EXPERIMENTAL EVOLUTION OF THE GENETIC LOAD AND ITS IMPLICATIONS FOR THE GENETIC BASIS OF INBREEDING DEPRESSION

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The degree to which, and rapidity with which, inbreeding depression can be purged from a population has important implications for conservation biology, captive breeding practices, and invasive species biology. The degree and rate of purging also informs us regarding the genetic mechanisms underlying inbreeding depression. We examine the evolution of mean survival and inbreeding depression in survival following serial inbreeding in a seed-feeding beetle, *Stator limbatus*, which shows substantial inbreeding depression at all stages of development. We created two replicate serially inbred populations perpetuated by full-sib matings and paired with outbred controls. The genetic load for the probability that an egg produces an adult was purged at ~0.45–0.50 lethal equivalents/generation, a reduction of more than half after only three generations of sib-mating. After serial inbreeding we outcrossed all beetles then measured (1) larval survival of outcrossed beetles and (2) inbreeding depression. Survival of outcrossed beetles evolved to be higher in the serially inbred populations for all periods of development. Inbreeding depression and the genetic load were significantly lower in the serially inbred than control populations. Inbreeding depression affecting larval survival of *S. limbatus* is largely due to recessive deleterious alleles of large effect that can be rapidly purged from a population by serial sib-mating. However, the effectiveness of purging varied among the periods of egg/larval survival and likely varies among other unstudied fitness components. This study presents novel results showing rapid and extensive purging of the genetic load, specifically a reduction of as much as 72% in only three generations of sib-mating. However, the high rate of extinction of inbred lines, despite the lines being reared in a benign laboratory environment, indicates that intentional purging of the genetic load of captive endangered species will not be practical due to high rates of subpopulation extinction.

KEY WORDS: Genetic load, inbreeding load, sib-mating, *Stator limbatus*.

Inbreeding reduces fitness in a majority of plants and animals for which it has been studied (Darwin 1892; Wright 1977; Lande and Schemske 1985; Schemske and Lande 1985; Charlesworth and Charlesworth 1987; Hedrick and Kalinowski 2000). Inbreeding depression, and the extent to which it can be purged from a population, have important implications for conservation biology, captive breeding practices, and invasive species biology. The extent of purging and its rapidity will impact the persistence times of consistently or transiently small populations by influenc-ing the ability of these populations to "shrug off" the effects of inbreeding depression and either remain near-carrying capacity (for naturally small populations) or grow rapidly after the bot-tleneck (avoiding the "extinction vortex") (O'Grady et al. 2006; Reed 2008). For captive breeding programs or domestic species, controlled inbreeding could theoretically be used to purge the genetic load and produce captive populations with higher mean fitness than the outbred populations from which they were formed (Templeton and Read 1984), although this is controversial (Ballou

1997; Willis and Wiese 1997; Kalinowski et al. 2000; Hedrick and Kalinowski 2000). The effectiveness of purging could also influence the ability of introduced species to recover from the initial small founding sizes and eventually become invasive (Barrett et al. 2008). However, the degree to which, and rapidity with which, inbreeding depression can be purged from a population is a topic of substantial debate.

Inbreeding depression is a consequence of an increase in homozygosity across the genome (Kristensen and Sorensen 2005). Two different mechanisms are responsible for the relationship between increased homozygosity and inbreeding depression (Deng 1998; Charlesworth and Charlesworth 1999; Crnokrak and Barrett 2002; Roff 2002; Carr and Dudash 2003; Kristensen and Sorensen 2005). For some traits in some organisms, heterozygotes have higher fitness than do homozygotes (overdominance) such that the reduction in mean heterozygosity caused by inbreeding reduces fitness (Karkkainen et al. 1999; Charlesworth and Hughes 2000; Crnokrak and Barrett 2002; Weeks 2004). However, the primary mechanism causing inbreeding depression appears to be the increased expression of deleterious recessive alleles that are partially masked by dominant alleles in outbred, and thus generally heterozygous, individuals (Carr and Dudash 2003). These alleles are more often homozygous (and thus expressed) in inbred individuals. These deleterious mutations are maintained in an outbreeding population at a frequency determined by the selection–mutation balance and represent the genetic load (mutational or segregating load) of the population.

The degree and rate of purging of inbreeding depression depends on the genetic mechanism underlying inbreeding depression. Thus, experimental studies measuring rates of purging of inbreeding depression can inform us about the genetic mechanism underlying inbreeding depression. If inbreeding depression is due to recessive or partially recessive deleterious alleles, then serial-inbreeding could increase the efficiency of selection against these alleles because of the increase in homozygosity that results from inbreeding. The resulting population would be expected to have a reduction in its genetic load, a reduction in inbreeding depression, and possibly an increase in mean fitness (Crow and Kimura 1970; Husband and Schemske 1996; Charlesworth and Charlesworth 1999; Reed and Bryant 2001). This theoretical result—that serial-inbreeding can increase mean population fitness—is what gives rise to the suggestion that captive populations can have their genetic loads purged through intentional inbreeding, improving the mean fitness of these populations (Templeton and Read 1984; review in Hedrick and Kalinowski 2000). Purging of deleterious alleles via inbreeding is often observed in captive animal populations, but its impact is relatively small and highly variable (Ballou 1997; Boakes et al. 2007).

Purging has been more clearly demonstrated in laboratory experiments (Charlesworth and Charlesworth 1999; Crnokrak and

Barrett 2002). However, the degree to which, and rapidity with which, recessive deleterious alleles can be purged from a population is a topic of substantial debate (Glémin 2003; Jamieson et al. 2006). This is partly because many studies have failed to observe purging in serially inbred populations (Byers and Waller 1999; Carr and Dudash 2003; Pico et al. 2007; Leberg and Firmin 2008), because the magnitude of purging can vary substantially among species (Lacy and Ballou 1998) and even among families within populations (Dudash et al. 1997), and because highly inbred populations may go extinct before their genetic loads can be purged (Frankham et al. 2001; Reed et al. 2003b; O'Grady et al. 2006). Also, theory tells us that deleterious alleles of small effect are under too weak of selection, and experience too much drift, to be easily purged by inbreeding (Lynch et al. 1995; Glémin 2003) such that purging of deleterious alleles could be too slow to be of significant conservation value (Charlesworth and Charlesworth 1987, 1999; Hedrick 1994; Wang et al. 1999; Wang 2000), especially in small populations where alleles are being purged primarily by drift (Crow and Kimura 1970; Glémin 2003). In contrast, deleterious alleles of large effect (including lethal alleles) should be purged very quickly from a serially inbred population (Lande and Schemske 1985; Charlesworth et al. 1990; Hedrick 1994; Wang et al. 1999; Kirkpatrick and Jarne 2000), although only if population sizes are large enough that drift does not swamp selection (Glémin 2003). It is still generally unknown to what extent these two types of alleles (those of small versus large effect) contribute the most to the inbreeding depression observed in natural populations (Dudash and Carr 1998).

