survival and aboveground biomass yield during a single growth season. We chose to include biomass in the cumulative performance measure instead of first-year reproductive investment, because biomass is a reasonable predictor of the lifetime survival and fecundity in the perennial *A. lyrata* (Løe, 2006; Sandring & Ågren, 2009). A previous study on the same species, set in a single garden environment, calculated cumulative performance in a similar way, but used multiyear flower or fruit production instead of vegetative biomass (Willi, 2013), and found inbreeding-depression estimates that were even lower than ours ($0.26 < \delta < 0.35$ in our study vs. $\delta = 0.18$ in Willi, 2013). However, neither of the two studies estimated fecundity in terms of seed set and performance of the progeny. Using biomass as a proxy for fecundity may bias estimates of inbreeding depression downward. Therefore, to determine to what extent such biases may exist, future studies should assess inbreeding depression in terms of reproductive success in natural environments with open pollination.

Such field studies would also avoid potential downward biases in estimates of inbreeding depression due to benign greenhouse or garden conditions that do not expose plants to the challenges met in natural environments (Armbruster & Reed, 2005). Although we acknowledge the need for such field-based studies, our experimental design circumvents some of the classical criticisms on greenhouse-based studies, as it also included less benign conditions (i.e. stress treatments). Our stress treatments had a clear negative effect on growth: drought reduced the reproductive biomass and inducing defence (with jasmonic acid) reduced vegetative growth and reproduction (but provided better defence against a powdery mildew infestation, see Appendix S7). However, stress did not magnify our estimates of inbreeding depression. This suggests that greenhouse-based estimates of inbreeding depression can actually give realistic predictions of levels of inbreeding depression under natural conditions, at least if one assumes that biomass is a good proxy of lifetime performance (Løe, 2006; Sandring & Ågren, 2009).

Early-acting inbreeding depression (during the seed stage)

Studies like ours that attempt to estimate inbreeding depression in self-incompatible species have to rely on methods to bypass the self-incompatibility response in order to obtain 'self-' seeds. These methods most commonly involve bud self-pollination (e.g. Busch, 2005; Sletvold *et al.*, 2013; Willi, 2013) or self-pollination in a CO_2-enriched environment (e.g. Stift *et al.*, 2013 and this paper). In *A. lyrata*, such self-pollinations usually do not yield as many seeds as cross-pollinations, with reductions of up to 61% (Table 2) or even higher (Sletvold *et al.*, 2013). This could reflect early inbreeding depression (i.e. during seed development), but may also be a consequence of incomplete bypassing of the self-incompatibility response. We are unaware of any studies that have attempted to disentangle these two effects.

Bud self-pollination in North American *A. lyrata* resulted in increased failure of pollen tube growth, which suggests that there may indeed have been some residual effects of self-incompatibility (Oakley *et al.*, 2015). Residual effects of self-incompatibility could also explain our finding that selfing in CO_2-enriched air leads to a strong reduction in seed set in outcrossing populations (45 %, Table 2), whereas the proportion of normal seeds only decreased by 4.4 % (Table 2). In other words, the decrease in seed set was not due to an increase in the proportion of late aborted seeds (i.e. aborted seeds that can still be recognized in mature siliques), but could of course still be explained by abortion during seed development (i.e. early-acting inbreeding depression). The only way to reliably disentangle the relative importance of early inbreeding depression vs. residual effects of self-incompatibility would be through microscopic examination of the ratio between unfertilized ovules, fertilized but aborted ovules, and regularly developing seeds in (premature) siliques. Like all previously published studies (Sletvold *et al.*, 2013; Willi, 2013; Oakley *et al.*, 2015), we avoided prematurely

Table 3 Likelihood-ratio tests (model comparisons) for the effect of mating system (MS), cross type (CT), jasmonic acid (JA) and drought (D) on vegetative biomass, cumulative vegetative performance, proportion of flowering plants and reproductive biomass in *Arabidopsis lyrata*.

