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ORIGINAL PAPER

Inbreeding-environment interactions for fitness: complex relationships between inbreeding depression and temperature stress in a seed-feeding beetle

Charles W. Fox · R. Craig Stillwell · William G. Wallin · Carrie L. Curtis · David H. Reed

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Abstract It is commonly argued that inbred individuals should be more sensitive to environmental stress than are outbred individuals, presumably because stress increases the expression of deleterious recessive alleles. However, the degree to which inbreeding depression is dependent on environmental conditions is not clear. We use two populations of the seed-feeding beetle, *Callosobruchus maculatus*, to test the hypotheses that (a) inbreeding depression varies among rearing temperatures, (b) inbreeding depression is greatest at the more stressful rearing temperatures, (c) the degree to which high or low temperature is stressful for larval development varies with inbreeding level, and (d) inbreeding depression is positively correlated between different environments. Inbreeding depression (δ) on larval development varied among temperatures (i.e., there was a significant inbreeding-environment interaction). Positive correlations for degree of inbreeding depression were consistently found between all pairs of temperatures, suggesting that at least some loci affected inbreeding depression across all temperatures examined. Despite variation in inbreeding depression among temperatures, inbreeding depression did not increase consistently with our proxy for developmental stress. However, inbreeding changed which environments are benign versus stressful for beetles; although 20°C was not a stressful rearing temperature for outbred beetles, it became the most stressful environment for inbred larvae. The finding that inbreeding-environment interactions can cause normally benign environments to become stressful for inbred populations has important

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C. W. Fox (✉) · R. C. Stillwell · W. G. Wallin · C. L. Curtis · D. H. Reed
Department of Entomology, University of Kentucky, Lexington, KY 40546-0091, USA
e-mail: fox@uky.edu

Present Address:
R. C. Stillwell
Department of Entomology, University of Arizona, Tucson, AZ 85721, USA

Present Address:
D. H. Reed
Department of Biology, University of Louisville, Louisville, KY 40292, USA

consequences for many areas of evolutionary genetics, artificial breeding (for conservation or food production), and conservation of natural populations.

Keywords Genetic load · Inbreeding depression · Stress · Temperature

Introduction

Inbreeding depression is a reduction in the fitness of inbreed versus outbreed offspring and is widespread among plant and animal taxa (Charlesworth and Charlesworth 1987). Inbreeding depression results from increases in average homozygosity levels across the genome (Crnokrak and Barrett 2002). Environmental conditions can have large effects on gene expression (Kondrashov and Houle 1994; Shabalina et al. 1997; Szafraniec et al. 2001; Fry and Heinsohn 2002) and numerous studies have shown that inbreeding depression varies along environmental gradients (Armbruster and Reed 2005). Thus, environmental sensitivity of gene expression can affect the fitness consequences of inbreeding (Armbruster and Reed 2005). However, results of experimental studies vary substantially among species, among the types of environmental conditions tested, and even among fitness components in individual studies (Marr et al. 2006; review in Armbruster and Reed 2005), so that the degree to which inbreeding depression is dependent on environmental conditions is not yet clear.

Understanding the extent to which environmental conditions affect inbreeding depression is of substantial evolutionary and conservation significance for at least three reasons. First, if environmental conditions have a large effect on the magnitude of inbreeding depression, then inbreeding depression estimated in nature cannot easily be compared among species or populations within species since environmental conditions inevitably also vary (Waller et al. 2008). For example, inbreeding depression estimated in controlled laboratory conditions, including captive rearing facilities, may not reflect inbreeding depression in nature (Dudash 1990; Pray et al. 1994; Joron and Brakefield 2003). Second, without an understanding of how inbreeding depression varies with environmental conditions we have little understanding of how the risk of population extinction will vary among environmental conditions when there is inbreeding (Bijlsma et al. 2000). Inbreeding-environment interactions can influence the amounts and kinds of genetic variation maintained in a population, limit evolutionary potential through negative genetic correlations to different stresses influencing population growth (Vieira et al. 2000; Weinig et al. 2003; Harbison et al. 2004; Zhong et al. 2005), impact population dynamics (Reed et al. 2007), and significantly reduce the expected time to extinction by exacerbating population declines under stressful conditions (Liao and Reed 2009). Third, our poor understanding of environmental effects on inbreeding depression limits our ability to predict how purging of deleterious recessives, either in captivity or due to natural bottlenecks, will affect populations as they recover from ecological crises; the degree to which the genetic load is purged may depend on environmental conditions (Bijlsma et al. 1999; Dahlgaard and Hoffmann 2000; Swindell and Bouzat 2006). We thus need more studies examining how a diversity of environmental conditions affect inbreeding depression in plants and animals.

It is commonly argued that inbred individuals should be more sensitive to environmental stress than are outbred individuals (Armbruster and Reed 2005). This is presumably because stress increases the expression of deleterious recessive alleles (Lynch and Walsh 1998). However, some models predict that environmental conditions primarily change the

variance of mutational fitness effects rather than their average effect or their net expression level; i.e., stress should affect which alleles are expressed, and the variance in effect size among alleles, but not the average effect size of deleterious alleles, and thus not the average effect of the genetic load (Martin and Lenormand 2006). Experimental studies of how stress affects inbreeding depression are inconsistent; many do indeed find the inbreeding depression increases with exposure to stressors such as temperature stress (Bijlsma et al. 1999), desiccation (Dahlgaard and Hoffmann 2000), chemical stressors (Bijlsma et al. 1999), resource and sexual competition (Meagher et al. 2000), or food stress (Keller et al. 2002; Killick et al. 2006) in animals, and nutrient limitation (Hayes et al. 2005) in plants. However, many studies fail to find an increase in inbreeding depression with increasing stress (Martin and Lenormand 2006; Willi et al. 2007; Jasnos et al. 2008) and a few have even found that inbreeding depression is greatest in benign environments (Waller et al. 2008; review in Armbruster and Reed 2005). However, few studies have been performed on any specific environmental stressor in taxa other than *Drosophila*. Also, the degree to which inbreeding depression in different environments reflects expression of the same loci and alleles, or different loci and alleles, is still largely unknown (Armbruster and Reed 2005).

