

## Environmental conditions during early life determine the consequences of inbreeding in *Agrostemma githago* (Caryophyllaceae)

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### Abstract

In an inbred population, selection may reduce the frequency of deleterious recessive alleles through a process known as purging. Empirical studies suggest, however, that the efficacy of purging in natural populations is highly variable. This variation may be due, in part, to variation in the expression of inbreeding depression available for selection to act on. This experiment investigates the roles of life stage and early-life environment in determining the expression of inbreeding depression in *Agrostemma githago*. Four population-level crosses ('self', 'within', 'near' and 'far') were conducted on 20 maternal plants from a focal population. Siblings were planted into one of three early environmental treatments with varying stress levels. Within the focal population, evidence for purging of deleterious recessive alleles, as well as for variation in the expression of inbreeding depression across the life cycle was examined. In addition, the effect of early environment on the expression of inbreeding depression and the interaction with cross-type was measured. We find that deleterious recessive alleles have not been effectively purged from our focal population, the expression of inbreeding depression decreases over the course of the life cycle, and a stressful early environment reduces the variance in inbreeding depression expressed later in life, but does not consistently influence the relative fitness of inbred versus outcrossed individuals.

### Introduction

Inbreeding depression is a serious hazard for small populations that results in diminished vigour or fecundity as a consequence of an increase in the frequency of homozygosity (Fisher, 1965; Husband & Schemske, 1996; Keller & Waller, 2002). As the frequency of homozygosity increases, a reduction in performance results from either dominance, the unmasking of deleterious recessive alleles (Lande & Schemske, 1985; Charlesworth & Charlesworth, 1990), or overdominance, a selective advantage for heterozygotes (Charlesworth & Charlesworth, 1987; Ziehe & Roberds, 1989).

If inbreeding depression is due to the unmasking of deleterious recessive alleles, natural selection may

effectively purge the population of these alleles, which would reduce inbreeding depression in subsequent generations (Templeton & Read, 1984). However, the efficacy of purging in natural populations remains a matter of some debate (Husband & Schemske, 1996; Byers & Waller, 1999; Willis, 1999; Kristensen *et al.*, 2008). If purging is effective in removing deleterious recessive alleles from a population, we might expect that species with high rates of selfing should show low rates of inbreeding depression. Although this appears to be the case for many studies (Barrett & Charlesworth, 1991; McCall *et al.*, 1994; Carr & Dudash, 1995), there are numerous exceptions in which selfing species show no decline in inbreeding depression (Ashman, 1992; Agren & Schemske, 1993; Koelewijn, 1998). The inconsistencies in these results may, in part, reflect the fact that purging is expected to be ineffective in populations with extremely high levels of inbreeding. In these populations, deleterious alleles quickly become fixed at

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random before natural selection can effectively remove them, which can result in further population decline (Byers & Waller, 1999; Keller & Waller, 2002).

This issue is further complicated by the difficulty in demonstrating purging in natural populations. A decline in inbreeding depression may in fact reflect the fixation of deleterious recessive alleles. These fixed deleterious alleles are known as the drift load (Glémin *et al.*, 2003). As Byers & Waller (1999) emphasize, the same conditions necessary for purging to be effective will also tend to accelerate the fixation of mildly deleterious alleles. As more deleterious alleles become fixed in a population, the number of the genes that have the potential to differ between inbred and outbred individuals declines. As a result, selfed lines may falsely appear to be purged of deleterious alleles, as a consequence of this reduced population-wide genetic variation. Purging and fixation, however, can be distinguished experimentally by comparing the inbreeding depression expressed relative to within-population versus among-population crosses. If a decline in the expression of inbreeding depression within a population is the result of the fixation of deleterious recessive alleles, then among-population crosses should increase the expression of inbreeding depression. If, however, a decline in the expression of inbreeding depression is due to the past purging of deleterious recessive alleles, among-population crosses should not greatly impact the expression of inbreeding depression (Keller & Waller, 2002).

The degree of purging, across species with different levels of selfing, may also be affected by the timing of inbreeding depression across life stages (Husband & Schemske, 1996). It is important to estimate inbreeding depression at multiple life stages, as studies that focus on only one life stage may miss the expression of inbreeding depression during other periods (Byers, 1998; Grueber *et al.*, 2010). Further, as inbreeding depression has been shown to vary greatly across life stages, inbreeding depression estimated at one stage is not necessarily indicative of the inbreeding depression likely to be expressed at other life stages (Carr & Dudash, 1995).

The environment may impact the expression of inbreeding depression and this impact may have carry-over effects across life stages. A mild environment may decrease the expression of inbreeding depression (Dudash, 1990; Hedrick & Kalinowski, 2000; Armbruster & Reed, 2005), while reducing the strength of selection. A stressful environment may increase the expression of inbreeding depression (Dudash, 1990; Hedrick & Kalinowski, 2000; Armbruster & Reed, 2005). This increase in inbreeding depression may result in selection against deleterious recessive alleles, and subsequent decreased levels of inbreeding depression in future generations. (Swindell & Bouzat, 2006). However, while theory suggests that the expression of inbreeding depression should increase in stressful environments, the many

studies testing this theory show mixed results (Armbruster & Reed, 2005). Understanding how environment impacts the expression of inbreeding depression and patterns of selection at different life stages may help to clarify this issue. In a natural environment, conditions change over time, and can vary within and across life stages. There may also be carry-over effects such that the patterns of selection across the life cycle can be affected by environmental differences occurring at early life stages (Donohue, 2002; Goodrich, 2009). These early-life environmental carry-over effects can have selective consequences even when individuals share a common late-life environment (c.f. Bennington & McGraw, 1995; see also Goodrich, 2009).

This study uses crosses within and among populations of *Agrostemma githago*, as well as three different early-life environmental stress treatments, to address the following questions: First, is there evidence for past purging of deleterious alleles in a natural population of *A. githago*? Second, how does the expression of inbreeding depression change over the course of a life cycle? And finally, how does early-life environment affect the expression of inbreeding depression across these life stages?

