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ORIGINAL ARTICLE

Environmental effects on sex differences in the genetic load for adult lifespan in a seed-feeding beetle

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We have little understanding of how environmental conditions affect the expression of the genetic load for lifespan and adult mortality rates, or how this environmental dependence affect tests of models for the evolution of senescence. We use the seed-feeding beetle, *Callosobruchus maculatus*, as a model to explore how the inbreeding load (L) affecting adult lifespan varies with rearing conditions (diet and temperature), and how rearing conditions affect tests of the mutation accumulation model of senescence. When reared under benign conditions, there was a large sex difference in inbreeding depression (δ) and the inbreeding load ($L = 0.51$–0.86 lethal equivalents per gamete for females $L = \sim 0$ for males). This sex difference in L was dependent on temperature, but not on rearing host or heat shock. At both high and low temperatures (relative to intermediate temperature) L increased for males, and L converged for the

sexes at low temperature ($L = 0.26$–0.53 for both sexes). Correlations were small for L between pairs of temperatures, indicating that the genes responsible for the inbreeding load differed between temperatures. In contrast to predictions of the mutation accumulation model of senescence, the age-specific inbreeding load for the adult mortality rate ($L_{u(t)}$) did not increase with age in any rearing environment. The genetic load underlying lifespan and adult mortality rates, and large sex differences in the genetic load, is highly dependent on environmental conditions. Estimating the genetic load in benign laboratory environments may be insufficient to predict the genetics underlying lifespan variation in nature where environmental variation is the norm.
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Introduction

Senescence is a decline in function and fitness, generally resulting in an increase in mortality rate with increasing age. It evolves because the effectiveness of selection declines with age (Charlesworth, 2000; Flatt and Promislow, 2007). However, there is tremendous variation in mortality rates and patterns of senescence at all taxonomic levels (Promislow, 1991). Understanding this variation requires an understanding of both how sources of death and selection on lifespan, reproduction and other fitness traits varies among taxa (Bonduriansky *et al.*, 2008; Monaghan *et al.*, 2008; Ricklefs, 2008) and how the genetics underlying patterns of mortality, and relationships between reproduction and mortality, varies among taxa (Wilson *et al.*, 2008). Recent studies of lifespan, primarily in *Drosophila melanogaster* (Mackay, 2002; Poirier and Seroude, 2005; Mackay and Anholt, 2006) but also in non-model systems (Munch *et al.*, 2008; Wilson *et al.*, 2008), have shown that the genetics underlying variation in adult lifespan (for example, number and effects of genes and degree of allelic and

genic interactions) can be quite complex. For example, the genetic architecture underlying variation in lifespan differs between the sexes and depends on the environmental conditions in which individuals are reared (Mackay, 2002; Burger and Promislow, 2004; Poirier and Seroude, 2005; Mackay and Anholt, 2006; Tower, 2006).

Environmental conditions can affect both the rate at which mutations occur (Foster, 2007; Baer, 2008; but see Baer *et al.*, 2006; Baer, 2008) and their subsequent expression and fitness effects in a population. Numerous studies have shown that the expression of the genetic load (the pool of deleterious recessive homozygotes) for fitness-related traits is dependent on environmental conditions (Kondrashov and Houle, 1994; Shabalina *et al.*, 1997; Fry and Heinsohn, 2002) and frequently increases with the degree of stress encountered during development (Szafraniec *et al.*, 2001; Armbruster and Reed, 2005; Martin and Lenormand, 2006; Jasnos *et al.*, 2008). This environmental dependence of gene expression, and sex differences in the environmental dependence, can affect the evolutionary dynamics of lifespan and senescence. It also limits our ability to test hypotheses for the evolution of senescence as experimental results will vary with the chosen environmental conditions (Fry and Heinsohn, 2002). Most experimental studies are carried out in benign, and often the most suitable, environments for juvenile and adult growth and survival, conditions that are not representative of stresses encountered in nature. Results from studies in controlled laboratory environments may thus not be predictive of

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how gene expression varies in nature, and thus not predictive of evolutionary dynamics that occur in nature (Roles and Conner, 2008; Wilson *et al.*, 2008). However, the degree to which environmental conditions affect the expression of genes underlying senescence and how this environmental dependence varies among the sexes are still poorly understood, even for the best studied model system (*Drosophila*; see Vermeulen and Bijlsma (2004).