Fixed effect	Vegetative biomass ($Y^{0.5}$, $n = 1008$)*,†		Cumulative vegetative performance ($Y^{0.5}$, $n = 1027$)*,‡		Flowering proportion (binomial, $n = 1009$)†,§		Reproductive biomass ($Y^{0.25}$, $n = 512$)*,¶	
	χ^2	P	χ^2	P	χ^2	P	χ^2	P
Mating system (MS)	0.005	0.94	0.29	0.59	0.0025	0.96	0.0070	0.93
Cross type (CT)	**103.1**	**<0.001**	**164.7**	**<0.001**	0.29	0.59	**7.18**	**0.0074**
Jasmonic acid (JA)	**66.1**	**<0.001**	**50.4**	**<0.001**	**5.78**	**0.016**	**13.3**	**<0.001**
Drought (D)	0.62	0.43	0.83	0.36	0.51	0.48	**3.85**	**0.0497**
MS: CT	**5.06**	**0.025**	2.22	0.14	1.78	0.18	0.096	0.76
MS: JA	0.12	0.73	0.24	0.63	0.76	0.38	0.26	0.61
MS: D	0.032	0.86	0.054	0.82	0.0011	0.97	1.44	0.23
CT: JA	0.12	0.73	0.32	0.57	0.49	0.49	2.49	0.11
CT: D	0.21	0.65	0.063	0.80	1.40	0.24	**4.19**	**0.041**
JA: D	1.79	0.18	1.46	0.23	0.77	0.38	*3.60*	*0.058*
MS: CT: JA	0.043	0.84	0.0027	0.96	0.93	0.33	0.33	0.57
MS: CT: D	1.10	0.29	0.46	0.50	0.23	0.63	0.49	0.49
MS: JA: D	0.75	0.39	0.22	0.64	0.52	0.47	0.0004	0.99
CT: JA: D	0.37	0.54	0.054	0.82	0.51	0.48	0.0050	0.94
MS: CT: JA: D	0.00063	0.98	0.074	0.79	0.058	0.81	1.48	0.22

Random effect	St. dev.	St. dev.	St. dev.	St. dev.
Mother	0.13	0.15	1.43	0.14
Population	0.11	0.13	1.26	0.15
Greenhouse compartment	0.0078	0.00	0.62	0.055

Chi-square test statistics (χ^2) and corresponding *P*-values (bold if <0.05) are shown for fixed effects and interactions. All tests had d.f. = 1 (as all fixed effects had two levels). The mixed models included the random-effects *mother*, *population* and *greenhouse*, for which the standard deviations (St. dev.) they explained are indicated.
*Analysed with linear mixed-effects models (*lmer*) with a Gaussian distribution, after transformation as indicated.
†Analysis on the subset of experimental plants that survived until harvest (minus one case for which the bag with biomass was lost).
‡Analysis included all experimental plants (minus one case for which the bag with biomass was lost). Cumulative vegetative performance was calculated as the product of germination proportion, survival and vegetative biomass.
§Data were analysed with generalized linear mixed-effects models (*lmer*) with the indicated distribution.
¶Analysis on the subset of experimental plants that flowered.

harvesting siliques, to maximize the number of seeds available for our experiment. Therefore, to which extent abortion during early seed development contributes to lifetime inbreeding depression in self-incompatible species remains to be investigated.

Evidence for purging in North American selfing populations

In the North American populations that have evolved higher selfing rates, we estimated a mean inbreeding depression of $\delta_{\text{per seed}} = 0.26$ under benign greenhouse conditions ($0.27 < \delta_{\text{per seed}} < 0.31$ under stress) and $\delta_{\text{per pollination}} = 0.30$ ($0.31 < \delta_{\text{per pollination}} < 0.36$ under stress; Appendix S5). These estimates are only just significantly lower than the lower bound of the inbreeding-depression estimates for outcrossing populations. In other words, our results only show weak evidence for purging, if reduced seed set after enforced selfing in