To accurately assess the effectiveness of inbreeding in purging deleterious recessives, and to detect recessive deleterious alleles of large effect, most studies either track changes in mean fitness of inbred lines following sequential generations of inbreeding (Saccheri et al. 1996; Lacy and Ballou 1998) or they outbreed serially inbred lines at the end of the experiment and compare mean population fitness with ancestral or control population fitness (Barrett and Charlesworth 1991), or both. The meta-analysis of Crnokrak and Barrett (2002) suggests that rebounds in fitness following sequential inbreeding are often the result of adaptation to laboratory/experimental conditions (Latter and Mulley 1995). Thus, unless purging experiments compare directly to an outbred control (rather than initial population fitness) any increases in fitness that occur relative to the ancestral population may be due to laboratory adaptation independent of inbreeding effects. To accurately measure the effect of purging on the genetic load it is thus necessary to both (1) outbreed a population following serial inbreeding and measure the change in population mean fitness in serially inbred lines relative to control (outbred) lines (Dudash et al. 1997; Roff 2002) and (2) quantify the degree to which inbreeding depression has declined due to purging by reinbreeding the population following at least one

generation of outcrossing (Charlesworth and Charlesworth 1999; Willis 1999c), with both control and serially inbred populations reared and tested simultaneously (to control for laboratory adaptation). Few studies meet this ideal (Crnokrak and Barrett 2002). This current study improves upon the previous work by maintaining inbred and outbred lines simultaneously and controlling for adaptation to the laboratory environment.

Here we examine the evolution of both mean population fitness and inbreeding depression following serial inbreeding in a seed-feeding beetle, *Stator limbatus* (Coleoptera: Chrysomelidae: Bruchinae). We created replicate serially inbred populations, and followed the change in genetic load through four (replicate A) or three (replicate B) generations of sib-mating. At the end of the experiment we outcrossed all beetles, then measured outcrossed population mean survival and inbreeding depression (analogous to the experimental design of Willis 1999c). We test two hypotheses that will be true if alleles of large effect contribute to inbreeding depression in *S. limbatus*. First, because deleterious alleles are being purged from our serially inbred populations more efficiently than in the control populations [those of effect size larger than $1/(2N_e)$](Kristensen and Sorensen 2005), the fitness of outbred beetles will be greater in our serially inbred populations (at the end of the experiment) than in our control populations. Second, because the alleles that are being removed by selection at a faster rate from the serially inbred populations than the control populations are recessive (dominant and strictly additive alleles are expressed equally in both populations) the magnitude of inbreeding depression should evolve in the serially inbred populations to be significantly lower than inbreeding depression in the control populations.

Methods

THE BIOLOGY OF *STATOR LIMBATUS*

Stator limbatus is a seed-feeding beetle native to the southwestern United States and distributed through dry forests and deserts all the way south to northern South America (Johnson and Kingsolver 1976; Johnson et al. 1989; Morse and Farrell 2005a,b). Its entire life cycle takes place on or near seeds. Eggs are glued to the surface of host seeds and larvae complete development inside a single seed, emerging after pupation as adults. Adults reproduce using larval resources; they require neither food nor water making them a very practical laboratory model. *Stator limbatus* uses >70 host species throughout its large geographic range. For these experiments we raised beetles on seeds of *Albizia julibrissin*. *Albizia julibrissin* is not a native host; it is invasive in the United States, but is closely related to a native host. It is readily colonized by beetles in the field and is a better host for beetles (i.e., beetles have lower mortality) than many of their native hosts. Also, we can purchase large supplies of seeds from horticultural suppliers.

Inbreeding depression has been detected in every fitness component measured in *S. limbatus*. Eggs from full-sib matings are 22–41% less likely to produce an adult offspring (depending on the population) than are eggs from outbred matings (Fox and Scheibly 2006; Fox et al. 2007). This effect of inbreeding on survival occurs across all stages of development—eggs produced by sib-mated parents are 0–8% less likely to develop, 10–21% less likely to hatch, and larvae hatching from these eggs are 11–27% less likely to survive to adult (Fox and Scheibly 2006). Although inbred offspring mature at the same size as outbred offspring, they develop more slowly (larval development time is extended by ∼5%) and have ∼5% shorter adult life spans (Fox et al. 2006).

The study population of *S. limbatus* used here was collected along Mount Lemmon Hwy in Oracle, Pinal Co., Arizona, USA (32.61°N 110.77°W) from >20 *Acacia greggii* trees as larvae inside of seeds. Beetles emerging from these field-collected seeds were used to establish a laboratory colony (>200 beetles) that was maintained for six generations in the laboratory at >100 families per generation at 26–28°C, light:dark 15:9, prior to this experiment.

EXPERIMENTAL DESIGN

Our experimental design is illustrated in Figure 1. To initiate the lines we used a "block" design (Fig. 2). Each block was created by randomly pairing two families chosen from a large outbred
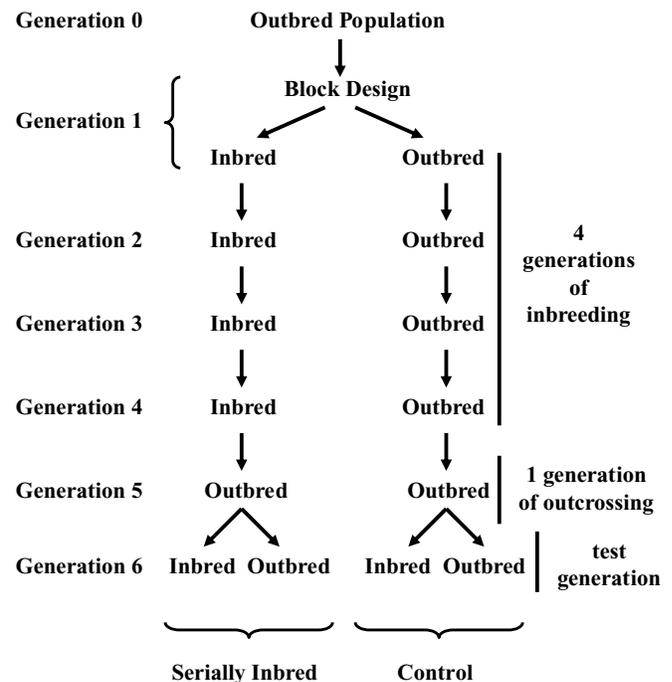
Figure 1. Design of the purging experiment. The generation numbers are as in replicate A for which beetles were serially inbred for four generations. Replicate B was serially inbred for only three generations.