## Materials and methods

### Study Species

*Agrostemma githago* (Caryophyllaceae) is a small (30–120 cm high), annual herb that blooms in late summer. The flowers, approximately 2.5 cm in diameter, open in the mornings and expose dehiscent anthers to pollinators (commonly Dipterans). As the anthers are lost, the stigma opens to receive pollen. Late in the day, a second set of anthers dehisces to allow for self-fertilization (Firbank, 1988). When compared with other members of Caryophylloideae, *A. githago* is categorized as a highly selfing species (Jurgens *et al.*, 2002; Jurgens, 2006). Each plant produces approximately 200 seeds, 2–5 mm in diameter (Miller, 2003). Seeds mature one month after fertilization (Firbank, 1988), and if given sufficient moisture, will germinate immediately (Thompson, 1973). The seeds have little or no innate dormancy in the soil (Holzner & Numata, 1982). Following germination, the plants overwinter as a rosette, and begin to gain height in spring (Firbank, 1988).

### Crossing Design

To address our first question of whether there was a history of purging in our focal population of *A. githago*, we needed a crossing design that would enable us to distinguish between historical purging and fixation of deleterious alleles. In addition, to address all of our questions, we needed a crossing design that generated individuals with a broad range of inbreeding coefficients. We satisfied both of these requirements by

producing selfed, one intra- and two inter-population crosses of maternal plants. To establish parental lines for these crosses, seed was collected from three populations of *A. githago* in western Virginia. In population A less than 20% of seed is produced by outcrossing (Goodrich, 2009). Population A and Population B were located 1.2 km apart on Route 602 in Newport, Virginia, whereas Population C was located 11.9 km from Population A on Route 658 in Maggie, Virginia. Populations A, B and C contained 1000, 75 and 250 individuals respectively. Population A was used as the focal population because this was the largest population and it was the population located closest to the common garden. The three populations showed signs of being genetically isolated from each other. In addition to low rates of pollinator-mediated outcrossing, the three populations showed significant differences in phenotypic characters when grown in a common greenhouse environment (Goodrich, 2009).

The crossing design was carried out in a greenhouse at the University of Virginia. Seeds from each population were grown in 4" pots. Blooming initiated in mid-August and crosses were made through late September. Twenty plants were randomly selected from population A to serve as pollen recipients (dams). Anthers on dams were removed with forceps the day before flowers opened. Four types of crosses were performed on each dam in a randomized order. 'Selfed' crosses were made between two flowers on the same plant; 'Within' crosses were made between two plants from population A; 'Near' crosses were made between a dam from population A and a sire from population B; and 'Far' crosses were made between a dam from population A and a sire from population C. A total of 1383 seeds from 20 dams were collected and weighed.

### Early Environment Treatments

To address the question of whether early environment alters the expression of inbreeding depression, seeds within each cross-type were evenly assigned to one of three early environment (EE) treatments representing different levels of stress. Individuals from all EE treatments then shared a common late-life environment. Seeds assigned to Early Environment 1 (EE1), the high stress treatment, were planted directly into the field. Seeds in Early Environment 2 (EE2), the medium stress treatment, were germinated in the greenhouse and transplanted to the field at the early rosette stage, when plants had 4–6 leaves. Seeds assigned to Early Environment 3 (EE3), the low stress treatment, were germinated in the greenhouse at the same time as EE2 and transplanted to the field at the late rosette stage when plants had 10–14 leaves. Each environmental treatment was composed of roughly 1/3 of the number of seeds per cross-type, ranging from 64 (Within, EE3) to 172 (Selfed, EE2).

### Field and Greenhouse Methods

The common garden field site for this experiment was in a cow pasture that had been used previously to cultivate wheat. It was located 0.7 km from population A. Prior to planting the seeds in the fall of 2006, the area was mown to the ground, leaving root systems and meristems of the plant community intact. No further clipping or mowing was performed prior to planting or transplanting. The mown area was divided into three spatial blocks (1 m × 7 m). Planting sites within blocks were separated by 10 cm. Individuals were evenly assigned to blocks, with respect to dam, cross and EE treatment.

Plants were moved from the greenhouse into the field at three different life history stages according to EE assignment. EE1 seeds were planted directly into the field in early November. Individual seeds were planted singly, 0.5 cm deep, and marked with swizzle sticks. Seeds assigned to EE2 and EE3 were planted singly in the greenhouse into 2.4 cm × 2.4 cm × 15 cm ConeTainers, filled to the top with MetroMix. Seeds were placed on a mist bench for 2 weeks, after which they were moved to a dry bench and hand watered every 2–3 days. To synchronize germination in the field and greenhouse, EE2 and EE3 were planted one week after EE1 was planted. EE2 and EE3 were transplanted into the field in early March and late April, respectively. Each rosette was removed from its ConeTainer and inserted into a 3 cm × 3 cm × 15 cm hole. To reduce stress associated with transplanting, each plant was watered twice a day, for three days following transplanting. Any mortality occurring within two weeks from transplanting was attributed to transplant shock and these individuals were deleted from the analysis.

### Census Data and Fitness Calculations

Censuses were conducted regularly over the course of the growing season. Germination of EE1 seeds was scored in the field every 3<sup>rd</sup> day for 27 days from mid November to late December. Germination of EE2 and EE3 seeds was scored daily in the greenhouse over the same period. For individuals in all treatments, survival, leaf number and length of the longest leaf were recorded every four weeks from November to August. Additionally, date of first flower was recorded daily for 5 weeks beginning in late May. At the conclusion of the experiment, each plant, including the taproot, was harvested, dried and weighed. The total number of seeds produced was counted.

This census data were used to calculate a number of fitness measurements for plants in each treatment. Above-ground biomass ('size') was estimated for each census date by multiplying leaf number by length of longest leaf. A previous greenhouse experiment using destructive sampling techniques demonstrated that this

estimation was highly correlated with above-ground biomass ( $r^2 = 0.88$ ,  $P < 0.0001$ ,  $n = 45$ ). Relative growth rate was calculated by dividing the difference in size between two consecutive censuses by the size of the individual at the earlier census (Hunt & Cornelissen, 1997). The mortality rate ( $q(x)$ ) was calculated by dividing the number of deaths within a treatment at the end of a census interval by the number alive at the beginning of that interval. This mortality calculation was independent for each census interval. All estimates of mortality were standardized to 28 days to account for uneven census intervals. Survival was calculated as the proportion of individuals alive at a given time relative to the number of seeds initially planted. An individual was determined to have survived to flower if a date of first flower was recorded. Likewise, survival to set seed refers to individuals that successfully produced mature seed, prior to death in the field. Fecundity, for all surviving plants, was defined as the total number of seeds a plant produced. Lifetime fitness, on the other hand, included values of zero for plants that died prior to reproduction. Variance for lifetime fitness was calculated for each maternal line-by-treatment combination. The variance statistic is appropriate to use in place of a coefficient of variance statistic because the means are small and do not significantly differ across treatments (Sokal & Rohlf, 1995).