One of the main genetic mechanisms proposed for the evolution of senescence is the accumulation in populations of late-acting deleterious alleles due to the declining force of selection with increasing age (mutation accumulation theory; Hughes and Reynolds, 2005; Wilson *et al.*, 2008). One method of testing this mutation accumulation hypothesis has been to quantify the inbreeding load (genetic or mutational load) affecting adult lifespan, or other fitness traits, and test whether the inbreeding load changes with age; the mutation accumulation hypothesis predicts that the inbreeding load will increase with age (Charlesworth and Hughes, 1996; Hughes and Reynolds, 2005). Studies of *Drosophila* using this approach, or comparing the ratio of dominance to additive genetic variance, have generally supported the mutation accumulation hypothesis (Snoke and Promislow, 2003; Swindell and Bouzat, 2006; Reynolds *et al.*, 2007). In contrast, a few recent studies of other model systems have found that the inbreeding load does not always increase with age (Fox *et al.*, 2006; Wilson *et al.*, 2008), and other studies have shown that the relationship between age and the inbreeding load varies between the sexes or among populations/lines (Lesser *et al.*, 2006; Reynolds *et al.*, 2007; Keller *et al.*, 2008). Understanding this diversity of experimental results requires analysis of the sources of variation in age-specific expression in the genetic load. For example, the accumulation of late-acting deleterious recessives, the expression of this genetic load and our ability to detect this genetic load could all be dependent on environmental conditions.

In this study, we examine how the inbreeding load affecting adult lifespan varies with rearing conditions (diet and temperature), and the implications of this for tests of the mutation accumulation model of senescence, in two populations of a seed-feeding beetle, *Callosobruchus maculatus*. This beetle is a commonly used model for demographic studies of aging, senescence and the cost of reproduction (Møller *et al.*, 1989a, b, 1990; Tatar *et al.*, 1993; Tatar and Carey, 1994, 1995; Messina and Slade, 1999; Fox and Moya-Laraño, 2003; Fox *et al.*, 2003a, b, 2004a, b, 2006). The genetic architecture underlying variation in adult lifespan of *C. maculatus* differs substantially between the sexes (Fox *et al.*, 2004c, 2006). Inbreeding studies within populations show that females have a much higher genetic load (inbreeding load) than do males (Fox *et al.*, 2006; Bilde *et al.*, 2009). Crosses between populations indicate that genes affecting lifespan are primarily autosomal and exhibit substantial dominance for long life in females, but not in males (Fox *et al.*, 2004b), consistent with results from within-population inbreeding studies. There is no evidence that expression of the inbreeding load increases with age in *C. maculatus*; instead, the trend is for expression of the inbreeding load to decrease with age (Fox *et al.*, 2006). However, these earlier studies were all conducted with beetles reared on high quality hosts and at benign temperatures (25–26 °C). Studies with *C. maculatus* have shown that higher and lower temperatures, and alternate

hosts, represent more stressful environments for beetle development (Lale and Vidal, 2003; Stillwell *et al.*, 2007), and that the inbreeding load for larval survival, development time and body size at emergence all vary with temperature. Mean adult lifespan for *C. maculatus* is dependent on larval diet (rearing host; Fox *et al.*, 2004a), and sex differences in a variety of traits (for example, development time and adult body mass) vary quite substantially with temperature (Stillwell and Fox, 2007; Stillwell *et al.*, 2007). It is thus clear that gene expression and sex differences in gene expression underlying variation in adult lifespan and patterns of adult mortality vary with environmental conditions.

In this study, we examine the effect of rearing and adult environment (diet, temperature and heat shock) on the genetic load affecting adult lifespan in *C. maculatus*. Specifically, we test the hypotheses that (a) the inbreeding load affecting adult lifespan varies with rearing host, temperature and adult heat shock; (b) the large sex difference in the inbreeding load observed in earlier studies is dependent on the environmental conditions; (c) the inbreeding load is independent of age (or declines with age), as observed in earlier studies of *C. maculatus*, regardless of environmental conditions; (d) the inbreeding load is influenced by the same genes/alleles, and those alleles have similar effects, across environments.