Family A   Family B

Outbred   ♀ ⟷ ♂

Outbred   ♂ ⟷ ♀
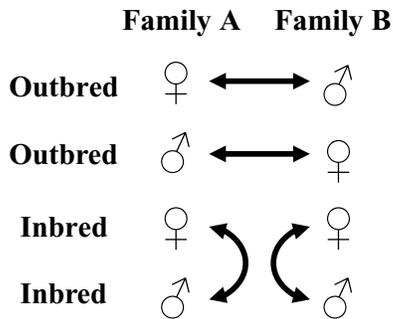
Inbred   ♀ ⤵ ⤴ ♀

Inbred   ♂ ⤴ ⤵ ♂

Figure 2. The block design used to create the serially inbred and control lines in generation 1 and used to measure inbreeding depression at the end of the experiment. Each block is created by crossing beetles from two unrelated families, creating two outbred matings (reciprocal crosses between the two families) and two inbred matings (crosses between full-siblings within each family).

population. From each family we randomly chose two female and two male offspring. We then crossed these two families as shown in Figure 2, creating two inbred and two outbred families. Eggs produced by these pairs are considered Generation 1 (Fig. 1; the outcrossed population from which families were chosen is generation 0). The advantage of this design is that it minimizes error in sampling alleles during line creation; all families contribute exactly the same number of offspring to the serially inbred and control populations. The effective population size at the start of the experiment is the same for both types of lines. The two inbred families became the "serially inbred" population and the two outbred matings became the "control" population.

Control populations

Pairs were confined in a 35-mm petri dish with 10 *A. julibrissin* seeds and allowed to lay >10 eggs. We randomly chose two eggs (generations 1–2 and 5–6) or three eggs (generation 3–4) per pair to produce offspring for the next generation. Larvae were allowed to develop at one egg per seed (excess eggs were scraped from the seed) and one seed per petri dish inside a temperature and photoperiod controlled growth chamber at 26°C, light:dark 15:9. Upon the emergence as an adult, offspring from these control population eggs were assigned a random nonsibling as a mate, confined in another 35-mm dish with 10 seeds and allowed to lay eggs. Beetles were mated at approximately the same age as serially inbred population beetles to ensure that age effects could not generate line differences (see below).

Serially inbred populations

The serially inbred populations were maintained similar to the control populations except that (1) females were paired with a sibling male (rather than a randomly chosen nonsibling) and (2) we raised offspring from 10 eggs per mated pair per generation

(rather than 3) because mortality was high (due to inbreeding depression) and families needed to produce at least one male and one female to create a sib-mating. To avoid inadvertent within-family selection on development time we allowed all offspring within the family to mature as adults before pairing one randomly chosen sister with a randomly chosen brother to create a sib-mated pair. The most significant problem we had in the serially inbred populations was families that produced offspring of only one sex. The absence of the opposite sex in some families was expected due to demographic stochasticity, exacerbated by inbreeding depression on egg-to-adult survival. To minimize loss of families we created a back-up sib-mating for each family. When our primary sib-family produced offspring of only one sex we substituted our back-up family for the extinct family. This back-up family was created in the same way as the primary family, from the same lineage, and shares the same inbreeding coefficient. This design reduces the magnitude of selection among families (by reducing lineage extinction) but increases the magnitude of selection within families, within the serially inbred treatment. This should reduce the effectiveness of selection in removing deleterious alleles (Wang 2000). However, we deemed it necessary as many families would have gone extinct despite moderately high survival within the family. For example, 12.5% of families (per generation) are expected to produce single-sex broods when four of the 10 offspring we raised survive to reproductive age in that family. Even with our protocol of using replacement families, inbreeding-related mortality was so high that 63% of lineages in the serially inbred population went extinct through the four generations of inbreeding in replicate A, and 60% went extinct in the three generations of replicate B.

Replication of the experiment

The experiment was replicated twice (called replicates A and B). Replicate A was initiated with 77 blocks (producing 142 outbred and 135 inbred families) and replicate B with 90 blocks (161 outbred and 173 inbred families) (the difference in family numbers reflects the failure of some females to lay eggs). The two replicates were otherwise maintained identically except that replicate B was started one generation after replicate A and was only inbred for three generations (rather than four). Replicates A and B were necessarily maintained in different growth chambers but, within each replicate, the serially inbred and control populations were maintained interspersed within a single growth chamber. Trays were rotated daily. Thus, differences between treatments within a replicate cannot be due to growth-chamber effects, but differences between replicates can be.

Population assessments after serial inbreeding

Following four (replicate A) or three (replicate B) generations of inbreeding we outbred all beetles in all lines for one generation (generation 5 for replicate A and generation 4 for replicate B;

Fig. 1), after which we paired families in blocks (as at the start of the experiment) to measure (1) mean fitness of serially inbred versus control populations when outbred, and (2) the magnitude of inbreeding depression in the serially inbred and control populations. We scored mortality, egg-to-adult development time, and adult longevity for both the control and serially inbred populations (this is the generation labeled "Generation 6" in Fig. 1). Matings for the fitness measures were created exactly as at the start of the experiment (Fig. 2). Females were confined with 10 seeds of *A. julibrissin* in a 35-mm petri dish and allowed to lay 8–10 eggs. Dishes were checked every 12 h for eggs. All seeds-bearing eggs were transferred to a new dish (one seed per dish). Larvae were raised to adult at one egg per seed, as in the previous generations. We checked for emerging beetles every 12 h (to estimate egg-to-adult development time). Emerging beetles were confined alone in a clean 35-mm Petri dish and checked every 12 h until death (to measure virgin adult life span). All eggs were classified to one of four fates; failed to develop, developed but did not hatch (a developing larva/embryo was visible inside the clear egg), hatched but did not emerge as an adult, or survived to adult. Our previous study (Fox and Scheibly 2006) showed that body size is not influenced by inbreeding, so we did not measure body mass in this experiment.

ANALYSES

Blocks (as depicted in Fig. 2) are the lowest level of independence in this design and thus block means were used in all analyses at both the start and end of the experiment. Each block contains four means, one for each sex × treatment combination (inbred male offspring, outbred males, inbred females, and outbred females). Block means were calculated first by averaging across offspring within a family and then by averaging between families within the block. Population means were calculated by averaging across blocks for each sex-by-treatment combination.