Here we present the second of a series of studies examining how environmental conditions affect expression of inbreeding depression in a model organism, the seed-feeding beetle, *Callosobruchus maculatus* (see also Fox and Stillwell 2009). *Callosobruchus maculatus* suffers substantial inbreeding depression throughout development. Eggs produced from inbred matings are less likely to develop, have lower hatch rates, and larvae hatching from these eggs have reduced hatch-to-adult survival. Eggs from full-sib matings are 17–21% less likely to produce an adult offspring than eggs from outbred matings in *C. maculatus* (Fox et al. 2007). Inbred offspring that survive to sexual maturity develop more slowly—larval development time is extended by $\sim$5% (>1 day) (Tran and Credland 1995; Fox et al. 2007). Inbreeding also affects adult lifespan of female, but not male, *C. maculatus* (Fox et al. 2006; Fox and Stillwell 2009). Similarly, inbreeding has been shown to affect female, but not male, adult body size (Tran and Credland 1995). Inbreeding also negatively affects female fecundity in both *C. maculatus* (Tran and Credland 1995) and its congener *C. chinensis* (Tanaka 1990, 1993).

In this study we manipulate rearing temperature from 20 to 35°C (in 5°C increments) and quantify the magnitude of inbreeding depression (δ) throughout development from egg laying to adult emergence (egg development, egg hatch, larval survival, development time and adult emergence mass). We test the hypotheses that (a) inbreeding depression varies among rearing temperatures, (b) inbreeding depression is greatest at the more stressful rearing temperature, i.e., at the temperature at which outbred larval mortality is greatest, (c) the degree to which high or low temperature is stressful for larval development varies with inbreeding level, and (d) inbreeding depression is positively correlated between different environmental conditions.

Materials and methods

The biology of *C. maculatus*

Callosobruchus maculatus (F.) is a cosmopolitan pest of grain legumes (Fabaceae), particularly beans of the genus *Vigna*. The life cycle of *C. maculatus* revolves around seeds.

Females cement their eggs to the surface of host seeds (Messina 1991). When eggs hatch first instar larvae burrow into the seed under the egg. Larval development and pupation are completed within a single seed—larvae do not move among seeds and are thus restricted to the seed chosen by their mother. Beetles emerge as reproductively mature adults and require neither food nor water as adults before mating and laying eggs (i.e., they are primarily capital breeders).

For this study we used two populations of *C. maculatus* that have been the subject of previous inbreeding experiments (Fox et al. 2006, 2007). The South India (SI) population was collected in 1979 from infested pods of mung bean, *Vigna radiata* (L.) Wilczek, and the closely related black gram, *Vigna mungo* (L.) Hepper, in Tirunelveli, India (Mitchell 1991). The Burkina Faso (BF) population was collected in 1989 from infested pods of cowpea, *V. unguiculata* (L.) Walp., in Ouagadougou, Burkina Faso (Messina 1993). These two populations differ in body size, lifetime fecundity, patterns of egg dispersion, oviposition preference, and adult longevity (Fox et al. 2004a, b; Messina 2004). Both populations were maintained in laboratory growth chambers on seeds of *V. radiata* (SI) or *V. unguiculata* (BF) at >1,000 adults per generation for >100 generations (BF) or >200 generations (SI), at ~25°C, prior to this experiment. Both populations harbor substantial amounts of genetic variation. They also exhibit substantial inbreeding depression when sib-mated and thus appear to maintain a large segregating load of deleterious recessive mutations (Bilde et al. 2009; Fox et al. 2006, 2007; Fox and Stillwell 2009).

Experimental design

To measure inbreeding depression we used a "block" design (Roff 1998), shown in Fig. 1. Blocks were created by randomly pairing two families chosen from an outbred population. From each family we randomly chose two females and two males, each reared at 26°C at one larva per seed, to become parents. We crossed these offspring from two families, creating two inbred and two outbred families per block. The advantage of this design is that it assures that inbred families are created from the same set of alleles as are the outbred families to which they are compared (Fox 2005).

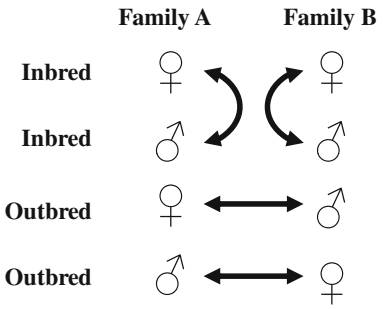

Fig. 1 The *block* design used to measure inbreeding depression. Each *block* is created by crossing beetles from two unrelated families, creating two outbred matings (reciprocal crosses between the two families) and two inbred matings (crosses between full-sibs within each family). Outbreds and inbreds within each block thus have, on average, the same set of alleles but differ in degree of homozygosity due to the mating treatment

Both populations of beetles have high and nearly equal survival on seeds of *V. radiata* (the host of the SI population), but not on *V. unguiculata* (the host of the BF population) (Stillwell et al. 2007). We thus used *V. radaiata* as our rearing host for beetles in this experiment to minimize confounding effects of host-associated mortality on estimates of inbreeding depression. Pairs were confined in a 35-mm Petri dish with 40 seeds of mung, *V. radiata*. Dishes were checked for eggs every 12 h. Females were allowed to lay eggs until they had produced at least 40 eggs. In the rare case where a pair did not lay eggs, both parents were replaced with a new mating of the exact same cross.

Seeds bearing eggs were replaced with fresh seeds at each 12 h check and the freshly laid eggs were evenly divided among four temperature treatments, 20, 25, 30 and 35 (all ± 0.5) °C. These temperatures were chosen to include a range of conditions from low stress (intermediate temperatures) to higher stress (the extreme temperatures); egg-to-adult survival for outbred beetles of these two populations is highest at temperatures between 25 and 30°C (Stillwell et al. 2007). The temperatures we used are within the normal range of temperatures at which *C. maculatus* can develop and reproduce (Chandrakantha and Mathavan 1986; Chandrakantha et al. 1987; Mbata et al. 2005). Females lay very few eggs, and those eggs often fail to develop, at temperatures approaching 40°C (Lale and Vidal 2003). The native climates of the BF and SI populations are very similar to each other (mean temperature difference between sites is ~ 0.4°C; National Climatic Data Center's Global Surface Summary of Day, Asheville, N.C.). Our 20°C low temperature treatment is below the average daily temperature for all months and below the average daily minimum temperature for most months (except 1–2 per site), at both sites. Likewise, our 35°C high temperature treatment is above the average daily temperature for all months and above the average daily high temperature for most months (except for 2–3 months per site), at both sites.