outcrossing populations is mainly due to residual effects of self-incompatibility (i.e. if the 'true' level of inbreeding depression is close to our lower bound estimates). This is a surprising finding, as inbreeding usually leads to substantial purging, even in studies that do not consider early-acting inbreeding depression (Crnokrak & Barrett, 2002). In *Leavenworthia alabamica,* for example where a breakdown of self-incompatibility also gave rise to several populations with high selfing rates, there was clear evidence for purging (Busch, 2005): outcrossing populations showed significant inbreeding depression (among others $\delta = 0.11$ for biomass), but selfing populations did not ($\delta = -0.01$ for biomass). Possibly, inbreeding depression in North American populations of *A. lyrata* is caused by many mutations of small effect, as these are difficult to purge (Lande & Schemske, 1985; Lynch *et al.*, 1995), or by heterozygote advantage, and cannot be purged (Charlesworth & Charlesworth, 1987). Alternatively, if reduced seed set after
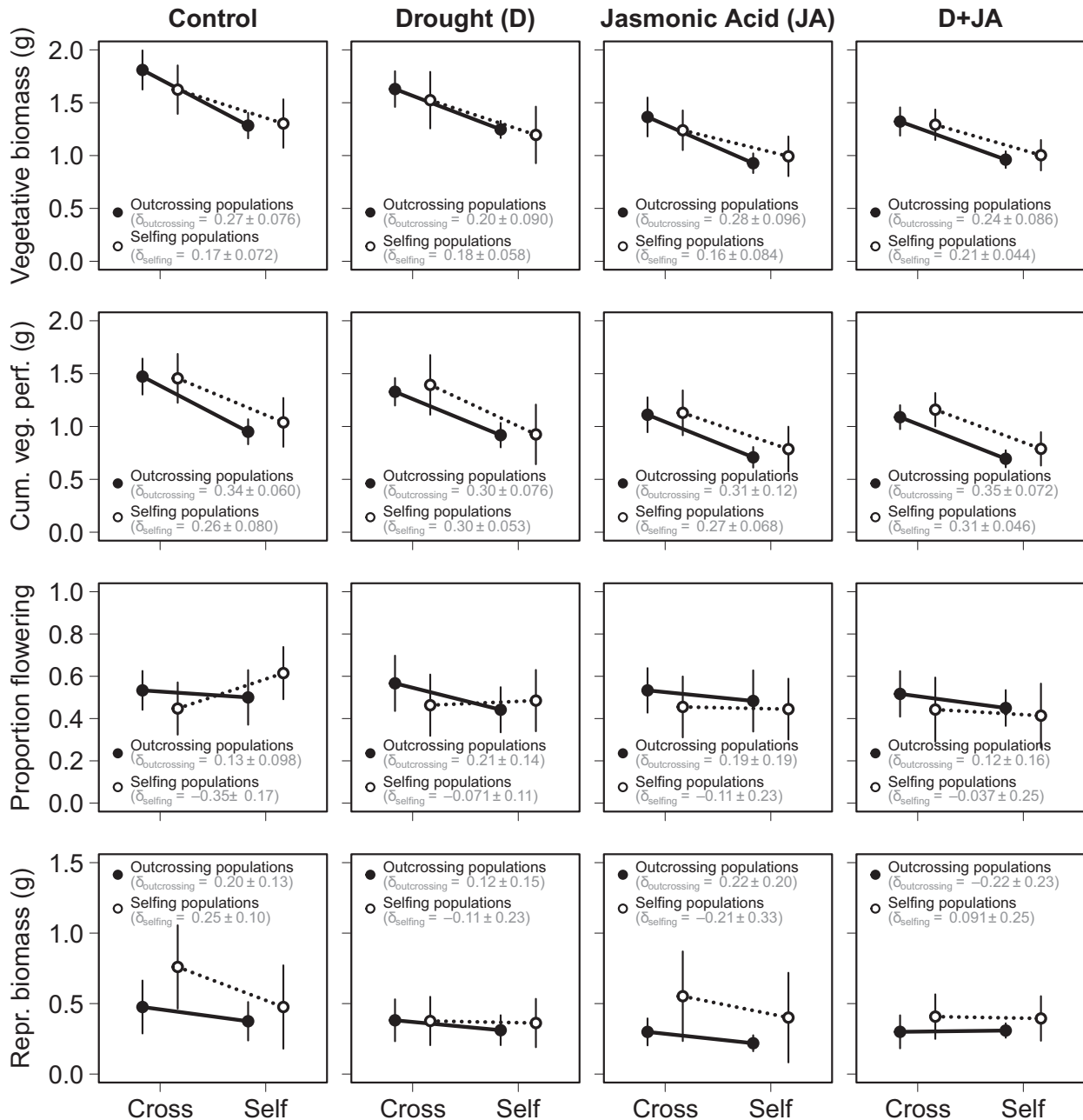
Fig. 2 Effect of cross type (cross vs. self) on vegetative biomass, cumulative vegetative performance (cum. veg. perf.), proportion of flowering plants and reproductive biomass, for outcrossing (black circles) and selfing (open circles) populations of North American *Arabidopsis lyrata*. The four different panels for each trait represent the treatments that the plants were exposed to: control, drought (D), jasmonic acid (JA) and a combination of drought and jasmonic acid (D+JA). Vertical lines indicate standard errors (SE) of the means of population trait means. The legend shows the mean ± SE of the population inbreeding-depression estimates (δ) for both mating systems (Appendix S5).