Inbreeding depression was calculated for each dam  $\times$  early environment  $\times$  cross treatment combination as

$$RP = (\text{outcross} - \text{self})/\text{maximum},$$

where RP = relative performance, maximum = outcross if outcross  $\geq$  self, and maximum = self when self  $>$  outcross (Agren & Schemske, 1994; Dudash *et al.*, 1997). The RP calculation of inbreeding depression allows estimates for each treatment to be summed and averaged without bias because it is restricted to values between 1 and  $-1$ . In contrast, a calculation of inbreeding depression in which the denominator is always the outcross value (Husband & Schemske, 1996) would produce estimates of inbreeding depression that could range from 1 to  $-\infty$ . Dam could be used as a blocking factor to control for maternal effects because all four cross-types were performed on each maternal plant.

## Analyses

We first asked whether there was past evidence of purging of deleterious alleles in our focal population. Using only individuals from EE1, we compared inbreeding depression for survival and fecundity within maternal lines across all cross treatments with an Analysis of Variance. We tested whether these inbreeding depression values were significantly different from zero using a *t*-test. If the fitness of selfed individuals within our focal population was lower than that of the within population

outcrossed individuals, then we could assume that selfing likely resulted in the unmasking of recessive deleterious alleles, and that effective purging has not yet been completed in this population. If the fitness of selfed individuals equalled the fitness of the within population outcrossed individuals, then this would suggest that it is possible that deleterious alleles have been effectively purged from the population. However, the fixation of deleterious alleles in a population also results in this same pattern of equal fitness between inbred and outbred individuals. Our crossing design allowed us to distinguish between these two possible causes. Individuals from population A were also outcrossed with individuals from the near and far populations. It is unlikely that these populations would share all of the same fixed deleterious alleles as population A. Therefore, if a lack of detected inbreeding depression in population A is caused by a fixation of deleterious recessive alleles, individuals outcrossed with population B or C should regain a masking of these deleterious recessive alleles and have a higher fitness than the selfed individuals. Alternatively, if a lack of detected inbreeding depression in population A is truly due to the past purging of deleterious recessive alleles, then individuals outcrossed with populations B and C should not be any more fit than the population A selfed individuals.

We then tested whether the expression of inbreeding depression changes over the course of a life cycle. We first calculated a change in inbreeding depression for maternal families in EE1 by subtracting inbreeding depression for seed weight from inbreeding depression for May size using maternal line means. May size was used because it was a late-life pre-reproductive census point. We then tested whether there was a correlation between the difference in inbreeding depression for seed weight and May size within a family and the proportion of survivors to seed set within that family. If we found a decline in inbreeding depression over time, and this decline was negatively correlated with the proportion of survivors within a family, then this correlation would suggest that the mortality of individuals expressing inbreeding depression early in life may reduce the expression of inbreeding depression later in life. Changes in inbreeding depression were not calculated for individuals in EE2 or EE3 because the environment was experimentally manipulated for these treatment groups. Estimates of inbreeding depression for seed weight and May size were normally distributed and did not require transformation (Shapiro–Wilk  $W = 0.97$  and  $0.98$  respectively).

Finally, we asked whether early environment affects the expression of inbreeding depression later in life. We first needed to establish whether field conditions were actually more stressful than greenhouse conditions, as some studies have found that laboratory or greenhouse conditions are more stressful than natural environments (Armbruster & Reed, 2005; Reed *et al.*, 2012).



To confirm that our EE treatments represented three different levels of stress, we tested for differences in survival to seed set among treatments. In addition, we tracked the mortality rate in each treatment across periods of experiment-wide dips and rises in relative growth rates. Periods of rapid investment in growth can be potentially stressful, so we tested whether the stress that typically follows this rapid growth was felt equally across treatments (Metcalf & Monaghan, 2001).

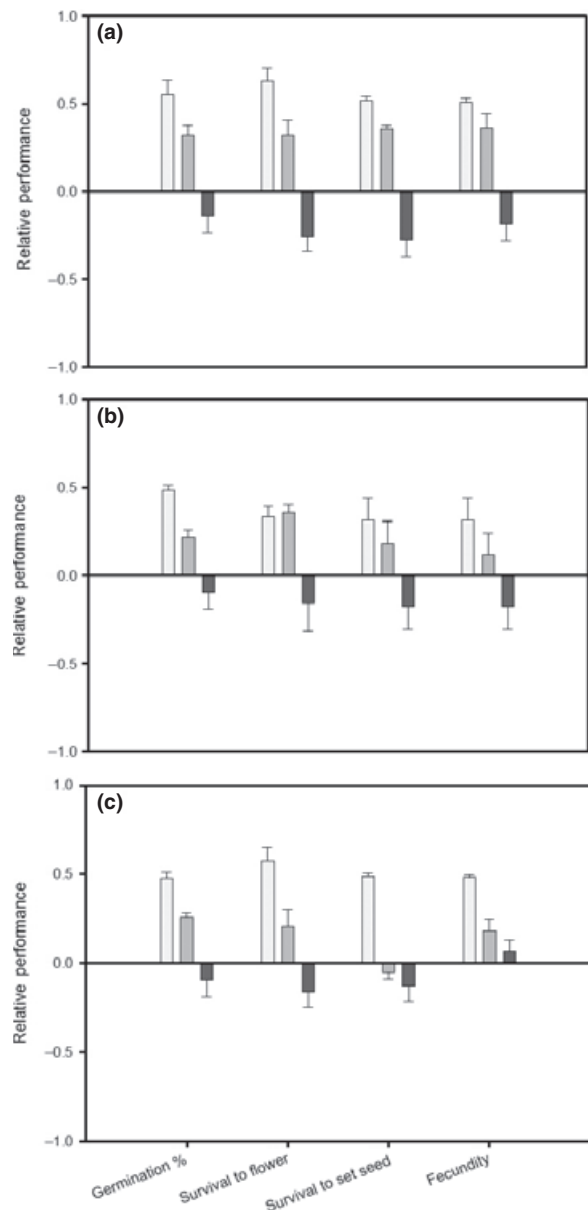
We next used Analysis of Variance to test how cross-type and early environment each independently affected inbreeding depression expressed in terms of both survival to flower and lifetime fitness. We also tested for the interaction effects of cross  $\times$  EE and cross  $\times$  dam. Dam was included as a random effect. Finally, because we found no major difference in survival between our two milder EE treatments (EE2 and EE3), we used Analysis of Variance to look more specifically at the difference in the variance for inbreeding depression for lifetime fitness between individuals in the harshest EE treatment (EE1) and individuals in the two milder treatments (EE2 and EE3). All statistical analyses were performed using SAS 9.1.2 (SAS Institute, 2000).