Larvae were reared at one egg per seed (excess eggs were scraped from the seed), one seed per dish, inside a temperature and photoperiod controlled growth chamber at Light:Dark 15:9. Dishes were checked twice per day for adult beetles that emerged from a seed.

We scored egg development, larval survival, egg-to-adult development time, and adult body mass for all offspring. All the eggs were classified to one of four fates; those that 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 emerged as an adult.

Sample sizes

In total, we created 46 blocks per population (92 total blocks). Each block consisted of two inbred and two outbred families (368 total families). Because mortality is greater for offspring of inbred matings, we reared more offspring for inbred families (10 eggs per temperature for outbred families and 14 eggs per family for inbred families). From these blocks we collected a total of 14,489 eggs (an average of 157.5 eggs per block, divided evenly among the four temperatures), from which 10,482 offspring survived to adult (113.9 offspring per block) and 10,414 offspring were weighed (113.2 offspring per block).

Analyses

Blocks are the lowest level of independence in this design and thus block means were used in all analyses. All block means were calculated first by averaging across offspring within a

family and then by averaging across families within the block and treatment. For survival data, each block contains two means for each temperature treatment (one mean each for inbred and outbred families). For development time and adult mass each block contains four means for each temperature treatment, one for each sex-by-inbreeding treatment combination (inbred male offspring, outbred males, inbred females and outbred females).Inbreeding depression for survival and offspring body mass was calculated as the proportional *reduction* in survival/mass,

$$\delta = \frac{\text{Mean}_{\text{outbred}} - \text{Mean}_{\text{inbred}}}{\text{Mean}_{\text{outbred}}}.$$

Inbreeding depression for development time was calculated as the proportional *increase* in development time

$$\delta = \frac{\text{Mean}_{\text{inbred}} - \text{Mean}_{\text{outbred}}}{\text{Mean}_{\text{outbred}}}.$$

δ was calculated separately for each block, and each estimate of δ was treated as a single independent data point.

Body mass data fit assumptions of standard general linear models and thus treatment effects on mass were analyzed using analysis of variance on block means. Development time was log transformed to eliminate the temperature effect on phenotypic variance. We used logistic regression to test for effects of population rearing temperature, and inbreeding treatment on egg/larval survival data (egg development, egg hatch, larval survival, and the probability that an egg produced an adult offspring).

Because the magnitude of inbreeding depression, δ, is calculated as a proportional change in survival/development time, testing for interactions in an analysis of variance is not appropriate for testing for variation in inbreeding depression among treatments; this is because interactions in an analysis of variance are based on linear effects and are thus not suitable for testing for changes in proportional effects (Stanton and Thiede 2005; Stillwell et al. 2007). We thus tested for rearing temperature effects on inbreeding depression using analysis of variance on δ, with each block treated as an independent data point. Despite being a ratio, the distribution of δ fit the assumptions of analysis of variance.

To test whether inbreeding depression was dependent on the quality of the environment, we needed a proxy for environmental quality. We chose the probability that an outbred egg produced an adult (which combines all periods of mortality) as our proxy under the assumption that the temperatures at which mortality is highest during development represent the poorest environmental conditions for larval development (Armbruster and Reed 2005). We used analysis of covariance to test for a significant relationship between mean δ for each population/temperature combination versus mean outbred egg-to-adult survival (model: δ = population + outbred survival). With only 8 data points (2 populations × 4 temperatures = 8 data points) this analysis is very conservative, but it does indicate the direction of relationship which, in this case, is adequate for our test of the hypothesis that inbreeding depression (δ) increases with decreasing environmental quality (see Results).

Because we used a split brood design (families evenly divided amongst the four temperature treatments) we can test whether inbreeding depression at one temperature was genetically correlated with inbreeding depression at other temperatures. We did this by calculating the correlation between δ for each pair of temperatures (with each block treated as a single data point).

Results

The effects of temperature on larval survival and development time, including differences between these two populations and between the sexes, are described thoroughly elsewhere (e.g., Stillwell and Fox 2007; Stillwell et al. 2007) and are thus not described in detail here. Likewise, patterns of inbreeding depression in these two populations of beetles are described elsewhere (Fox et al. 2006, 2007). Here we emphasize only the new contributions from this current study and a few additional results needed for interpreting treatment effects in the current study.

The effect of temperature on larval survival, development time and body mass of outbred beetles

Temperature affected the proportion of eggs developing and hatch-to-adult survival (solid circles in Fig. 2a, c; logistic regression, temperature effect for outbred beetles only; $\chi^2_3 = 9.1$, $P = 0.03$ and $\chi^2_3 = 8.5$, $P = 0.04$, respectively); the proportion of eggs developing and hatch-to-adult survival were lowest at 30°C. However, the effect of temperature on egg hatch was marginally non-significant (Fig. 2b; $\chi^2_3 = 7.1$, $P = 0.068$). The combined effects of temperature on two developmental stages resulted in a large effect of temperature on the proportion of eggs producing an adult offspring (Fig. 2d; $\chi^2_3 = 12.7$, $P = 0.005$). The temperature effect did not differ between populations for any period of larval development (population × temperature interactions; $\chi^2_3 < 1.30$, $P > 0.73$ for all