Materials and methods

The biology of *C. maculatus*
The life cycle of *C. maculatus* revolves around seeds. Females cement their eggs to the surface of host seeds (Messina, 1991, 2003). When eggs hatch, the 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. *C. maculatus* suffers substantial inbreeding depression throughout development (Tran and Credland, 1995; Fox *et al.*, 2007). Inbreeding has been shown to affect female, but not male, lifespan and adult body size (Tran and Credland, 1995; Fox *et al.*, 2006). Inbreeding also negatively affects female fecundity (Tran and Credland, 1995).

We used two populations of beetles for this study. The South Indian (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, from Tirunelveli, India (Mitchell, 1991). The Burkina Faso (BF) population was collected in 1989 from infested pods of cowpea, *V. unguiculata* (L.) Walp., from Ouagadougou, Burkina Faso (Messina, 1993). These two populations differ in body size, lifetime fecundity, patterns of egg dispersion, oviposition preference and adult longevity (Messina, 2004; Fox *et al.*, 2004a, b). Both populations were maintained in laboratory growth chambers on seeds of *V. radiata* (SI) or *V. unguiculata* (BF) at >1000 adults per generation for >200 (BF) or >250 generations (SI) before this experiment.

Experimental design
Our experimental design is illustrated in Supplementary Figure 1. To measure inbreeding depression, we used a

'block' design (Roff, 1998). Blocks were created by randomly pairing two families chosen from an outbred population. From each family, we randomly chose two female and two males to become parents. We crossed these 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).

We ran three sequential experiments. In experiment 1, we reared inbred and outbred beetles on two different rearing hosts, seeds of cowpea (*V. unguiculata*) and mung (*V. radiata*), to examine the effect of rearing host on the magnitude of inbreeding depression in adult lifespan and adult mortality rates. Cowpea and mung are the native hosts for the BF and SI populations, respectively. Larval egg-to-adult survival is generally much greater on mung seeds than on cowpea seeds, but the relative difference in suitability of these hosts differs between the two populations (Stillwell *et al.*, 2007). Experiment 2 was nearly identical to experiment 1 except that beetles were reared at one of four different temperatures. These temperatures span the range of daily mean temperatures found in the native ranges of the two populations (National Climatic Data Center's Global Surface Summary of Day, Asheville, NC, USA) and include a range of stressfulness for larval development. Larval egg-to-adult survival for these two populations of *C. maculatus* is highest at temperatures between 25 and 30 °C (Stillwell *et al.*, 2007), but females lay very few eggs, and those eggs often fail to develop, at temperatures close to 40 °C (Lale and Vidal, 2003). Lastly, in experiment 3, we expose females to a brief period of high temperature (45 °C for 60 min) 24 h after their emergence as an adult, and examine the effects of this heat shock on adult lifespan and the inbreeding load for lifespan.

Experiment 1: effect of diet on inbreeding depression
Pairs of beetles created using the block design were confined in a 35-mm Petri dish with 35 seeds of either mung or in a 60-mm Petri dish with 35 seeds of cowpea, *V. unguiculata*. Each block was randomly assigned to only one host. Dishes were checked for eggs every 12 h until females had laid at least one egg on each of 20 seeds (this usually occurred within 12 h of pairing). Larvae were allowed to develop at one egg per seed (excess eggs were scraped from the seed), one seed per 35-mm dish, inside a temperature- and photoperiod-controlled growth chamber at 27 °C, light:dark 15:9. Dishes were checked twice per day for adult beetles that emerged from a seed. Emerged beetles were transferred as virgins to a clean 35-mm Petri dish, one beetle per dish, and checked twice per day for their time of death.

In total, we scored adult lifespan of 3442 beetles: 855 females and 1002 males from 32 blocks per host (64 total) from the BF population and 784 females and 801 males from 30 and 33 blocks (reared on cowpea and mung bean, respectively) from the SI population.

Experiment 2: effect of temperature on inbreeding depression
Pairs created using the block design were confined in a 35-mm Petri dish with 40 mung seeds until they had laid at least 40 eggs. Dishes were checked for eggs every 12 h. At each check, eggs were evenly divided among four temperature treatments, 20, 25, 30 and 35 °C (all ± 0.5), and these seeds bearing eggs were replaced with fresh seeds for female oviposition. Larvae were reared to adult, and adult lifespan was scored as in experiment 1. Beetles were maintained as adults at the same temperature at which they were raised (that is, this study does not disentangle larval from adult experiences).