enforced selfing in outcrossing populations is due to early-acting inbreeding depression (i.e. if the 'true' level of inbreeding depression is close to our upper bound estimates), our data would suggest that the transition to selfing has led to considerable purging. However, as

discussed above, our data cannot tease apart whether the substantial reductions in seed set after enforced selfing of self-incompatible plants are due to residual self-incompatibility or due to early-acting inbreeding depression. This again highlights the need for future

studies to distinguish seed abortion due to inbreeding depression from reduced seed formation.

Conclusion

Our results show that inbreeding depression was relatively low in North American *A. lyrata* populations (in comparison with estimates based on the same traits in the European subspecies). This low barrier to the evolution of selfing may help explain why selfing could evolve in some North American populations. Our data, however, cannot conclusively answer whether there is evidence for purging in populations with naturally high selfing rates, because of the unknown contribution of early seed abortion to inbreeding depression in outcrossing populations. Finally, stress imposed by drought and induction of defence did not magnify our inbreeding-depression estimates compared to those obtained under benign greenhouse conditions. Whereas this supports the robustness and generality of our inbreeding-depression estimates to environmental influences, future work should integrate measures of plant fecundity under open pollination and elucidate the potential contribution of early seed abortion to lifetime inbreeding depression.

Acknowledgments

Conflict of interest

Authors declare no conflict of interest.

References

Ågren, J. & Schemske, D.W. 1993. Outcrossing rate and inbreeding depression in two annual monoecious herbs, *Begonia hirsuta* and *B. semiovata*. *Evolution* **47**: 125–135.

Al-Shehbaz, I.A. & O'Kane, S.L. Jr 2002. Taxonomy and phylogeny of *Arabidopsis* (Brassicaceae). *Arabidopsis Book* **1**: e0001.

Armbruster, P. & Reed, D.H. 2005. Inbreeding depression in benign and stressful environments. *Heredity* **95**: 235–242.

Baldwin, I.T. 1998. Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proc. Natl. Acad. Sci. USA* **95**: 8113–8118.

Barrett, S.C.H. 2002. The evolution of plant sexual diversity. *Nat. Rev. Genet.* **3**: 274–284.

Bates, D., Maechler, M., Bolker, B. & Walker, S. 2014. lme4: Linear mixed-effects models using Eigen and S4. R package version 1.1-7.

Bellanger, S., Guillemin, J.P., Touzeau, S. & Darmency, H. 2015. Variation of inbreeding depression in *Centaurea cyanus* L., a self-incompatible species. *Flora* **212**: 24–29.

Busch, J.W. 2005. Inbreeding depression in self-incompatible and self-compatible populations of *Leavenworthia alabamica*. *Heredity* **94**: 159–165.

Charlesworth, D. & Charlesworth, B. 1987. Inbreeding depression and its evolutionary consequences. *Annu. Rev. Ecol. Syst.* **18**: 237–268.

Charlesworth, D. & Willis, J.H. 2009. The genetics of inbreeding depression. *Nat. Rev. Genet.* **10**: 783–796.