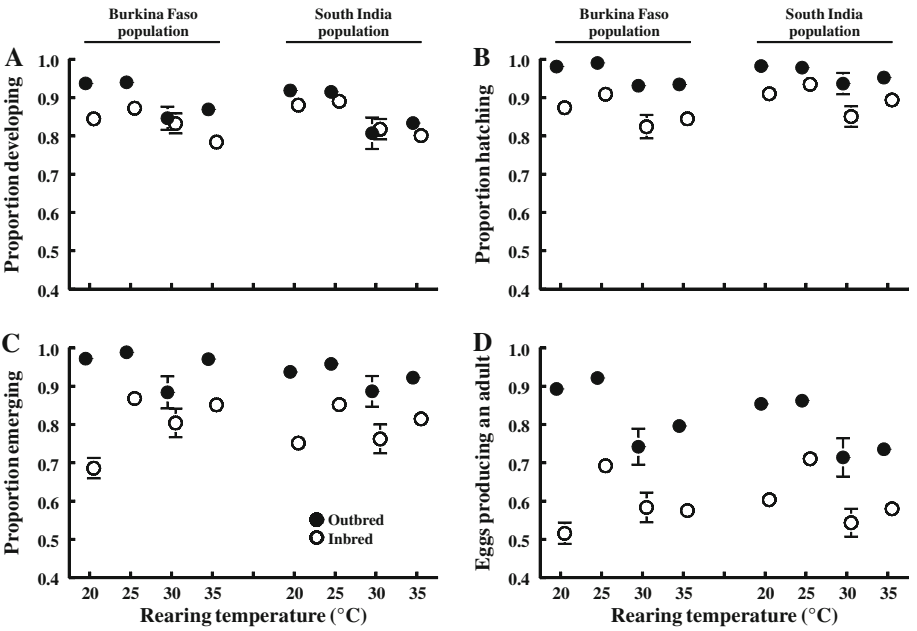

Fig. 2 The proportion of eggs that develop (**a**), egg hatch (**b**), hatch-to-emergence survival (**c**), and the proportion of all eggs that gave rise to an adult offspring (**d**) for two populations of *Callosobruchus maculatus* reared at four temperatures. Means (±SEM) are calculated first by averaging across families in a block, then across blocks. *Closed circles* are eggs produced from outbred mating, *open circles* are eggs produced from sib-matings

periods of development). For both populations, eggs were more likely to produce an offspring when reared at 25°C; 92 ± 1 and 86 ± 2% of eggs (BF and SI populations, respectively) produced an adult offspring when reared at this intermediate temperature (25°C), which was significantly higher than the proportion of eggs producing an adult offspring at either of the higher temperatures (linear contrasts: 25 vs. 30°C, $\chi^2_1 = 8.4$, $P = 0.004$; 25 vs. 35°C, $\chi^2_1 = 5.4$, $P = 0.005$) but not significantly different from 20°C (20 vs. 25°C, $\chi^2_1 = 0.2$, $P = 0.7$). Thus, based on the cumulative probability that an outbred egg produces an adult (across all developmental stages), 25°C is the least stressful temperature for larval development, consistent with results of Stillwell et al. (2007), with 20°C not significantly different from 25°C.

Beetles took longer to reach sexual maturity (Fig. 3a, b) and were larger (Fig. 3c, d) when reared at lower temperature (temperature effect in analysis of variance; development time: $F_{1,693} = 2{,}787$, $P < 0.001$, mass: $F_{1,693} = 1{,}355$, $P < 0.001$), as is typical of ectotherms (Atkinson 1994; Kingsolver and Huey 2008) and this particular species (Stillwell et al. 2007). The effect of temperature on body mass differed significantly between the populations (Fig. 3c, d; population × temperature interaction; $F_{1,693} = 22.9$, $P < 0.001$), but the effect on development time did not (Fig. 3a, b; $F_{1,693} = 3.6$, $P = 0.06$). Although females were larger than males at all temperatures, the degree of dimorphism varied with temperature ($F_{3,340} = 91.8$, $P < 0.001$); dimorphism was smallest at the lowest temperature (20°C) and generally highest at 30°C, although the details of the pattern differed between the populations ($F_{3,340} = 3.33$, $P = 0.02$) as previously observed (Stillwell and Fox 2007).

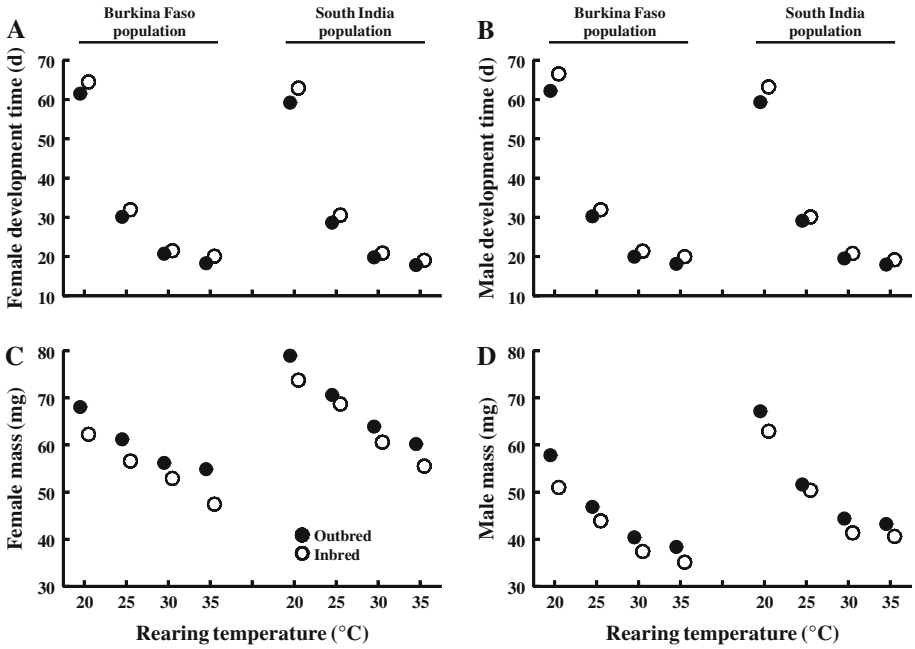

Fig. 3 Egg-to-emergence development time (**a**, **b**) and body mass at emergence (**c**, **d**) for males and females in two populations of *Callosobruchus maculatus* reared at four temperatures. Means (±SEM) are calculated first by averaging both families in a *block*, then across *blocks*. *Closed circles* are eggs produced from outbred mating, *open circles* are eggs produced from sib-matings