This experiment differed from experiment 1 in that (a) we manipulated temperature instead of rearing host, (b) we used a split-family design in which offspring within each family were split evenly among the four temperature treatments and (c) adult beetles were all weighed within 12 h of their emergence as an adult.

We scored adult lifespan for 10 269 beetles: 2558 females and 2781 males from 46 blocks in the BF population, and 2415 females and 2515 males from 46 blocks in the SI population.

Experiment 3: effect of heat shock on inbreeding depression
Pairs created using the block design were confined with 25 mung seeds until they had laid ~20 eggs. Larvae (10 eggs per dish) were reared to adult at 26 °C, light:dark 15:9. Emerged beetles were transferred to a clean 35 mm Petri dish for 24 h. After 24 h, 40% of the adults (randomly chosen) were transferred to a 0.5-dram vial and heat shocked for 60 min at 45 °C in a temperature-controlled water bath. This temperature is high enough to incapacitate adult beetles, but does not cause immediate death. These heat-shocked beetles were then transferred to a new 35-mm dish (at 26 °C) and, along with control beetles, were checked once per day for their time of death.

We scored adult lifespan for 4491 beetles: 971 females and 1084 males from 60 blocks in the BF population, and 1172 females and 1264 males from 66 blocks in the SI population, with ~40% heat shocked per population.

Analyses
Blocks are the lowest level of independence in this design. Each block contains four means, one for each sex-by-inbreeding 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 the families within the block.

Lifespan data (block means) were log-transformed to fit assumptions of standard general linear models (to remove heterogeneity of variances among treatments) and then analyzed using analysis of variance with log (lifespan) as the response variable.

The magnitude of inbreeding depression was measured as the proportional decrease in lifespan due to inbreeding, $\delta = (\text{Mean}_{\text{outbred}} - \text{Mean}_{\text{inbred}}) / \text{Mean}_{\text{outbred}}$. The inbreeding load ($L$) affecting the age-specific mortality rate was estimated as the number of lethal equivalents per gamete (Simmons and Crow, 1977; Lynch and Walsh, 1998; Charlesworth and Charlesworth, 1999) following the methods in Swindell and Bouzat (2006). The inbreeding load ($L_{u(t)}$) affecting age specific

mortality, $u(t)$, was calculated as

$$L_{u(t)} = \frac{1}{F}\ln\left(\frac{u(t)_{\text{inbred}}}{u(t)_{\text{outbred}}}\right),$$

where $u(t)$ is the age-specific mortality rate, $u(t) = -\ln[p(t)]$ (where $p(t)$ is the probability of surviving from the beginning of age t to the beginning of age $t+1$) and F is the inbreeding coefficient (0.25 in our experiment). The age-specific mortality rate $u(t)$ was calculated using WinModest (Pletcher, 1999). The inbreeding load for genes affecting total adult lifespan was estimated by regressing lifespan on the inbreeding coefficient, which, for our two treatment case, is

$$L_{\text{Lifespan}} = \frac{-[\ln(\text{Lifespan}_{\text{inbred}}) - \ln(\text{Lifespan}_{\text{outbred}})]}{F}.$$

L_{Lifespan} was calculated separately for each block, and then averaged across blocks within a population/species/sex. Both δ and L were normally distributed, and thus analyzed with analysis of variance.

Results

Experiment 1: rearing host affects adult lifespan, but not inbreeding depression
Rearing host affects lifespan of outbred beetles: In contrast to earlier experiments, we detected no difference
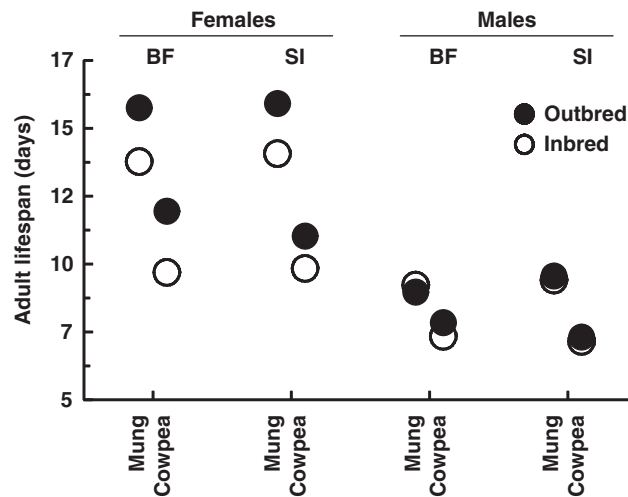