For each trait we calculated the proportional reduction in fitness due to inbreeding depression. For survival estimates and adult life span this is $\delta = (\text{Mean}_{outbred} - \text{Mean}_{inbred})/\text{Mean}_{outbred}$, the proportional reduction in survival or life span, a standard measure of inbreeding depression (Fox 2005). For development time, $\delta = (\text{Mean}_{inbred} - \text{Mean}_{outbred})/\text{Mean}_{outbred}$ (the proportional increase in development time). δ was calculated separately for each block. We then used nonparametric analyses (nonparametric analysis of variance and Wilcoxon signed-rank tests) to test the hypotheses that δ differed from zero, differed between replicates (A vs. B), and differed between treatments (serially inbred vs. control). In the tables we present standard errors for all estimates of δ. However, δ is a ratio and thus very sensitive to error in the estimation of the denominator and thus standard errors should be interpreted with caution.

The genetic load (inbreeding load) for genes affecting egg development, egg hatch, hatch-to-adult survival, and the proportion of eggs producing an adult offspring were estimated as

$$L_{Survival} = \frac{-[\ln(Survival_{inbred}) - \ln(Survival_{outbred})]}{f} \quad (1)$$

(Lynch and Walsh 1998), where f is the inbreeding coefficient. At the start and end of the experiment, inbreeding load was calculated separately for each block (Fig. 2; $f = 0.25$ for sib-matings) and then averaged across blocks within a replicate/treatment. However, although the block design was used for creating the serially inbred and control populations, it was not used for generations 2–4. Thus, to quantify the change in inbreeding load during serial inbreeding we used the survival of beetles in the control populations as our estimate of outbred fitness. Inbreeding load was calculated separately for each family, relative to mean outbred survival, then averaged across families ($f = 0.375, 0.50$, and 0.594 in generations 2, 3, and 4, respectively).

Results

INBREEDING DEPRESSION IN GENERATION 1

In our first generation of inbreeding we observed significant inbreeding depression in the proportion of eggs that developed ($\delta = 0.02$–0.05), the proportion of developed eggs that hatched ($\delta = 0.14$), and larval hatch-to-adult survival ($\delta = 0.13$–0.14) (Wilcoxon signed-rank tests comparing inbred vs. outbred means with each block treated as a single datapoint; $P < 0.002$ for each; the sign test for $\delta > 0$ was also significant for every trait, $P < 0.001$ for each). Combining all three periods of mortality, eggs produced from sib-matings were 26% less likely to produce an adult offspring than were eggs from outbred matings (Table 1). There was no evidence that inbreeding depression at the start of the experiment differed between replicates A and B for any measure of mortality (Table 1; Mann–Whitney U-tests; $\chi_1^2 < 0.37$, $P > 0.54$ for all traits).

EVOLUTION OF INBREEDING LOAD WITH SERIAL INBREEDING

Mortality during all three periods of development (egg development, egg hatch, and the larval period) stayed fairly constant in the control populations through the first four generations of the experiment—there was no detectable heterogeneity among generations for any of the three periods of mortality for either replicate A or B (nonparametric analysis of variance on family means; $\chi_3^2 < 3.4$, $P > 0.34$ [replicate A] and $\chi_2^2 < 2.6$, $P > 0.28$ [replicate B] for all periods of mortality) except for the probability of egg hatch in Replicate B for which there was significant (although small) variation among generations ($\chi_2^2 = 7.2$, $P > 0.03$ (online Supplementary Fig. S1).

Table 1. The inbreeding load (*L*) and magnitude of inbreeding depression (δ) for the proportion of eggs developing, egg hatch, larval hatch-to-adult survival, and the total proportion of eggs producing an adult offspring, for generation 1 of the purging experiment. These estimates were calculated by first calculating *L* or δ for each block and then averaging across blocks within a replicate; they are not calculated from the overall treatment means. Because both *L* and δ are ratios, the standard errors should be interpreted with caution (i.e., neither *L* nor δ are normally distributed). All estimates are significantly greater than 0 (Sign test, *P*<0.001 for all tests), including egg development for Replicate A (the low estimates are due to three blocks that that had very low egg development for outbreds but have little effect on the nonparametric analysis).

Trait	Inbreeding load *L*		Inbreeding depression δ[1]	
	Replicate A	Replicate B	Replicate A	Replicate B
Egg development	0.20±0.11	0.30±0.11	0.02±0.03	0.05±0.02
Egg hatch	0.70±0.11	0.73±0.10	0.14±0.02	0.14±0.02
Larval hatch-to-adult survival	0.65±0.11	0.71±0.12	0.13±0.02	0.14±0.03
Proportion of eggs producing an adult	1.55±0.19	1.60±0.20	0.26±0.03	0.26±0.04
N	76 blocks[2]	90 blocks[2]	76 blocks[2]	90 blocks[2]

[1]δ has an expectation of 0 when there is no inbreeding depression, and can vary from positive to negative depending on whether inbreds have lower or higher performance than outbreds.

[2]Each block consists of two inbred and two outbred families; see Figure 2.

In contrast, in the serially inbred populations there was significant heterogeneity in mortality among generations for both egg hatch and larval hatch-to-adult survival ($\chi^2_3 > 8.2$, $P < 0.05$ for both traits in both replicates) but not for the probability that an egg developed ($P > 0.11$ in both replicates) (online Supplementary Fig. S1). Variation in larval mortality among generations is expected in the serially inbred populations because the inbreeding coefficient is increasing in each generation (inbreeding coefficient, $f = 0.25, 0.375, 0.50$, and 0.594 for generations 1, 2,

3, and 4, respectively). If deleterious alleles are being removed by selection more efficiently from the serially inbred populations then we expect the estimated inbreeding load to decrease through the experiment. Using the control populations as our measure of outbred beetle survival, we calculated the inbreeding load each generation for both the replicates. Consistent with the predictions of purging of deleterious recessive alleles, the inbreeding load decreased significantly for egg hatch and larval hatch-to-adult survival (Fig. 3B, C; analysis of covariance, $F > 24.6$, $P < 0.001$
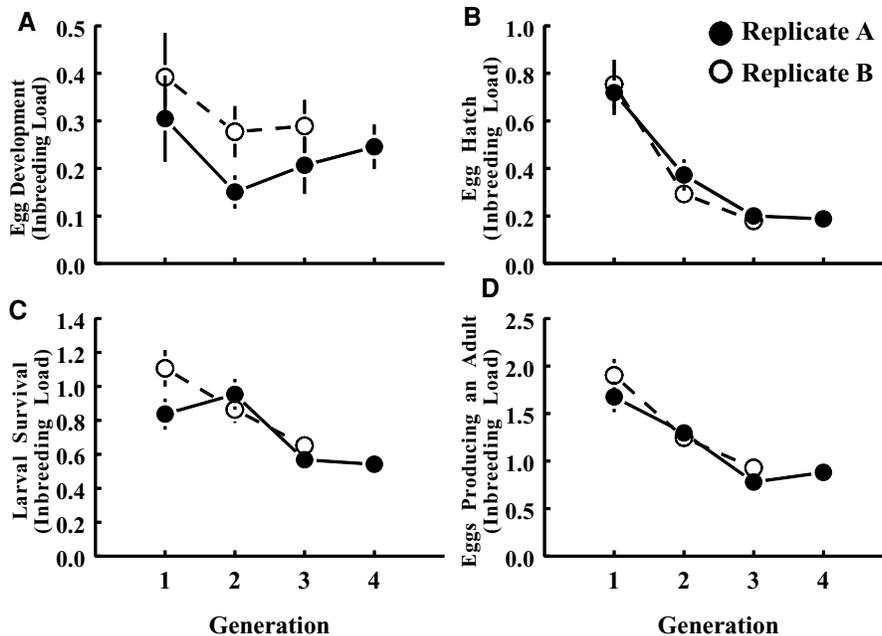
Figure 3. The evolution of the genetic load (inbreeding load) in serially inbred populations of *Stator limbatus* over four generations (replicate A; ●) or three generations (replicate B; ○) of full-sib mating.