The effect of inbreeding on larval survival, development time and body mass

On average (across populations and temperatures) inbreeding reduced the proportion of eggs that hatched by 8% (Fig. 2b; treatment effect in logistic regression; $\chi^2_1 = 17.1$, $P < 0.001$; Supplemental Material Table 1) and reduced the proportion of hatched eggs that produced an adult offspring by 13% (Fig. 2c; $\chi^2_1 = 34.3$, $P < 0.001$). However, the effect of inbreeding on the proportion of eggs developing was generally small (3%) and not statistically significant (Fig. 2a; $\chi^2_1 = 2.9$, $P = 0.09$), indicating that the detrimental effects of inbreeding occur primarily during larval development and not during embryonic development. Because of the large effects of inbreeding on egg hatch and hatch-to-adult survival, inbreeding reduced the proportion of eggs producing an adult offspring by, on average across populations and temperatures, 22% (Fig. 2d, Supplemental Material Fig. S1). The magnitude of these effects is similar to that found in previous studies of inbreeding depression on these beetles (Fox et al. 2007). The magnitude of inbreeding depression (δ) differed between populations for egg development ($F_{1,356} = 4.81$, $P = 0.03$) but not for egg hatch or larval survival ($F < 2.01$, $P > 0.16$ for both).

Inbred beetles took 3–10% longer to develop to adult, and were 2–13% smaller as adults, compared to outbred beetles (treatment effect in analysis of variance; development time: $F_{1,1378} = 384$, $P < 0.001$, mass: $F_{1,1378} = 226$, $P < 0.001$). There was no difference between the sexes in the magnitude of inbreeding depression on development time or adult body mass (non-significant sex × treatment interaction for δ; development time: $F_{1,678} = 0.00$, $P = 0.96$; mass: $F_{1,678} = 0.04$, $P = 0.84$), in contrast to results found for a different population of *C. maculatus* (Tran and Credland 1995).

The effect of temperature on inbreeding depression

Temperature did not affect the magnitude of inbreeding depression (δ) for the proportion of eggs that developed or the proportion of developing eggs that hatched (Fig. 4a, b; $F_{3,356} = 2.39$, $P = 0.07$ and $F_{3,356} = 0.54$, $P = 0.66$, respectively). However, temperature did have a large effect on δ for hatch-to-adult survival (Fig. 4c; $F_{3,356} = 11.56$, $P = < 0.001$) and thus on the proportion of eggs producing an adult offspring (Fig. 4d; $F_{3,356} = 8.38$, $P < 0.001$). This large temperature effect in the ANOVA was largely because inbred beetles performed very poorly at the lowest temperature (20°C), and thus inbreeding depression was greater at 20°C than at any other temperature (compare inbred versus outbred beetles in Fig. 2d). On average (between populations) eggs produced from sib matings were 34% less likely to produce an adult offspring than were eggs from outbred matings when offspring were reared at 20°C, compared to inbreeding depression of only 20, 13 and 21% when offspring were reared at 25, 30 and 35°C, respectively (linear contrasts, 20°C versus each other temperature, $F_{1,355} > 9.1$, $P < 0.003$ for all three contrasts; no other contrasts were significant). There were no significant differences in δ between any other pairs of temperatures (linear contrasts, $P > 0.11$ for each contrast). The temperature effect on inbreeding depression did not differ between populations for any period of mortality (population × temperature interaction; $F_{3,356} < 1.55$, $P > 0.20$ for all periods of mortality).

Temperature also had a significant effect on δ for development time ($F_{3,678} = 13.3$, $P < 0.001$) and adult body mass ($F_{3,678} = 8.11$, $P < 0.001$). The effect of temperature on inbreeding depression differed between populations for development time (temperature by population interaction; $F_{3,678} = 3.49$, $P = 0.02$) but not for body mass ($F_{3,678} = 1.71$, $P = 0.16$), whereas the effect of temperature differed between males and females for body

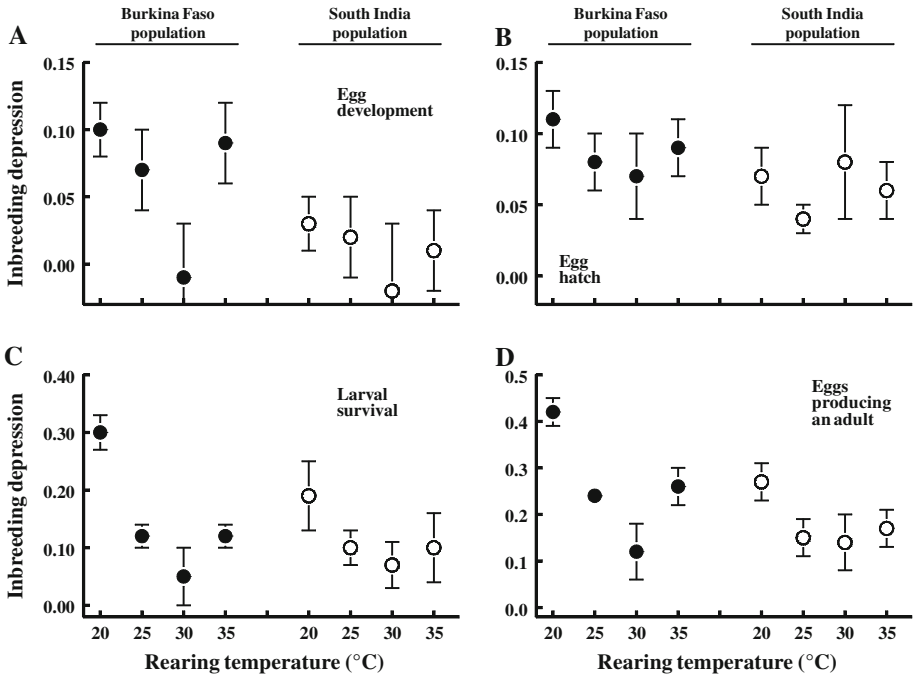

Fig. 4 The magnitude of inbreeding depression (δ) on proportion of eggs that develop (**a**), egg hatch (**b**), hatch-to-emergence survival (**c**), and the proportion of all eggs that gave rise to an adult offspring (**d**) for two populations of *Callosobruchus maculatus* reared at four temperatures. δ ($\pm$SEM) are calculated separately for each *block*, then across *blocks* within a population