Crnokrak, P. & Barrett, S.C.H. 2002. Purging the genetic load: a review of the experimental evidence. *Evolution* **56**: 2347–2358.

Cruzan, M.B. & Barrett, S.C.H. 2016. Postpollination discrimination between self and outcross pollen covaries with the mating system of a self-compatible flowering plant. *Am. J. Bot.* **103**: 568–576.

Fisher, R.A. 1941. Average excess and average effect of a gene substitution. *Ann. Eugen.* **11**: 53–63.

Fox, C.W. & Reed, D.H. 2011. Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. *Evolution* **65**: 246–258.

Foxe, J.P., Stift, M., Tedder, A., Haudry, A., Wright, S.I. & Mable, B.K. 2010. Reconstructing origins of loss of self-incompatibility and selfing in North American *Arabidopsis lyrata*: a population genetic context. *Evolution* **64**: 3495–3510.

Goodwillie, C., Kalisz, S. & Eckert, C.G. 2005. The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annu. Rev. Ecol. Evol. Syst.* **36**: 47–79.

Griffin, P.C. & Willi, Y. 2014. Evolutionary shifts to self-fertilisation restricted to geographic range margins in North American *Arabidopsis lyrata*. *Ecol. Lett.* **17**: 484–490.

Husband, B.C. & Schemske, D.W. 1996. Evolution of the magnitude and timing of inbreeding depression in plants. *Evolution* **50**: 54–70.

Igic, B., Lande, R. & Kohn, J.R. 2008. Loss of self-incompatibility and its evolutionary consequences. *Int. J. Plant Sci.* **169**: 93–104.

Johnson, M.T., Smith, S.D. & Rausher, M.D. 2009. Plant sex and the evolution of plant defenses against herbivores. *Proc. Natl. Acad. Sci. USA* **106**: 18079–18084.

Johnston, M.O., Porcher, E., Cheptou, P.-O., Eckert, C.G., Elle, E., Geber, M.A. *et al.* 2009. Correlations among fertility components can maintain mixed mating in plants. *Am. Nat.* **173**: 1–11.

Joschinski, J., van Kleunen, M. & Stift, M. 2015. Costs associated with the evolution of selfing in North American populations of *Arabidopsis lyrata*? *Evol. Ecol.* **29**: 749–764.

Kondrashov, A.S. 1985. Deleterious mutations as an evolutionary factor. II. Facultative apomixis and selfing. *Genetics* **111**: 635–653.

Lande, R. & Schemske, D.W. 1985. The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. *Evolution* **39**: 24–40.

Lloyd, D.G. 1979. Some reproductive factors affecting the selection of self-fertilization in plants. *Am. Nat.* **113**: 67–79.

Løe, G. 2006. Ecology and evolution of resistance to herbivory: trichome production in *Arabidopsis lyrata*. PhD dissertation, Uppsala University, Uppsala, Sweden.

Lynch, M., Conery, J. & Burger, R. 1995. Mutational meltdowns in sexual populations. *Evolution* **49**: 1067–1080.

Mable, B.K. & Adam, A. 2007. Patterns of genetic diversity in outcrossing and selfing populations of *Arabidopsis lyrata*. *Mol. Ecol.* **16**: 3565–3580.

Mable, B.K., Robertson, A.V., Dart, S., Di Berardo, C. & Witham, L. 2005. Breakdown of self-incompatibility in the perennial *Arabidopsis lyrata* (Brassicaceae) and its genetic consequences. *Evolution* **59**: 1437–1448.

Mable, B.K., Hagmann, J., Kim, S.-T., Adam, A., Kilbride, E., Weigel, D. *et al.* 2017. What causes mating system shifts in plants? *Arabidopsis lyrata* as a case study. *Heredity* **118**: 52–63.

Moore, J.P., Paul, N.D., Whittaker, J.B. & Taylor, J.E. 2003. Exogenous jasmonic acid mimics herbivore-induced systemic increase in cell wall bound peroxidase activity and reduction in leaf expansion. *Funct. Ecol.* **17**: 549–554.

Nakanishi, T., Esashi, Y. & Hinata, K. 1969. Control of self-incompatibility by CO_2 gas in *Brassica*. *Plant Cell Physiol.* **10**: 925–927.