Figure 1 Adult lifespan for two populations of *Callosobruchus maculatus* reared on two different hosts. Means are calculated first by averaging across families in a block, then across blocks. All standard errors are all smaller than the points, and thus not visible on the figure. Burkina Faso (BF) and South Indians (SI) are the two study populations.

in adult lifespan of either sex between the two study populations of *C. maculatus* (Figure 1; comparison of outbreds only; analysis of variance, population effect: $F_{1,250} = 0.53$, $P = 0.47$; sex-by-population interaction; $F_{1,250} = 0.72$, $P = 0.40$). However, rearing host had a large effect on adult lifespan of outbred beetles—beetles had shorter adult lifespan when reared on cowpea seeds than when reared on mung seeds (Figure 1; $F_{1,250} = 155.6$, $P < 0.001$). This host effect differed between populations (Figure 1; host-by-population interaction; $F_{1,250} = 5.17$, $P = 0.02$); the two populations had similar adult lifespans when beetles were reared on mung seeds ($F_{1,126} = 1.38$, $P = 0.24$), whereas BF beetles (which are adapted to cowpea) lived longer than SI beetles when reared on cowpea ($F_{1,124} = 3.96$, $P = 0.049$).

Inbreeding depression: As observed in our earlier studies, the effect of inbreeding on adult lifespan differed substantially between males and females (sex effect on δ; $F_{1,246} = 20.4$, $P < 0.001$). There was no detectable effect of inbreeding on the adult lifespan of males in either population, regardless of rearing host (Table 1; t-tests for $\delta \neq 0$, $|t| < 1.74$, $P > 0.09$ for all population–host combination). In contrast, inbreeding reduced the adult lifespan of females by ~ 10–17% (Table 1); δ was significantly >0 for all population-by-host combinations, except SI females on cowpea, for which δ was similar to the other population/host combinations but for which the standard error was large (s.e. $= 0.06$). This inbreeding depression translated into estimates of the genetic load affecting adult lifespan of $L = 0.40$–0.86 for females and $L \leqslant 0.28$ for males (Table 1).

The magnitude of inbreeding depression did not differ between the two populations for either sex of beetles (males: $F_{1,123} = 0.11$, $P = 0.74$; females: $F_{1,122} = 1.07$, $P = 0.30$). Rearing host had no effect on the magnitude of inbreeding depression on adult lifespan for either males ($F_{1,123} = 1.63$, $P = 0.20$) or females ($F_{1,122} = 0.69$, $P = 0.41$).

Change of inbreeding load with age: There was no evidence that genetic load (L) calculated from the adult mortality rate ($u(t)$) increased with age for either population or rearing host (Figure 2). Instead, the genetic load tended to decline with age, in contrast to predictions from mutation accumulation models; when treating each population-by-host-by-sex combination as a single replicate, there was a significant negative effect

Table 1 The effect of inbreeding on adult lifespan of *Callosobruchus maculatus* reared on two different host species

Population/host	N	Inbreeding depression, δ		Inbreeding load, L	
		Females	*Males*	*Females*	*Males*
Burkina Faso					
Reared on cowpea	32	0.17 ± 0.04	0.05 ± 0.03	0.86 ± 0.17	0.28 ± 0.13
Reared on mung	32	0.12 ± 0.02	-0.04 ± 0.03	0.53 ± 0.11	-0.12 ± 0.11
South Indian					
Reared on cowpea	30	0.11 ± 0.06	-0.01 ± 0.05	0.40 ± 0.18	0.11 ± 0.20
Reared on mung	33	0.10 ± 0.04	-0.00 ± 0.03	0.51 ± 0.16	0.06 ± 0.15