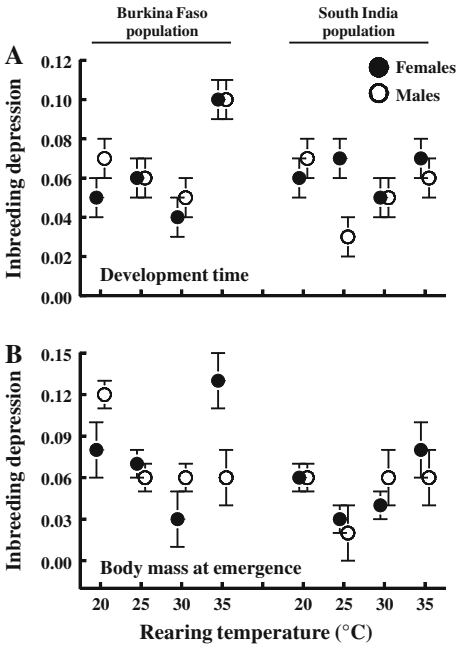

Fig. 5 The magnitude of inbreeding depression (δ) on egg-to-adult development time (**a**) and body mass at adult emergence (**b**) for two populations of *Callosobruchus maculatus* reared at four temperatures. δ ($\pm$SEM) are calculated separately for each *block*, then across *blocks* within a population

mass (temperature by sex interaction; $F_{3,678} = 2.97$, $P = 0.03$) but not for development time ($F_{3,678} = 2.31$, $P = 0.08$). Overall, inbreeding depression was lowest for the intermediate temperatures and highest at the two extremes, but the effect was small and variable among population/sex combinations (Fig. 5).

Does inbreeding depression increase with decreasing environmental quality?

We found no evidence that inbreeding depression (δ) was greatest at the temperatures that are most stressful for outbred beetles (Fig. 6). Treating each population/temperature combination as a single data point, we found no significant relationships between our proxy for environmental stress and δ for any trait ($P > 0.05$ for all tests). This analysis has low power to detect patterns because we have only 8 data points (two populations $\times$ four temperatures). However, the direction of effect was not suggestive of a significant relationship between stress and δ for any dependent variable. It is possible that the temperatures that are most stressful vary among periods of development. However, repeating the analysis comparing δ during each period of development with larval survival during that same period of development (e.g., regressing δ for egg hatch versus average outbred egg hatch) likewise failed to detect evidence that inbreeding depression increased with developmental stress.

Although inbreeding depression was not greater at more stressful temperatures (when stress was defined as a function of the performance of outbred beetles), it is clear that inbreeding changed which temperatures are stressful for beetles. Specifically, 20 and 25°C were equally benign temperatures for outbred beetles—outbred beetles had similar survivorship at all stages of development when reared at these two temperatures—and only 30 and 35°C could be considered relatively stressful (Fig. 2; logistic regression, linear contrast for the probability that an egg produces an adult, non-significant between 20 and 25°C [$P = 0.66$] and non-significant between 30 and 35°C [$P = 0.54$] but all other contrasts significant at $P < 0.05$). In contrast, 20°C was not a benign temperature for inbred beetles. Instead, eggs reared at 20°C were less likely to develop, and less likely to hatch (relative to 25°C), and larvae reared at 20°C had the lowest survivorship (Fig. 2) of all treatments

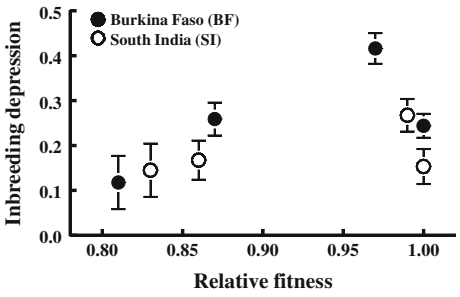

Fig. 6 The relationship between inbreeding depression (δ) on fitness (egg-to-adult survival) and mean relative fitness. Relative fitness was calculated separately for each population, as egg-to-adult survival of outbred beetles for each treatment relative to the treatment at which outbred beetles survived best (25°C for both populations). Data are those from Fig. 4d (*y*-axis) and Fig. 2d (*x*-axis). Data are not presented for other periods of mortality, egg-to-adult development time and adult body mass because all relationships are similarly positive or flat, and none are consistent with the hypothesis that δ increases with decreasing environmental quality (which predicts a negative relationship)

(linear contrasts, larval survival at 20°C was lower than survival at 25°C [$P = 0.02$] but not significantly different than survival at 30 or 35C [$P > 0.06$ for both].

Thus, 20°C was a relatively benign temperature for egg and larval development when beetles were outbred, but 20°C was a very stressful, and possibly the most stressful, temperature when larvae were inbred.

Correlation between inbreeding depression across treatments

For the four periods of larval mortality, all estimates (except one) of the correlations between δ for pairs of temperatures were positive (47 of 48 positive, sign test, $P < 0.001$), and 24 of 48 were significantly greater than 0 ($P < 0.001$ against the expected 5% frequency of false positives) (Table 1). The mean correlation for the blocks among temperatures for overall fitness (probability an egg produces an adult offspring) was a highly

Table 1 Between-temperature Pearson moment correlations for inbreeding depression (δ)

	20°C	25°C	30°C	35°C
Eggs developing[a]				
20°C	–	0.63***	0.20ns	0.53***
25°C	0.46**	–	0.35*	0.61***
30°C	0.30*	0.25ns	–	0.32*
35°C	0.56***	0.40**	0.21ns	–
Eggs hatching[b]				
20°C	–	0.41**	0.27ns	0.44**
25°C	0.04ns	–	0.16ns	0.31*
30°C	0.56***	−0.02ns	–	0.30ns
35°C	0.20ns	0.13	0.12ns	–
Larval survival[c]				
20°C	–	0.61***	0.33*	0.28ns
25°C	0.13ns	–	0.26ns	0.39**
30°C	0.18ns	0.03ns	–	0.26ns
35°C	0.44**	0.09ns	0.10ns	–
Probability of an adult[d]				
20°C	–	0.63***	0.19ns	0.49***
25°C	0.39*	–	0.31*	0.37*
30°C	0.32ns	0.30*	–	0.24ns
35°C	0.68***	0.35*	0.22ns	–