## Results

### Is there evidence for past purging of deleterious alleles?

If population A had already purged its deleterious alleles, then the fitness of selfed individuals would equal that of intra population outcrossed individuals. This, however, was not the case. Selfed progeny showed the lowest survival across life stages when compared to within and near population crosses (Fig. 1a). Percent germination was 17.1% higher for outcrossed individuals from within population crosses, and 12.4% higher for outcrossed individuals from near population crosses relative to selfed individuals. Survival to flower was 22.7% higher for outcrossed individuals from within population crosses, and 15.6% higher for outcrossed individuals from near population crosses. Similarly, survival to set seed was 18.2% higher for outcrossed individuals from within population crosses and 13.9% higher for outcrossed individuals from near population crosses. Inbreeding depression for fecundity was observed for both the within-population ( $0.50 \pm 0.04$  SE) and the near-population ( $0.36 \pm 0.06$ ) levels of comparison with inbred individuals.

Selfed progeny had higher fitness than progeny from far population crosses, suggesting possible outbreeding depression. Percent germination was 4.0% higher among selfed progeny than among far population crosses. Survival to flower and survival to set seed were 6.1% and 5.7% higher, respectively, among selfed individuals than progeny from far population crosses.



**Fig. 1** Inbreeding depression ( $\pm$  SE) for survival and fecundity for plants in EE1 (Panel (a)), EE2 (Panel (b)) and EE3 (Panel (c)). All estimates differ significantly from zero ( $t$ -test, maximum  $P$ -value = 0.02). Estimates of inbreeding depression for outcrossed progeny from within population crosses (white bars), near population crosses (light grey bars) or far population crosses (dark grey bars) were calculated relative to progeny from selfed crosses. Positive values of inbreeding depression indicate lower survival among selfed progeny. Note that each survival benchmark, including fecundity, is a statistically independent estimate.

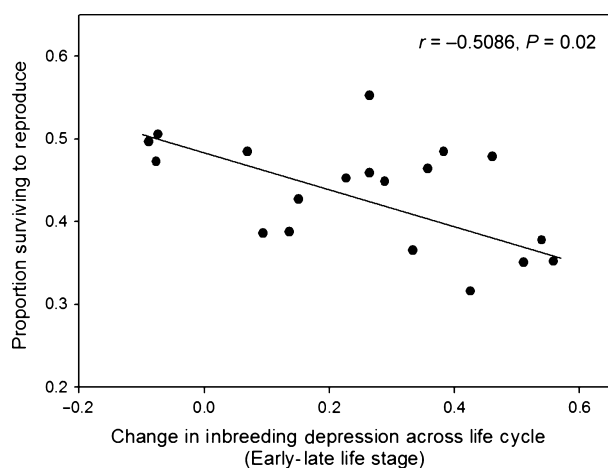
Mild outbreeding depression for fecundity was observed in the far population ( $-0.18 \pm 0.06$ ). All estimates of relative performance were significantly different from zero (maximum  $P$ -value = 0.02).

### How does the expression of inbreeding depression change over the course of a life cycle?

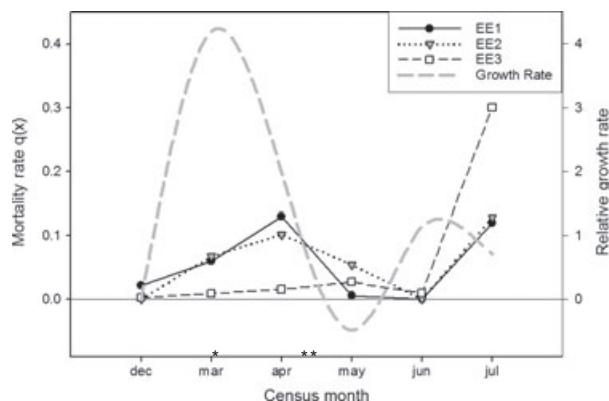
Within maternal lines, inbreeding depression tended to decrease over the life cycle (Fig. 2). The difference between inbreeding depression for seed weight and May size within a family was negatively correlated with the proportion of survivors within that family (Fig. 2,  $r = -0.509$ ,  $P = 0.02$ ). This indicates that inbreeding depression at later life stages diminished the most among maternal lines with lowest survival. However, three families had slightly higher inbreeding depression for May size than for seed weight, and thus showed negative values for a change in inbreeding depression (Fig. 2). These families also had above average survival, suggesting that they may have had a lower mutational load than other families and, therefore, were not as susceptible to inbreeding depression. Among survivors to reproduce, there were no significant differences between maternal lines in inbreeding depression for reproductive output ( $F_{20,798} = 1.28$ ,  $P = 0.18$ ).