for both traits). However, the inbreeding load did not decrease for the proportion of eggs that developed (Fig. 3A; $F < 1.5, P > 0.22$). This evolution of inbreeding load was particularly striking for egg hatch—L for egg hatch declined more than 72% by Generation 3 (Fig. 3D). This is a decline of 0.26 ± 0.05 lethal equivalents per generation in replicate A, and 0.31 ± 0.05 per generation in replicate B. The proportional decline in L for larval hatch-to-adult survival was less substantial, 32 and 41% (replicates A and B, respectively) over the first three generations (Fig. 3B), equivalent to 0.13 ± 0.06 lethal equivalents per generation in replicate A and 0.23 ± 0.06 in replicate B. These significant changes in inbreeding load led to an overall reduction in L of >51% for the proportion of eggs that produced an adult offspring, from 1.7 and 1.9 lethal equivalents (replicates A and B, respectively) to less than 0.93 lethal equivalents, in just three generations of sib-mating. This is a decline of 0.45 ± 0.10 and 0.50 ± 0.10 lethal equivalents per generation (replicates A and B, respectively; Fig. 3D).

The change in the inbreeding load across generations did not differ between replicates (analysis of covariance, nonsignificant replicate-by-generation interaction, $F < 1.30, P > 0.25$ for all four traits).

MEAN POPULATION FITNESS OF SERIALLY INBRED VERSUS CONTROL POPULATIONS AFTER OUTBREEDING

We scored mortality, egg-to-adult development time, and adult longevity for both the control and serially inbred populations using the block design at the end of the experiment (this is the generation labeled "Generation 6" in Fig. 1). As predicted, the proportion of eggs developing (Replicate A only; Fig. 4A), the proportion of eggs hatching (both replicates; Fig. 4B), and hatch-to-adult larval survival (both replicates; Fig. 4C) in outbred families were all significantly higher in the serially inbred than in control populations, leading to a substantial increase in the proportion of eggs that produced adult offspring in serially inbred relative to control populations (Fig. 4D; compare filled circles between control and serially inbred populations). The treatment effect (serially inbred vs. control) was statistically significant for all traits in both replicates (Mann–Whitney U-test on block means; $\chi_1^2 > 5.3, P < 0.021$ for each) except for the proportion of eggs developing in replicate B (Fig. 4A). The proportion of eggs failing to develop decreased by >65% in the serially inbred relative to control populations, and the proportion of eggs failing to hatch decreased by >60% for both replicates (Table 2). In contrast, alleles affecting whether
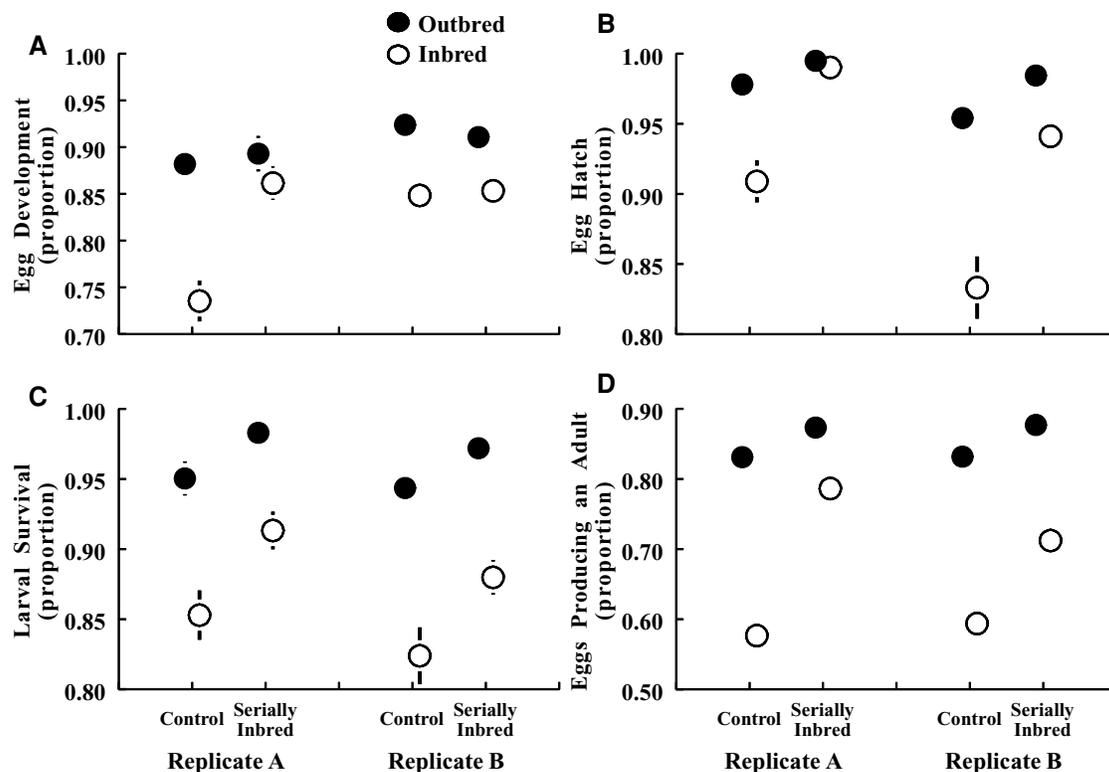
Figure 4. The effect of inbreeding (full-sib mating) on (A) the proportion of eggs developing, (B) the proportion of developed eggs hatching, (C) the proportion of hatched eggs producing an adult offspring, and (D) the total proportion of eggs laid that produced an adult offspring (including all sources of egg and larval mortality) in the serially inbred and control populations after four (replicate A) or three (replicate B) generations of full-sib mating.