Oakley, C.G., Spoelhof, J.P. & Schemske, D.W. 2015. Increased heterosis in selfing populations of a perennial forb. *AoB Plants* **7**: plv122.

Porcher, E. & Lande, R. 2005. Loss of gametophytic self-incompatibility with evolution of inbreeding depression. *Evolution* **59**: 46–60.

Pujol, B., Zhou, S.R., Vilas, J.S. & Pannell, J.R. 2009. Reduced inbreeding depression after species range expansion. *Proc. Natl. Acad. Sci. USA* **106**: 15379–15383.

R Core Team. 2016. R: a language and environment for statistical computing, R Foundation for Statistical Computing. R Core Team, Vienna, Austria.

Razanajatovo, M., Maurel, N., Dawson, W., Essl, F., Kreft, H., Pergl, J. *et al.* 2016. Plants capable of selfing are more likely to become naturalized. *Nat. Commun.* **7**: 13313.

Reed, D.H., Briscoe, D.A. & Frankham, R. 2002. Inbreeding and extinction: the effect of environmental stress and lineage. *Conserv. Genet.* **3**: 301–307.

Ross-Ibarra, J., Wright, S.I., Foxe, J.P., Kawabe, A., DeRose-Wilson, L., Gos, G. *et al.* 2008. Patterns of polymorphism and demographic history in natural populations of *Arabidopsis lyrata*. *PLoS One* **3**: e2411.

Sandner, T.M. & Matthies, D. 2016. The effects of stress intensity and stress type on inbreeding depression in *Silene vulgaris*. *Evolution* **70**: 1225–1238.

Sandring, S. & Ågren, J. 2009. Pollinator-mediated selection on floral display and flowering time in the perennial herb *Arabidopsis lyrata*. *Evolution* **63**: 1292–1300.

Schemske, D.W. & Lande, R. 1985. The evolution of self-fertilization and inbreeding depression in plants. II. Empirical observations. *Evolution* **39**: 41–52.

Sicard, A. & Lenhard, M. 2011. The selfing syndrome: a model for studying the genetic and evolutionary basis of morphological adaptation in plants. *Ann. Bot.* **107**: 1433–1443.

Sletvold, N., Mousset, M., Hagenblad, J., Hansson, B. & Agren, J. 2013. Strong inbreeding depression in two scandinavian populations of the self-incompatible perennial herb *Arabidopsis lyrata*. *Evolution* **67**: 2876–2888.

Stebbins, G.L. 1957. Self fertilization and population variability in the higher plants. *Am. Nat.* **91**: 337–354.

Stift, M., Hunter, B.D., Shaw, B., Adam, A., Hoebe, P.N. & Mable, B.K. 2013. Inbreeding depression in self-incompatible North-American *Arabidopsis lyrata*: disentangling genomic and S-locus-specific genetic load. *Heredity* **110**: 19–28.

Tedder, A., Carleial, S., Gołębiewska, M., Kappel, C., Shimizu, K.K. & Stift, M. 2015. Evolution of the selfing syndrome in *Arabis alpina* (Brassicaceae). *PLoS One* **10**: e0126618.

Willi, Y. 2013. Mutational meltdown in selfing *Arabidopsis lyrata*. *Evolution* **67**: 806–815.

Wright, S.I., Kalisz, S. & Slotte, T. 2013. Evolutionary consequences of self-fertilization in plants. *Proc. R. Soc. Lond., B, Biol. Sci.* **280**: 20130133.

Zuur, A.F., Hilbe, J. & Ieno, E.N. 2013. *A Beginner's Guide to GLM and GLMM with R: A Frequentist and Bayesian Perspective for Ecologists*. Highland Statistics, Newburgh, UK.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1 Sample sizes.

Appendix S2 Side effects of CO_2.

Appendix S3 Confirmation of induced defence.

Appendix S4 Analysis of seed weight.

Appendix S5 Inbreeding-depression coefficients.

Appendix S6 Analysis of cumulative vegetative performance per pollination.

Appendix S7 Powdery mildew infestation.