### Does early environment alter the expression of inbreeding depression later in life?

Survival to seed set differed greatly between the more severe EE treatment and the two milder treatments ( $\chi^2 = 24.264$ , d.f. = 2,  $P < 0.0001$ ). Only 31% survived to reproduce in the EE1 treatment, while 63% and 68% survived to reproduce in the EE2 and EE3 treatments respectively. Early environment also affected patterns of mortality. Specifically, increases in the rate of mortality were related to the environment during periods of peak growth (Fig. 3). In March, plants in all three environmental treatments underwent a period of rapid growth. EE1 and EE2 experienced this growth



**Fig. 2** Correlation between the proportion of individuals surviving to reproduction and the decrease in inbreeding depression between early life (seed weight) and late life (May size). Each point represents the mean estimate for a maternal line in EE1.



**Fig. 3** Plot of monthly estimates of mortality for each environmental treatment (symbols) and mean relative growth rate for all plants averaged across all environments (grey dashed line). Mean relative growth rate is presented as a spline curve to give a smoothed average over time. Filled circles (solid line) represent monthly mortality rates for plants that were reared entirely in the field environment (EE1). The grey triangles (dotted line) represent monthly mortality rates for EE2 (\* on x-axis indicates transplant to field). Open squares (dashed line) represent monthly mortality rates for EE3 (\*\* on x-axis indicates transplant to field). Horizontal line represents estimates of zero for both mortality rate and relative growth rate.

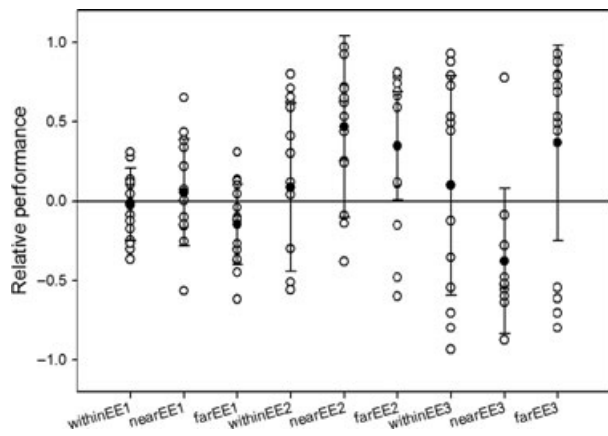
peak in the field environment, while EE3 did so in the greenhouse. The following month, EE1 and EE2 showed an increase in the mortality rate, while mortality for EE3 remained low. In June, a second peak in growth rate was followed by increases in mortality in all three environmental treatments in July, when all plants were in the field. The differences in survival and mortality rate across treatments confirm that the field was a more stressful environment than the greenhouse.

Early environment had a large effect on the expression of inbreeding depression for lifetime fitness, but no effect on the expression of inbreeding depression for survival to reproduce (Table 1). The variance in dam means for lifetime fitness was significantly greater in the two treatments that experienced milder early environments (ANOVA with 'field' vs. 'greenhouse',  $F_{1, 2} = 11.54$ ,  $P = 0.01$ , Fig. 4 and Table 1). After experiencing a mild early environment, maternal lines expressed both higher and lower average levels of inbreeding depression than plants germinated in the field. Mean variance of inbreeding depression for lifetime fitness within maternal lines was 0.066 in EE1, 0.21 in EE2 and 0.37 in EE3.

For the mild early environment treatments (EE2 and EE3), survival to seed set within maternal lines was not correlated with inbreeding depression for seed weight ( $n = 48$ ,  $r = 0.01$ ,  $P = 0.9$ ), May size ( $n = 24$ ,  $r = 0.13$ ,  $P = 0.4$ ) or lifetime fitness ( $n = 20$ ,  $r = 0.44$ ,  $P = 0.06$ ). However, for EE1, there was a negative correlation between survival to reproduce and inbreeding depression

**Table 1** Analysis of variance for main effects of cross-type, early environment (EE), dam, and environmental interactions on inbreeding depression for survival to flower and lifetime fitness.

|            | d.f. | Survival to Flower |         | Lifetime Fitness |         |
|------------|------|--------------------|---------|------------------|---------|
|            |      | F                  | P-value | F                | P-value |
| Cross      | 1    | 6.04               | 0.043   | 6.52             | 0.037   |
| EE         | 2    | 1.41               | 0.305   | 14.33            | 0.003   |
| Dam        | 18   | 1.02               | 0.521   | 2.36             | 0.127   |
| Cross × EE | 2    | 1.17               | 0.365   | 0.71             | 0.523   |
| Dam × EE   | 17   | 1.32               | 0.369   | 3.53             | 0.049   |



**Fig. 4** Estimates of inbreeding depression for lifetime fitness for each environment × cross treatment combination. The horizontal line represents an inbreeding depression estimate of zero. The open circles represent estimates for maternal lines, and the filled circles represent the mean values for each treatment. Interval bars represent the standard deviation associated with each mean.

for seed weight ( $n = 20$ ,  $r = -0.37$ ,  $P = 0.04$ ) and lifetime fitness ( $n = 15$ ,  $r = -0.18$ ,  $P = 0.02$ ). There was also a negative correlation between survival to reproduce and May Size, but this was not statistically significant ( $n = 19$ ,  $r = -0.21$ ,  $P = 0.08$ ).

There was no significant interaction between early environment and cross treatment means for survival to flower or lifetime fitness, which indicates that the more stressful early environment treatments did not increase the expression of inbreeding depression (Table 1).

## Discussion

Our study found no evidence of historical purging in the *A. githago* population. Selfed individuals performed worse than individuals from within and near population crosses at all life history stages. If inbreeding is caused by the unmasking of deleterious recessive alleles, then theory suggests that selection should purge these alleles from the population and subsequently reduce inbreeding depression. Yet, in a review of 52

studies comparing inbreeding depression among plant populations with varied degrees of past inbreeding, Byers & Waller (1999) found that purging did not reliably reduce inbreeding depression. Although strong evidence for purging was found in some studies, results varied widely.

There are a number of reasons why selection may be ineffective at reducing inbreeding depression in a population. First, purging is ineffective when populations demonstrate extremely high rates of inbreeding because in these populations deleterious alleles become randomly fixed at a rate faster than natural selection can effectively act to remove them (Byers & Waller, 1999; Keller & Waller, 2002). The deleterious alleles in our focal population, however, have not yet been fixed, as demonstrated by our detection of inbreeding depression when comparing individuals outcrossed within the population relative to selfed individuals.