BF correlations are above the diagonal, SI correlations are below the diagonal

ns non-significant

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (before correction for multiple comparisons)

[a] The proportion of eggs producing a visible embryo

[b] The proportion of developing eggs that hatch (hatching is defined to have occurred if the larvae begins digging into the seed)

[c] The proportion of hatched eggs that produce an adult offspring that successfully emerges from the seed; offspring that pupated but failed to emerge from a seed are counted as part of larval mortality

[d] The total probability that an egg produces an adult offspring that successfully emerges from the seed

Table 2 Between-temperature Pearson moment correlations for inbreeding depression (δ)

	Females				Males			
	20°C	25°C	30°C	35°C	20°C	25°C	30°C	35°C
Development time								
20°C	–	0.52***	0.49***	0.40***	–	0.05ns	0.22ns	0.50***
25°C	−0.11ns	–	0.08ns	0.29*	0.29ns	–	0.09ns	0.03ns
30°C	0.36*	0.06ns	–	0.27ns	0.41*	0.39*	–	0.03ns
35°C	0.25ns	−0.06ns	0.12ns	–	0.25ns	0.29ns	0.40*	–
Adult mass								
20°C	–	0.31*	0.06ns	0.13ns	–	0.50***	0.40*	−0.08ns
25°C	0.08ns	–	0.28ns	0.21ns	0.25ns	–	0.46**	0.28ns
30°C	0.07ns	0.13ns	–	0.23ns	0.11ns	−0.10ns	–	0.18ns
35°C	0.16ns	0.18ns	−0.05ns	–	0.30*	0.12ns	0.08ns	–

BF correlations are above the diagonal, SI correlations are below the diagonal

ns non-significant

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (before correction for multiple comparisons)

significant, but rather modest, 0.37 ± 0.05. For female development time, 10 of 12 correlations were positive and five of them significantly so, while for male development time 12 of 12 correlations were positive and four of them significantly so (Table 2). The average correlation between temperatures (among blocks; lumping males and females) was significant but small (0.23 ± 0.04). Correlations for mass were almost entirely positive (12 of 12 in females and 11 of 12 in males), with one significant correlation for females and three for males (Table 2). As with development time the average correlation for body size between pairs of temperatures (among blocks) was significant but small ($r = 0.18 \pm 0.03$).

Discussion

It is commonly argued that the fitness consequences of exposure to environmental stressors should vary with inbreeding level and, reciprocally, that the magnitude of inbreeding depression should be greater in stressful environments. For *C. maculatus*, we found that inbreeding depression (δ) on larval development varied among temperatures (i.e., there was a significant inbreeding-by-environment interaction), being particularly large at 20°C (the lowest experimental temperature) relative to all other temperatures. Despite the variance in inbreeding depression among temperatures, we have no evidence that inbreeding depression increases consistently with our proxy for developmental stress. However, inbreeding changed which environments are perceived by *C. maculatus* as being benign versus stressful, as has been observed previously in a few studies of Drosophila (e.g., Kristensen et al. 2003). Specifically, 20°C was not a stressful rearing temperature for outbred beetles (survivorship of outbred beetles at 20°C did not differ from survivorship at the most benign temperature, 25°C) whereas both 30 and 35°C were relatively stressful for outbred beetles (survivorship was significantly lower at these high temperatures than at 25°C). In contrast, 20°C became very stressful—the most stressful of those environments studied here—after only one generation of full-sib mating. This is an important observation with relevance for understanding the interplay between genetics and environmental

stochasticity in nature. For example, it is often appreciated that the loss of genetic variation that accompanies reduced population size can affect demography and contribute to extinction risk. However, our results indicate that increased homozygosity associated with reduced population size can also change once benign environments into stressful environments (Kristensen et al. 2003), accentuating the risk of extinction by further exacerbating declines caused by the effects of inbreeding estimated from a constant environment.

Our conclusion that inbreeding depression does not increase with the degree to which an environment is stressful for outbred beetles is necessarily affected by our proxy for stress. Following the widely used proxy used in studies of mutational effects we defined stressfulness of an environment as a function of the mean fitness of individuals in that environment relative to other environments (Armbruster and Reed 2005; Martin and Lenormand 2006). Specifically, we used the probability that an outbred egg produces an adult as our proxy for fitness, though our results do not change if we use survival during each individual period of development as the fitness proxy for measuring stress during that particular period of development.

One reason why we might fail to detect a significant relationship between stress and inbreeding depression, even if such a relationship exists, is that stressful environments lower fitness and, because fitness has a lower bound of zero, the most deleterious of environmental conditions will have a truncated distribution of fitness effects and could cause a statistical artifact suggesting little or no additional inbreeding depression (Armbruster and Reed 2005). However, this is unlikely to be the case here because even the most stressful environment in this study reduced fitness less than 20% and thus, compared to other studies and stressful conditions seen in nature (Reed et al. 2003b), the stresses experienced by these beetles were relatively mild and unlikely to lead to statistical problems due to truncation of a bounded distribution.

Some studies of how environmental stress interacts with inbreeding may be confounded by the effects of stress on density (stress affects survival which in turn reduces density) and subsequent effects of density on inbreeding depression (Armbruster and Reed 2005). However, stress effects on larval density cannot confound the results of our study because all beetles were reared at one beetle per seed, with one seed per dish (larvae cannot move among seeds and cannot interact with larvae in different seeds). However, larval interactions, including both contest and scramble competition, are common in nature, in this and other seed beetle species (e.g., Messina 1991, 2004). Because inbreeding affects juvenile (larval) mortality, and thus juvenile density, some negative effects of inbreeding could be offset by positive effects (e.g., due to reduced competition) at later stages of development. Few studies have examined how competition affects inbreeding depression (Van Noordwijk and Scharloo 1981), so it is not yet possible to generalize and make predictions about how larval competition would have changed our results.