Table 2. Proportion decrease in egg failure/larval mortality of serially inbred relative to control populations after two generations of outcrossing. These values are calculated as (Mean$_{control}$ − Mean$_{seriallyinbred}$)/Mean$_{control}$. Positive numbers indicate that the serially inbred populations had lower mortality than control populations.

Trait	Replicate A	Replicate B
Percent decrease in egg failure to develop	9.4	−17.2
Percent decrease in egg failure to hatch	76.9	66.1
Percent decrease in larval mortality	65.7	60.3
Percent decrease in the proportion of eggs failing to produce an adult offspring	25.0	26.9

eggs develop appear to have evolved very little, with the failure of eggs to develop decreasing by only 9% in serially inbred relative to control populations in replicate A and actually increasing (by 17%) in replicate B, consistent with the lack of change in inbreeding load seen in Figure 3A.

There was no consistent difference between serially inbred and control populations for development time of outbred beetles; development time actually increased in the serially inbred relative to the control populations in replicate A ($\chi_1^2 > 66$, $P < 0.001$ for both males and females) whereas there was no difference between

the serially inbred and control populations in replicate B ($\chi_1^2 < 0.04$, $P > 0.84$ for both males and females). Adult longevity of outbred did not differ between the serially inbred and control populations for either sex in either replicate (nonparametric analysis of variance comparing group mean life span between inbred and outbred beetles, with each block mean treated as a single datapoint; $\chi_1^2 < 3.7$, $P > 0.05$ for both males and females in both replicates).

THE MAGNITUDE OF INBREEDING DEPRESSION IN SERIALLY INBRED VERSUS CONTROL POPULATIONS

Following one generation of outbreeding we again quantified the magnitude of inbreeding depression in both the control and serially inbred populations (labeled "Generation 6" in Fig. 1). In Figure 4, the magnitude of inbreeding depression is the proportional difference between filled circles (mortality of outbred beetles) and open circles (mortality of inbred beetles); estimates of δ and *L* are presented in Table 3. The magnitude of inbreeding depression (δ) and the estimates of inbreeding load (*L*) for mortality were significantly lower in the serially inbred relative to control populations for both egg development (replicate A only; Fig. 4A) and egg hatch (both replicates; Fig. 4B). These translated into large reductions in δ and *L* for the proportion of eggs that produce an adult offspring (Fig. 4D). Interestingly, there was no difference in the magnitude of inbreeding depression for larval hatch-to-adult survival in the serially inbred relative to control populations (Fig. 4C) despite our previous observation that the

Table 3. The inbreeding load (*L*) and magnitude of inbreeding depression (δ) in serially inbred and control populations of *Stator limbatus* at the end of the experiment. Both *L* and δ are calculated separately for each block and then averaged across blocks. The χ_1^2 is for a nonparametric analysis of variance testing for a significant difference between serially inbred and control populations with each block treated as a single datapoint per population.

Trait	Inbreeding load *L*			Inbreeding depression δ		
	Control populations	Serially inbred populations	χ_1^2	Control populations	Serially inbred populations	χ_1^2
Egg development						
Replicate A	0.80±0.15	0.08±0.11	13.9***	0.13±0.04	0.00±0.03	13.9***
Replicate B	0.36±0.09	0.29±0.09	0.1 *ns*	0.07±0.02	0.05±0.02	0.1 *ns*
Egg hatch						
Replicate A	0.35±0.10	0.02±0.02	15.0***	0.07±0.02	0.00±0.00	15.0***
Replicate B	0.49±0.10	0.19±0.04	9.4**	0.12±0.03	0.04±0.01	10.8***
Larval hatch-to-adult survival						
Replicate A	0.44±0.12	0.31±0.07	1.5 *ns*	0.07±0.03	0.07±0.01	1.5 *ns*
Replicate B	0.60±0.13	0.42±0.07	0.2 *ns*	0.12±0.02	0.09±0.02	0.4 *ns*
Proportion of eggs producing an adult offspring						
Replicate A	1.57±0.22	0.39±0.14	22.5***	0.23±0.07	0.06±0.04	22.4***
Replicate B	1.33±0.19	0.90±0.13	3.9*	0.26±0.04	0.17±0.03	4.8*

*$P<0.05$; **$P<0.01$; ***$P<0.001$; *ns* $P>0.05$.

inbreeding load declined significantly during serial sib-mating (Fig. 3C).

We observed significant inbreeding depression for both development time (inbred beetles look longer to reach adult) and adult longevity (inbred beetles lived shorter adult lives; $F_{1,1141} = 11.9$, $P < 0.001$), both as observed in generation 1. However, there was no evidence that the magnitude of inbreeding depression in development time decreased in the serially inbred relative to control populations (nonparametric analysis of variance comparing δ between serially inbred and control lines; $\chi_1^2 < 2.6$, $P > 0.10$ for development time of both males and females in both replicates). Inbreeding depression in adult life span was lower in serially inbred than control populations for male offspring in replicate A ($\chi_1^2 = 6.4$, $P = 0.01$) but was not different between serially inbred and control populations for males in replicate B nor for females in either replicate ($P > 0.05$ for each).

Discussion

The results of this experiment demonstrate rapid and efficient purging of the genetic load in experimental populations of seed beetles using nonrandom (full-sib) mating. The purging was effective enough that it not only significantly reduced the segregating load of mutations and decreased the effects of future inbreeding (which is expected due to both fixation and purging of deleterious alleles; Reed and Bryant 2001), but also increased the fitness of populations formed from the hybridization of serially inbred lines. Further, the study sheds light on the two mechanisms responsible for the genetic basis of inbreeding depression: (1) the substantial inbreeding depression observed in *S. limbatus* is due to increased expression of recessive deleterious alleles rather than a reduction in overdominance in inbred individuals relative to outbred individuals, and (2) the loci involved are few in number and had major effects on the fitness components. Additionally, the high extinction rate suffered by inbred lines and variation in the efficacy of purging for different stages of development argues against purposeful inbreeding as a conservation strategy. This study has important implications for the dynamics of genetic load in wild populations and captive populations of conservation interest. We elaborate on each of these findings and conclusions below.