Secondly, while selection may be effective at purging deleterious recessive mutations of major effect from an inbred population, deleterious recessive mutations of minor effect are likely to be fixed through drift (Wang *et al.*, 1999; Carr & Dudash, 2003). Some research shows that inbreeding depression is primarily caused by these mutations of minor effect (Willis, 1999). From one highly outcrossed *Mimulus guttatus* source population, Willis (1999) conducted a crossing design that allowed him to compare inbreeding depression in a population that had been experimentally purged of mutations of major effect with a population that maintained mutations of both major and minor effect. There was little difference between the inbreeding depression expressed in these two populations, suggesting that inbreeding depression is primarily caused by mutations of minor effect. As we found that inbreeding depression was expressed relative to within population crosses, the alleles responsible for inbreeding depression in our focal population could not have been fixed. These mutations of minor effect still, however, may be responsible for the inbreeding depression we detected.

Finally, we cannot exclude the possibility that inbreeding depression in our focal population is caused at least in part by overdominance. Overdominance refers to a selective advantage for heterozygotes. When heterozygotes have a fitness advantage over homozygotes, inbreeding depression may be caused by homozygosity itself, instead of by the unmasking of deleterious recessive alleles in the homozygous state (Carr & Dudash, 2003). Whereas inbreeding depression caused by the unmasking of deleterious recessive alleles may be reduced over time through purging, inbreeding depression caused by overdominance increases over time as the frequency of homozygosity increases with inbreeding (Charlesworth & Charlesworth, 1990; Carr & Dudash, 2003). However, there is currently a consensus that overdominance does not play a large role in inbreeding depression, so this explanation for our lack

of purging seems unlikely (Charlesworth & Charlesworth, 1987; Willis, 1999; Waller *et al.*, 2008).

For our focal population, the benefits of outcrossing decreased with increasing distance from the maternal plant, with crosses with the far population resulting in outbreeding depression. When plants are crossed between populations, there is a trade-off between the benefit of the masking of deleterious recessive alleles and the cost of the breakup of locally adapted genotypes (Byers, 1998; Whitlock *et al.*, 2000; Hufford & Mazer, 2003). A model created by Lynch (1991) showed that the cost of the breakup of co-adapted gene complexes would need to be twice as great as the benefit of between-population dominance for outbreeding depression to be expressed in  $F_1$  crosses. For our focal population, this model suggests that the cost of outbreeding with the far population was severe.

This trend of a decreased benefit of outcrossing with increased distance from maternal plant can also give us insight into the underlying mutational load in our populations. Glémin *et al.* (2003) developed a model to investigate inbreeding depression and mutational load in subdivided populations. They suggest that measures of inbreeding depression within a population give us an estimate of the mutational load caused by segregating deleterious alleles. However, measures of heterosis between populations give us an estimate of the underlying 'drift load', which is the mutational load due to the fixation of deleterious alleles in small populations. The greater the heterosis, the greater the drift load is expected to be in the subdivided populations. As heterosis is greatest for our within population cross, this suggests that inbreeding depression in our focal population is primarily caused by segregating deleterious alleles that have not yet been fixed in the population. Glémin *et al.* (2003) acknowledge, however, that adaptation to local environmental conditions in each population should decrease the magnitude of heterosis, such that most estimates of drift load will be conservative.

The expression of inbreeding depression for individuals in EE1 declined across the life cycle, as there was a greater expression of inbreeding depression for seed weight than for May size. There are a number of explanations for this decline. First, there may be different alleles affecting the expression of inbreeding depression at different life history stages. Carr & Dudash (1995) examined the expression of inbreeding depression in *Mimulus guttatus* across multiple life history stages and found that inbreeding depression for early life history traits was not correlated with inbreeding depression expressed later in life. Similarly, in a review of the inbreeding depression literature, Husband & Schemske (1996) found that species that predominately self-fertilize most commonly express inbreeding depression later in the life cycle, whereas species that predominately outcross expressed inbreeding depression in a variety of life history stages. They interpret these results

to mean that inbreeding depression early in the life cycle is due to recessive lethals that can be easily purged from a population through selection, while inbreeding depression expressed later in life is primarily due to recessive deleterious mutations that are difficult to purge. Our results are characteristic of a population that has not yet purged the recessive lethals expressed early in the life cycle.

Alternatively, if inbreeding depression is caused in part by genes with pleiotropic effects throughout the life cycle, then a reduction in inbreeding depression over time could be indicative of within generation purging. Our study found that maternal lines with high levels of inbreeding depression at the seed stage show lower rates of survival. Individuals suffering the most from inbreeding were selectively removed at an early life stage. It remains unknown whether these same individuals, had they survived, would have increased the expression of inbreeding depression detected later in the life cycle.

We found no significant interaction between cross treatment and early environment, despite the fact that variance among maternal lines for inbreeding depression in lifetime fitness was significantly greater in the milder environmental treatments. This finding indicates that the expression of inbreeding depression was consistent across environments. Our results are in agreement with many other studies that found a stressful environment did not consistently increase the expression of inbreeding depression (Nason & Ellstrand, 1995; Mayer *et al.*, 1996; Laws *et al.*, 2010). For example, Waller *et al.* (2008) tested for changes in the expression of inbreeding depression for selfed seeds relative to cross-fertilized seeds of *Brassica rapa* under density and saline stresses. They observed inbreeding depression for a number of traits including survival and seed production, and both stress treatments independently reduced plant fitness. These stresses, however, did not increase the expression of inbreeding depression, and even reduced it for some traits such as flower number and height.

Still, many studies have found that the expression of inbreeding depression decreases under mild environmental conditions (Dudash, 1990; Hedrick & Kalinowski, 2000; Armbruster & Reed, 2005). For example, a harsh competitive environment greatly increased the expression of inbreeding depression in *Impatiens capensis* plants grown from selfed cleistogamous seed relative to those produced by outcrossed chasmogamous seed (Schmitt & Ehrhardt, 1990). Armbruster & Reed (2005) found that roughly half of the studies of inbreeding depression across environments of varying stress levels found a statistically significant increase in the expression of inbreeding depression in the more stressful environments. However, in nearly one quarter of the studies, inbreeding depression actually decreased in the more stressful environments. Contrary to what theory



might predict, a stressful environment does not consistently impact the expression of inbreeding depression.