Only a few studies have examined the effect of temperature on inbreeding depression, and results have varied among these studies. Bijlsma et al. (1999, 2000) found high temperatures (30̊ C) to be a significant stress for *Drosophila melanogaster*, producing significantly greater inbreeding depression when compared to the benign environment and a larger number of lethal equivalents than three other environmental stressors (crowding, DDT exposure, ethanol exposure). Likewise, Kristensen et al. (2003, 2008) found significant increases in inbreeding depression at fluctuating temperatures compared to constant temperatures in *Drosophila buzzatii* and *D. melanogaster*, as did Reed and Bryant (2001) using the housefly (*Musca domestica*) as a model system. Unfortunately, since these studies used both high and low temperatures in combination (fluctuating between the two extremes), it is impossible to tease apart whether one extreme or a combination of both led

to the inbreeding-stress interaction. In contrast to these examples and the current study, Fowler and Whitlock (2002) found no significant increases in inbreeding depression using cold temperatures (18̊ C) or crowding as stressors in *D. melanogaster*. Reed and Bryant (2001) illustrate that which environment is stressful can vary substantially among lines and in wild versus laboratory-adapted strains of flies. In their study, outbred lines recently derived from the wild had slightly higher fitness in the variable temperature compared to the constant temperatures, while inbred lines had significantly lower fitness in the variable temperatures than in the constant. However, laboratory adapted lines did not show this trend, having lower fitness in the variable environment regardless of inbreeding level (see below). Interestingly, how temperature affects inbreeding depression for adult lifespan differs substantially between males and females in *C. maculatus*; females show generally high inbreeding depression in adult lifespan across all temperatures, whereas inbreeding depression in male lifespan is undetectable at intermediate temperatures (25–27°C) but significantly greater than 0 at the extremes (especially at 20°C) (Fox and Stillwell 2009). However, sex ratios of emerging adults did not vary with temperature (Fox and Stillwell 2009), suggesting that this substantial sex × temperature × inbreeding interaction observed for adult lifespan does not similarly occur during larval development and thus cannot explain the patterns observed in the current study.

Numerous studies have demonstrated that which QTLs affect a trait, and the degree to which specific QTLs affect a trait, vary with temperature (Fry et al. 1998; Vieira et al. 2000; Dilda and Mackay 2002), and that gene expression (Kristensen et al. 2005) and metabolic processes (Pedersen et al. 2005) are impacted by inbreeding. There also appears to be an interaction between inbreeding and temperature stress at the molecular level, at least in *Drosophila melanogaster* (Kristensen et al. 2006). Thus, the magnitude of inbreeding depression should depend on the specific deleterious recessive alleles fixed during inbreeding, the specific genetic architecture of the particular trait (e.g., Dahlgaard et al. 1995; Dahlgaard and Loeschcke 1997), and the environmental conditions (e.g., temperature) in which a trait is expressed. However, the reason why the fitness consequences of these genes should vary so much with temperature in *C. maculatus*—e.g., whether due to changes in expression of genes that repair/reduce tissue and genomic damage (Conti 2008), changes in expression of genes involved in metabolism (Pedersen et al. 2008) and general stress resistance (Kristensen et al. 2005, 2006), or due to the presence of conditional lethal alleles (Vermeulen and Bijlsma 2004; Vermeulen et al. 2008)—is not known.

One predicted pattern is that there should be stronger inbreeding depression and a greater potential for inbreeding-stress interactions in novel environmental conditions, to which the organism is not adapted, and in which the organism has had little or no opportunity to purge their genetic load. Some recent studies support the notion that whether animals are adapted, or even acclimated, to the conditions in which they are being raised is a significant factor affecting inbreeding depression (Kristensen et al. 2003). It is therefore possible that the greater inbreeding depression at colder temperatures of *C. maculatus* might be due to its origins as a tropical species (Smartt 1985; Shade et al. 1999). However, these particular study populations have been in laboratory culture for greater than 100 generations and reared at ∼25–27°C. Since the laboratory colonies never experience temperatures as low as 20°C or as high as 35°C, recessive mutations expressed only at these extreme temperatures would be free to accumulate in the genome, possibly explaining the observed increase in δ and L at these temperatures.

Contradicting the novel environment hypothesis is the evidence from *D. melanogaster* that the effects of new mutations are highly and positively correlated between the benign environment and an otherwise identical environment where low temperature constitutes a

stress. In one study, cross-environment correlations for viability among mutation accumulation lines in *D. melanogaster* had genetic correlation coefficients of 0.80 ± 0.06 (Fry and Heinsohn 2002). If new mutations in *C. maculatus* have a similar correlation structure to this, then the much lower correlations for standing genetic variation suggest that drift has fixed, or selection has weeded out, the majority of the genetic variation that is largely deleterious in both cold and more benign temperatures leaving those that have negative sign or those that are too weakly deleterious (or too recent) to be removed efficiently.

The consistently positive, but weak to moderate, correlations among temperatures for blocks in this experiment suggests that at least some loci affect inbreeding depression across the full range of temperatures examined here. This is interesting in context of the sparse literature on cross-environment correlations for inbred families of various model organisms which have generally found little or no correlation between performances across different stressors (Pray et al. 1994). Dahlgaard and Hoffmann (2000) exposed inbred populations of *D. melanogaster* to acetone, desiccation, and heat stress. Cross-environment correlations were not significant and averaged −0.04 ± 0.07. Reed and Bryant (2001) found non-significant negative correlations among 24 inbred lines of *M. domestica* for thermal stress and dietary stress (removal of yeast from the standard media), $r = -0.13$. Haag et al. (2003), using *Daphnia magnia*, found a positive but non-significant correlation, among fitness of 28 clonal lines, after infection by two different parasites ($r = 0.27$). Reed et al. (2003a) found a positive but non-significant correlation ($r = 0.02$) among 72 inbred lines of *D. melanogaster* for chemical and dietary stresses. It should be noted that the two most positive correlations come from the current study, where a continuum of a single stress (temperature) was used, and from Haag et al. (2003), where two parasites were used as the stress. Less homogeneous sources of stress would be expected to have weaker positive (or negative) correlations and there is a trend in that direction. Thus, from these limited data, cross-environment correlations among inbred lines to different stresses appear to be zero or only very weakly positive, whereas for *C. maculatus* we found generally positive, and often highly positive, correlations for inbreeding depression among temperatures.

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