PURGING OF THE GENETIC LOAD

Our results for *S. limbatus* provide some of the most convincing evidence that inbreeding depression in survival can be purged within only a few generations of full-sib mating. Although numerous studies have demonstrated purging of deleterious recessives, the amount of purging is generally small (Byers and Waller 1999; Carr and Dudash 2003). For example, in a metaanalysis of serial inbreeding studies, Crnokrak and Barrett (2002) found that 72% of studied traits showed a decrease in inbreeding depression

during serial inbreeding, and 48% of studied traits show evidence of purging of deleterious alleles, measured as an increase in mean fitness of serially inbred lines, relative to controls, when all lines were outbred at the end of the experiment (as in our experiment). They found an overall decrease in f-adjusted inbreeding depression (adjusted to an inbreeding coefficient, f, of 1) from 0.73 to 0.38 during the course of serial inbreeding (note that this substantially inflates the estimates of δ relative to what would be observed for sib-mating, for which $f = 0.25$). For larval survivorship, this decline in inbreeding depression represents the purging of ∼0.4 lethal equivalents through the course of the experiment. For an average study with serial inbreeding of four or more generations, this represents the elimination of ∼0.1 lethal equivalents per generation. In comparison, the genetic load for egg hatch of *S. limbatus* was purged at the rate of ∼0.26–0.31 lethal equivalents per generation, and the genetic load for the probability that an egg produces an adult was purged at ∼0.45–0.50 lethal equivalents per generation. Both of these reflect a reduction in genetic load of more than half in only three generations of sib-mating. This represents some of the most rapid purging of deleterious recessive alleles yet observed in captive animal studies (Ballou 1997; Crnokrak and Barrett 2002). However, the effectiveness of purging clearly varied among the periods of *S. limbatus* mortality, being most effective for egg hatch and least effective for genes affecting larval hatch-to-adult survival. In concert with other studies of purging our results highlight the general result that the effectiveness of purging for removing deleterious recessive alleles varies substantially among taxa (Lacy and Ballou 1998), among traits within species (Lacy and Ballou 1998), and among stages of development (Remington and O'Malley 2000a).

We have no evidence in our experiment that sib-mating purged deleterious alleles that affect development time or adult longevity of *S. limbatus*. This result was expected because our design intentionally minimized or eliminated natural selection on development time and life span. Evolution of development time or life span, and evolution of the genetic loads of these traits, would have suggested that a mechanism other than purging (e.g., genetic drift) could have contributed significantly to the evolutionary responses observed for all traits (e.g., egg development, egg hatch and larval survival) during the experiment. The absence of detectable evolution of development time or life span does not demonstrate that genetic drift was insignificant during the experiment, but does suggest that effective population sizes were large enough that drift did not overwhelm responses to selection.

OVERDOMINANCE VERSUS DELETERIOUS RECESSIVES

Our results are consistent with the hypothesis that the substantial inbreeding depression observed in *S. limbatus* is due primarily to recessive deleterious alleles that have large effects on fitness, and

generally inconsistent with the hypothesis that overdominance contributes significantly to the inbreeding depression in *S. limbatus*, at least for egg development and egg hatch (see discussion of larval hatch-to-adult survival, below). Deleterious recessive alleles are widely suspected of contributing much more strongly to inbreeding than is overdominance, but some studies suggest that overdominance maybe more prevalent than assumed (e.g., Latter 1998). Symmetric overdominance would result in a gradual decline of mean population fitness with degree of inbreeding. However, with overdominance the inbreeding depression is due to decreased heterozygosity across all (or many) loci and there is no selection against specific deleterious or lethal alleles (Charlesworth and Charlesworth 1990). Thus, under the overdominance model outcrossing at the end of the experiment would recreate the heterozygosity of the original population and return mean fitness to its original value (i.e., not increase mean fitness) or even return fitness to a lower mean if genetic drift reduces the polymorphism that was maintained by selection in the original population (Carr and Dudash 2003). Instead, we found that mean population fitness increased in the serially inbred relative to control populations. Also, because the overdominance model posits that inbreeding depression is a consequence of reduced heterozygosity, no specific deleterious recessive alleles are purged and thus reinbreeding the population at the end of the experiment should recreate the same magnitude of inbreeding depression initially observed. We found that inbreeding depression decreased in the serially inbred relative to control populations. Thus, our data are inconsistent with both predictions of the overdominance model of inbreeding depression.

One contrary result we obtained was for larval hatch-to-adult survival (Figs. 3C and 4C). The genetic load (inbreeding load) for larval survival declined through our four generations of sib-mating at a rate of ~0.13 to 0.23 lethal equivalents each generation of sib-mating (Fig. 3C). However, when we remeasured inbreeding depression at the end of the experiment, after beetles had been outbred for one generation, we detected no difference in inbreeding depression or the genetic load between the serially inbred and control populations (Fig. 4C and Table 3). This latter result is expected if inbreeding depression is due to overdominance rather than increased expression of deleterious recessive alleles or if the decrease in inbreeding load in the inbred lines was due to the fixation of deleterious recessives rather than their elimination. However, both the observed decline in genetic load through the experiment and the evolution of increased mean larval hatch-to-adult survival of serially inbred relative to control populations are inconsistent with an overdominance explanation for inbreeding depression. Other studies have likewise found inconsistent results depending on the method of testing for purging. For example, Willis (1999c) demonstrated that mean fitness evolved in serially inbred populations of *Mimulus guttatus*, but inbreeding depression (δ) was unchanged for most traits relative to the ancestral population. Willis posits that his results may reflect adaptation to the laboratory/greenhouse environment. We controlled for differences between treatments in adaptation to the laboratory by simultaneously rearing both populations in a single growth chamber and testing all populations simultaneously at the end of the experiment. However, it is possible that inbreeding unmasked recessive beneficial alleles, exposing them to selection (Knowles et al. 1999; Reed et al. 2003a) and facilitating rapid adaptation to the laboratory in the serially inbred, but not control, populations. Alternatively, the changes in genetic architecture associated with inbreeding could increase genetic and phenotypic variance for fitness-related traits in the serially inbred populations allowing more rapid adaptation to the laboratory (e.g., facilitating peak shifts) in serially inbred relative to control populations (Willis and Orr 1993; Fowler and Whitlock 1999). Unfortunately, we cannot test these hypotheses with our current dataset.

MANY LOCI OF SMALL EFFECT VERSUS FEW LOCI OF LARGE EFFECT

The effectiveness of sib-mating in purging deleterious alleles from a population depends both on the effect size of the selection coefficient against the allele and on the degree of recessiveness. The larger the effect size (Lande and Schemske 1985; Hedrick 1994; Wang et al. 1999) and the more recessive the mutation (Glémin 2003) the more efficient is purging due to inbreeding. We thus interpret the rapid purging observed here as evidence that a small number of highly recessive alleles have large deleterious effects on the probability that an egg will develop and on the probability that a developing egg will hatch. Indeed, if the models of Wang (2000) and Glémin (2003) are correct, purging by intentional inbreeding could be effective in families of the size the beetles were limited to only if the selection coefficients were large [note that Glémin (2003) considered only a single locus, the results of which may not extend to multiple loci]. How large depends on several factors, especially the degree of recessiveness of the alleles, but almost certainly requires $s > 0.15$. However, as noted above, our results indicate that little purging occurred at loci affecting hatch-to-adult survival, suggesting either that inbreeding depression in survival at this stage of development is not due to deleterious recessive alleles or that those alleles have small effect. This could be because pleiotropic effects of recessive alleles on survival across stages of development would lead to earlier stages of development being more significantly impacted by negative effects of those alleles (because the frequency of homozygotes for these loci would decline through development as homozygotes die). Alternatively, selection may vary across the various stages of development, possibly because environmental stresses encountered vary among stages. Because the magnitude and architecture of the genetic load depends on the strength and type of selection

on those mutations, in addition to the mutation rate and the distribution of mutational effects (Reed 2008), a reduction in selection on earlier developmental stages (egg development and egg hatch) would allow accumulation of deleterious recessives in these stages such that the mechanism causing inbreeding depression changes through development.