In our study, the absence of an environmental impact on the expression of inbreeding depression in different cross treatments may reflect the fact that the mild environment treatments were restricted to early life stages and all individuals shared a harsh late-life environment. An alternative explanation is that there is relatively little plasticity in the expression of inbreeding depression in *A. githago*. Still, we cannot rule out the possibility that a different early environmental stress or more extreme stress might have increased the expression of inbreeding depression. Bijlsma *et al.* (1999) found that inbreeding depression in *Drosophila melanogaster* that had been experimentally purged under favourable environmental conditions was again expressed under specific stressful conditions. Not all stressful conditions, however, resulted in an increased expression of inbreeding depression. Although heat and ethanol treatments both resulted in decreased survival, only the heat treatment resulted in a statistically significant increase in the expression of inbreeding depression. In addition, two recent meta-analyses found that the severity of the stress imposed resulted in a linear increase in the expression of inbreeding depression observed (Fox & Reed, 2011; Enders & Nunney, 2012; Reed *et al.*, 2012). These studies suggest that it is possible that we could have found an increase in inbreeding depression under the more stressful field environment if the conditions in our field had been harsher.

In summary, our study examined the history of purging in a focal population of *A. githago*, as well as inbreeding depression expressed across the life cycle and under different early environmental treatments. It avoided potentially conflating fixation and purging by comparing multiple levels of outcrosses (within, near, and far) within maternal lines. We found that purging had not occurred in our focal population, inbreeding depression declined over the life cycle, and early environment impacted the variance of inbreeding depression expressed within maternal lines, but had no impact on the expression of inbreeding depression in different cross treatments. Our research underscores the need for further studies in which natural selection and inbreeding depression are monitored across the life cycle in natural environments. As the plants in this experiment flowered in the field, it was not feasible to control pollination to examine the levels of inbreeding in subsequent generations. Linking intra-generational patterns of natural selection with observations of the genetic load across generations remains a relatively unexplored area of study. In addition, there is much work to be done in identifying the alleles responsible for the expression of inbreeding depression at different life stages and in different environments. Continued research into these topics is critical because an understanding of the roles life history stage and environment play in determining both

the short and long-term selective consequences for inbred phenotypes is of paramount importance for small populations and threatened species.

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## References

- Agren, J. & Schemske, D.W. 1993. Outcrossing rate and inbreeding depression in two annual monoecious herbs, *Begonia hirsuta* and *B. semiovata*. *Evolution* **47**: 125–135.
- Agren, J. & Schemske, D.W. 1994. Evolution of trichome number in a naturalized population of *Brassica rapa*. *Am. Nat.* **143**: 1–13.
- Armbruster, P. & Reed, D.H. 2005. Inbreeding depression in benign and stressful environments. *Heredity* **95**: 235–242.
- Ashman, T.L. 1992. The relative importance of inbreeding and maternal sex in determining progeny fitness in *Sidalcea oregana* Ssp *spicata*, a gynodioecious plant. *Evolution* **46**: 1862–1874.
- Barrett, S.C.H. & Charlesworth, D. 1991. Effects of a change in the level of inbreeding on the genetic load. *Nature* **352**: 522–524.
- Bennington, C.C. & McGraw, J.B. 1995. Phenotypic selection in an artificial population of *Impatiens pallida*: the importance of the invisible fraction. *Evolution* **49**: 317–324.
- Bijlsma, R., Bundgaard, J. & Van Putten, W.F. 1999. Environmental dependence of inbreeding depression and purging in *Drosophila melanogaster*. *J. Evol. Bio.* **12**: 1125–1137.
- Byers, D.L. 1998. Effect of cross proximity on progeny fitness in a rare and a common species of *Eupatorium* (Asteraceae). *Am. J. Bot.* **85**: 644–653.
- Byers, D.L. & Waller, D.M. 1999. Do plant populations purge their genetic load? Effects of population size and mating history on inbreeding depression. *Annu. Rev. Ecol. Syst.* **30**: 479–513.
- Carr, D.E. & Dudash, M.R. 1995. Inbreeding depression under a competitive regime in *Mimulus guttatus* - consequences for potential male and female function. *Heredity* **75**: 437–445.
- Carr, D.E. & Dudash, M.R. 2003. Recent approaches into the genetic basis of inbreeding depression in plants. *Phil. Trans. R. Soc. Lond. B.* **358**: 1071–1084.
- Charlesworth, D. & Charlesworth, B. 1987. Inbreeding depression and its evolutionary consequences. *Annu. Rev. Ecol. Syst.* **18**: 237–268.
- Charlesworth, D. & Charlesworth, B. 1990. Inbreeding depression with heterozygote advantage and its effect on selection for modifiers changing the outcrossing rate. *Evolution* **44**: 870–888.