Inbreeding depression, δ, is the proportional decrease in adult lifespan of inbred relative to outbred beetles ($\pm$ s.e.m.). Both δ and L are calculated separately for each block and then averaged across blocks.
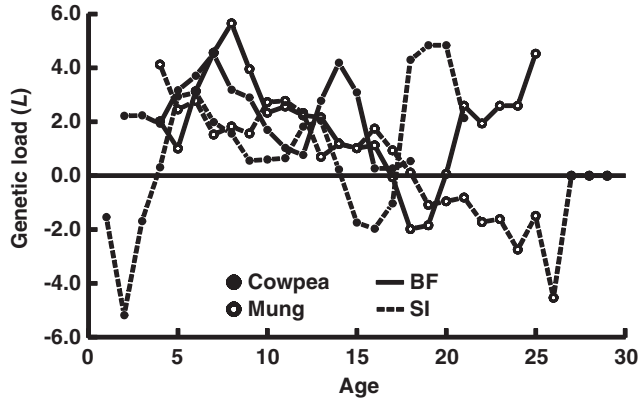
Figure 2 Age-specific inbreeding load for the mortality rate of female *Callosobruchus maculatus* reared on two hosts. The lines are smoothed by averaging 3-day intervals centered on the age on the x axis. Cowpea and mung are the two rearing hosts; the Burkina Faso (BF) and South Indians (SI) are the two study populations.

of age on the genetic load for the adult mortality rate ($F_{1,7} = 5.86$, $P = 0.046$; Figure 2). The average decline in the number of lethal equivalents expressed per day was 0.10 ± 0.09 for females and 0.06 ± 0.10 for males (neither of which was individually significantly different from 0). Because of the small number of lines (two populations-by-two hosts), this analysis did not have enough statistical power to detect significant differences between hosts or the sexes in the slope of the relationship between genetic load and age.

Experiment 2: temperature affects inbreeding depression on adult lifespan

Temperature affects the sex difference in lifespan of outbred beetles: Not surprisingly, both sex and temperature had very large effect on the adult lifespan of outbred beetles (Supplementary Figure 2; sex effect: $F_{1,685} = 671.6$, $P < 0.001$; temperature effect: $F_{3,685} = 2638$, $P < 0.001$). More interestingly, the effect of temperature on adult lifespan of outbred beetles differed substantially between the sexes (sex-by-temperature interaction: $F_{3,685} = 66$, $P < 0.001$); females lived only 18% longer than males when reared at 20 °C, but lived 35, 43 and 42% longer when reared at 25, 30 and 35 °C, respectively. This variation in lifespan dimorphism was concordant with the temperature effect on body size dimorphism; outbred females were 6, 44, 59 and 34% larger than males when reared at 20, 25, 30 and 35 °C, respectively. Dimorphism in body mass did explain some of the sex effect on variation in lifespan among temperatures (mass dimorphism effect on lifespan dimorphism; $F_{1,342} = 26.1$, $P < 0.001$). However, even after correcting for the temperature-by-sex interaction effect on body size, females lived only 14% longer than males at 20 °C, compared with living 43, 54 and 29% longer than males at 25, 30 and 35 °C, respectively.

Inbreeding depression: As observed in experiment 1, the effect of inbreeding on adult lifespan differed substantially between males and females ($F_{1,678} = 14.6$, $P < 0.001$; Figure 3; Table 2). For example, at the most benign temperature (25 °C), inbreeding depression was
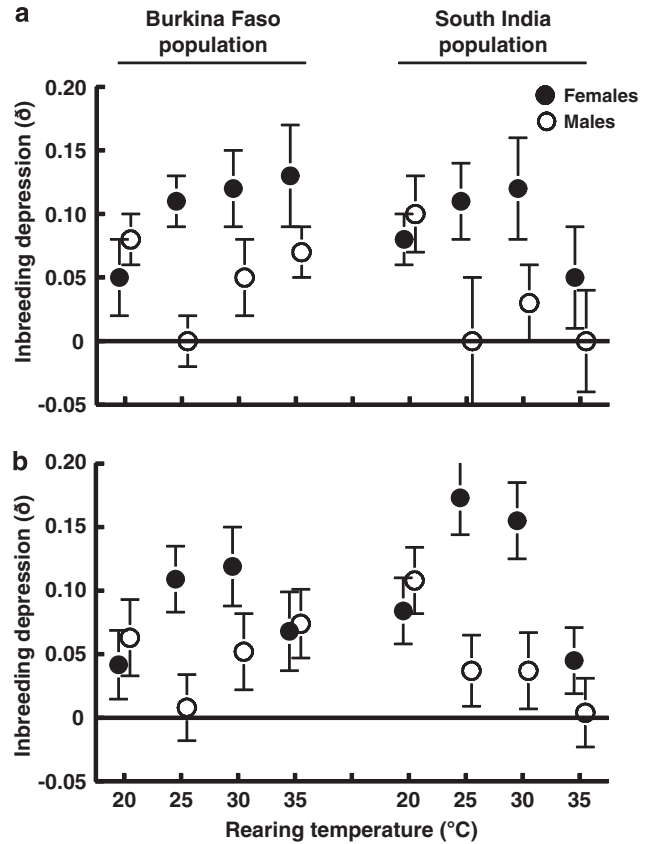