Most studies have shown that although highly recessive alleles that have large effect on inbreeding depression are common (Kuang et al. 1999; Willis 1999a,c; Remington and O'Malley 2000a,b) most mutations causing inbreeding depression are partially recessive (Willis 1999b) and have small effects on fitness (Latter 1998; Willis 1999a,c). Unfortunately, in contrast to studies of *Mimulus* where specific lethal or highly deleterious alleles have been identified (Carr and Dudash 2003), such as male sterility mutations (Willis 1993) and chlorophyll-deficient lethals (Willis 1992), we have no candidate genes of large effect in *S. limbatus*. QTL or genomic studies will be necessary to quantify the number of genes affecting inbreeding depression in *S. limbatus*, and their relative effect sizes (Remington and O'Malley 2000a,b; Carr and Dudash 2003). However, we can rule out male sterility mutations as a major factor causing inbreeding depression in egg development and egg hatch. Because we outbred all beetles for one generation at the end of purging and before remeasuring population mean fitness and inbreeding depression, we ensured that all parents of inbred beetles were themselves outbred. Thus, inbreeding depression observed at the end of the experiment reflects only differences in inbreeding coefficients between offspring, not their parents, and thus does not include an effect of inbreeding on fertility.

Our experimental design, in which we mated siblings to create many very small demes within our serially inbred population, increases the potential for selection against recessive alleles (due to increased homozygosity) but also increases the potential for genetic drift within inbred lines (Caballero and Hill 1992; Glémin 2003). Population mean fitness and inbreeding depression can thus evolve due to fixation of alleles via genetic drift (Charlesworth and Charlesworth 1999; Wang et al. 1999; Miller and Hedrick 2001). However, drift within demes (families) should fix both deleterious and advantageous alleles because even rare deleterious alleles will start at high frequency (at least 0.25) in the mated pair founding the deme; thus, this drift can increase or decrease population mean fitness (Miller and Hedrick 2001), whereas we observed that population mean survival increased for all traits in our serially inbred relative to control populations. Also, this within-line genetic drift should have little effect on overall allele frequencies in the serially inbred population once all families are outcrossed at the end of the experiment (Wang 2000); although individual *S. limbatus* families were small, the total size of the serially inbred populations are large enough that purging of alleles due to nonrandom mating (i.e., selection against

homozygotes) should be much more efficient than is purging due to genetic drift (Glémin 2003). We thus conclude that the majority of the evolution of population mean fitness and inbreeding depression is necessarily due to selection against deleterious alleles and not due to drift.

CONSERVATION RELEVANCE

Inbreeding depression is a major force affecting the fitness and viability of small populations in the wild and in captivity. It has been suggested for populations doomed to inbreeding through small population size, that purposeful increases in the inbreeding level through nonrandom mating might lessen the chances of eventual extinction by purging the population of its genetic load. In particular, serial inbreeding has been proposed as a potential strategy for improving population mean fitness of captive animals. Our data very clearly show why this is a very poor strategy. During the inbreeding process, drift will randomly fix both beneficial and deleterious alleles, whereas selection opposes the fixation of the deleterious alleles. The mostly stochastic balance of the fixation versus purging of the deleterious recessive alleles will determine the fate of the population (Reed and Bryant 2001). That 61.5% of lineages in this experiment went extinct, despite the benign laboratory environment, is the single best warning against intentionally trying to purge the genetic load of endangered species in captivity. Even the potential for population subdivision to maintain genetic diversity and increase fitness of the resulting panmictic population (as witnessed in this experiment) depends on there being no extinction among the subpopulations.

Further, purging seems to be highly variable among species, populations, and families within populations; as well as being at least partially environment specific. We found that serial inbreeding (sequential sib-mating) rapidly increased population mean survival in *S. limbatus*, suggesting that serial inbreeding may be effective strategy for rapidly purging deleterious alleles in some species. However, we emphasize that mean survival is only one component of total fitness and that our experiment was not designed to examine the effectiveness of purging for increasing other components of population fitness. Our results indicate that the effectiveness of purging varies among the three studied periods of egg/larval survival and thus likely also vary among the other unstudied fitness components. Also, it is unclear to what extent purging of deleterious alleles in one environment will affect population mean fitness and inbreeding depression in alternate environments, because deleterious recessive alleles often have environment-specific effects (Reed and Bryant 2001; Swindell and Bouzat 2006). This latter question is currently under investigation.

This experiment also provides insight into the potential for survival in bottlenecked populations of conservation or evolutionary interest, such as founder populations on true or

habitat islands, or reintroduced species. The fact that a large part of the genetic load was purged effectively in only a few generations and that nearly 40% of the lineages survived despite full-sib mating, suggests that a large number of bottlenecked populations, even in novel environments will be able to recover from inbreeding and attain viable sizes, provided the environment is suitable and enough such habitat exists. An apparent example of this can be found in Kaeuffer et al. (2006). They emphasized that the unexpected levels of heterozygosity found in an isolated population of island mouflon (a wild sheep) founded from a single pair; however, the population clearly struggled for six to seven generations after being introduced to the island before entering an exponential growth phase and then finally settling into a cyclic pattern of population growth. Anecdotally, it would appear that the population teetered on the verge of extinction because of inbreeding depression before being purged of its genetic load and then rapidly growing to carrying capacity.

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Supporting Information

The following supporting information is available for this article:

Figure S1 The proportion of eggs developing (A) and hatching (B), the proportion of larvae surviving to adult (C), and the cumulative probability that an egg produced an adult offspring (D) for serially inbred (serially sib-mated) and control (panmictic) populations of *Stator limbatus* through the four generations (replicate A) or three generations (replicate B) of the experiment.

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Supplemental Figure 1. The proportion of eggs developing (A) and hatching (B), the proportion of larvae surviving to adult (C), and the cumulative probability that an egg produced an adult offspring (D) for Serially-inbred (serially sib-mated) and Control (panmictic) populations of *Stator limbatus* through the four generations (replicate A) or three generations (replicate B) of the experiment.