- Donohue, K. 2002. Germination timing influences natural selection on life-history characters in *Arabidopsis thaliana*. *Ecology* **83**: 1006–1016.
- Dudash, M.R. 1990. Relative fitness of selfed and outcrossed progeny in a self-compatible, protandrous species, *Sabatia angularis* L. (Gentianaceae): a comparison in three environments. *Evolution* **44**: 1129–1139.
- Dudash, M.R., Carr, D.E. & Fenster, C.B. 1997. Five generations of enforced selfing and outcrossing in *Mimulus guttatus*: inbreeding depression variation at the population and family level. *Evolution* **51**: 54–65.
- Enders, L.S. & Nunney, L. 2012. Seasonal stress drives predictable changes in inbreeding depression in field-tested captive populations of *Drosophila melanogaster*. *Proc. R. Soc. B.* **279**: 3756–3764.
- Firbank, L.G. 1988. *Agrostemma githago* L. *J. Ecol.* **76**: 1232–1246.
- Fisher, R.A. 1965. *The theory of inbreeding*, 2nd edn. Academic Press, New York, NY.
- Fox, C.W. & Reed, D.H. 2011. Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. *Evolution* **65**: 246–258.
- Glémin, S., Ronfort, J. & Bataillon, T. 2003. Patterns of inbreeding depression and architecture of the load in subdivided populations. *Genetics* **165**: 2193–2212.
- Goodrich, S. H. 2009. The consequences of early life environment on phenotype and selection across the life cycle in *Agrostemma githago* (Ph.D. diss). Univ. of Virginia.
- Grueter, C.E., Laws, R.J., Nakagawa, S. & Jamieson, I.G. 2010. Inbreeding depression accumulation across life-history stages of the endangered Takahe. *Conserv. Biol.* **24**: 1617–1625.
- Hedrick, P.W. & Kalinowski, S.T. 2000. Inbreeding depression in conservation biology. *Annu. Rev. Ecol. Syst.* **31**: 139–162.
- Holzner, W. & Numata, M. 1982. *Biology and ecology of weeds*. Dr. W. Junk Publishers, Boston, MA.
- Hufford, K.M. & Mazer, S.M. 2003. Plant ecotypes: genetic differentiation in the age of ecological restoration. *Trends Ecol. Evol.* **18**: 147–155.
- Hunt, R. & Cornelissen, J.H.C. 1997. Components of relative growth rate and their interrelations in 59 temperate plant species. *New Phytol.* **135**: 395–417.
- Husband, B.C. & Schemske, D.W. 1996. Evolution of the magnitude and timing of inbreeding depression in plants. *Evolution* **50**: 54–70.
- Jurgens, A. 2006. Comparative floral morphometrics in day-flowering, night-flowering and self-pollinated Caryophyllaceae (*Agrostemma*, *Dianthus*, *Saponaria*, *Silene*, and *Vaccaria*). *Plant Syst. Evol.* **257**: 233–250.
- Jurgens, A., Witt, T. & Gottsberger, G. 2002. Pollen grain numbers, ovule numbers and pollen-ovule ratios in Caryophyllaceae: correlation with breeding system, pollination, life form, style number, and sexual system. *Sex. Plant Reprod.* **14**: 279–289.
- Keller, L.F. & Waller, D.M. 2002. Inbreeding effects in wild populations. *Trends in Ecol. Evol.* **17**: 230–241.
- Koelwijn, H.P. 1998. Effects of different levels of inbreeding on progeny fitness in *Plantago coronopus*. *Evolution* **52**: 692–702.
- Kristensen, T.N., Loeschcke, V. & Hoffmann, A.A. 2008. Linking inbreeding effects in captive populations with fitness in the wild: release of replicated *Drosophila melanogaster* lines under different temperatures. *Conserv. Biol.* **22**: 189–199.
- Lande, R. & Schemske, D.W. 1985. The evolution of self-fertilization and inbreeding depression in plants. I. *Genetic models. Evolution* **39**: 24–40.
- Laws, R.J., Townsend, S.M., Nakagawa, S. & Jamieson, I.G. 2010. Limited inbreeding depression in a bottlenecked population is age but not environment dependent. *J. Avian Biol.* **41**: 645–652.
- Lynch, M. 1991. The genetic interpretation of inbreeding depression and outbreeding depression. *Evolution* **45**: 622–629.
- Mayer, S.S., Charlesworth, D. & Meyers, B. 1996. Inbreeding depression in four populations of *Collinsia heterophylla* Nutt (Scrophulariaceae). *Evolution* **50**: 879–891.
- McCall, C., Waller, D.M. & Mitchell-Olds, T. 1994. Effects of serial inbreeding on fitness components in *Impatiens capensis*. *Evolution* **48**: 818–827.
- Metcalfe, N.B. & Monaghan, P. 2001. Compensation for a bad start: grow now, pay later? *Trends Ecol. Evol.* **16**: 254–260.
- Miller, S. 2003. *Pest Fact Sheet: Agrostemma githago*. North American Plant Protection Organization, Ontario.
- Nason, J.D. & Ellstrand, N.C. 1995. Lifetime estimates of biparental inbreeding depression in the self-incompatible annual plant *Raphanus sativus*. *Evolution* **49**: 307–316.
- Reed, D.H., Fox, C.W., Enders, L.S. & Kristensen, T.N. 2012. Inbreeding-stress interactions: evolutionary and conservation consequences. *Ann. N.Y. Acad. Sci.* **1256**: 33–48.
- SAS Institute. 2000. *SAS 8.1: Users' Guide*. SAS Institute, Cary, North Carolina, USA.
- Schmitt, J. & Ehrhardt, D.W. 1990. Enhancement of inbreeding depression by dominance and suppression in *Impatiens capensis*. *Evolution* **44**: 269–278.
- Sokal, R.R. & Rohlf, F.J. 1995. *Biometry: the principles and practice of statistics in biological research*, 3rd edn. W.H. Freeman and Co., New York, NY.
- Swindell, W.R. & Bouzat, J.L. 2006. Selection and inbreeding depression: effects of inbreeding rate and inbreeding environment. *Evolution* **60**: 1014–1022.
- Templeton, A.R. & Read, B. 1984. Factors eliminating inbreeding depression in a captive herd of spekes gazelle (*Gazella spekei*). *Zoo Biol.* **3**: 177–199.
- Thompson, P.A. 1973. The effects of cultivation on the germination character of the corn cockle (*Agrostemma githago*). *Ann. Bot.-London.* **37**: 133–154.
- Waller, D.M., Dole, J. & Bersch, A.J. 2008. Effects of stress and phenotypic variation on inbreeding depression in *Brassica rapa*. *Evolution* **62**: 917–931.
- Wang, J., Hill, W.G., Charlesworth, D. & Charlesworth, B. 1999. Dynamics of inbreeding depression due to deleterious mutations in small populations: mutation parameters and inbreeding rate. *Genet. Res., Camb.* **74**: 165–178.
- Whitlock, M.C., Ingvarsson, P.K. & Hatfield, T. 2000. Local drift load and the heterosis of interconnected populations. *Heredity* **84**: 452–457.
- Willis, J.H. 1999. The role of genes of large effect on inbreeding depression in *Mimulus guttatus*. *Evolution* **53**: 1678–1691.
- Ziehe, M. & Roberds, J.H. 1989. Inbreeding depression due to overdominance in partially self-fertilizing plant populations. *Genetics* **121**: 861–868.

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