Figure 3 The magnitude of inbreeding depression (δ) for adult lifespan of two populations of *Callosobruchus maculatus* reared at four temperatures. (**a**) δ For lifespan before correcting for effects of body mass. (**b**) δ Is least square means after removing the effect of inbreeding on adult body mass; note that the pattern changes little. δ Was calculated for each block, and then averaged across blocks; note that both δ and L for males are ~0 (and substantially less than females) when reared at 25 °C, but much greater than 0 (and not less than females) when reared at 20 °C.

Table 2 The estimated genetic load (L) for adult lifespan of *Callosobruchus maculatus* reared at four different temperatures

Temperature	Inbreeding load, L	
	Females	*Males*
Burkina Faso		
20 °C	0.26 ± 0.12	0.38 ± 0.10
25 °C	0.54 ± 0.10	0.05 ± 0.07
30 °C	0.61 ± 0.13	0.33 ± 0.19
35 °C	0.79 ± 0.21	0.36 ± 0.10
South Indian		
20 °C	0.40 ± 0.10	0.53 ± 0.13
25 °C	0.61 ± 0.15	0.06 ± 0.10
30 °C	0.63 ± 0.17	0.18 ± 0.12
35 °C	0.34 ± 0.15	0.19 ± 0.14

L was calculated separately for each block and then averaged across blocks.

substantial for female lifespan (δ = 0.11 for both BF and SI), but 0 for male lifespan (Figure 3a). This translated into a difference in the genetic load (L) of 0.54–0.64 lethal equivalents per haploid gamete for females (for BF and

SI, respectively), but only 0.05–0.06 lethal equivalents for males (Table 2). These results for 25 °C are consistent with the results of the experiment 1 (above) and of the earlier published studies (Fox *et al.*, 2006).

However, the difference in δ between the sexes was dependent on temperature (sex-by-temperature interaction on δ: $F_{3,678} = 14.6$, $P < 0.001$). Inbreeding depression on adult lifespan was largely unaffected by temperature for females (Figure 3; $F_{3,338} = 1.27$, $P = 0.29$), whereas inbreeding depression was highly variable among temperatures for males (Figure 3; $F_{3,338} = 3.90$, $P = 0.009$). As in all earlier studies for these two populations of *C. maculatus*, δ for male lifespan was not significantly different from 0, and was significantly lower than δ for females, when beetles were reared and maintained at the benign temperature of 25 °C. In contrast, δ for male lifespan was significantly > 0, and not significantly different from δ for females, when beetles were reared and maintained at 20 °C; *post hoc* contrasts showed that δ measured for male at 20 °C was significantly (or nearly significant) higher than δ measured at all other temperatures ($P < 0.064$ for all pairwise comparisons). For the two higher temperatures, δ for male lifespan tended toward being slightly positive, but was consistently less than δ for female lifespan, and in neither case was δ (at 30 or 35 °C) different from δ at 25 °C ($P > 0.2$ for each comparison).

Inbreeding depression on adult lifespan did not differ between the two populations ($F_{1,678} = 1.10$, $P = 0.29$). Also, all interactions including population were non-significant; despite the suggestion of a difference between SI and BF in the temperature effect on δ for male lifespan (Figure 3), this and all other interactions were non-significant (for example, population-by-temperature interaction, $F_{3,338} = 1.32$, $P = 0.27$), indicating that the inbreeding effect on lifespan, the sex-by-temperature interaction and the temperature effect on inbreeding depression for males were all similar between the two populations.

Inbred beetles were smaller as adults than were outbred beetles ($F_{1,1378} = 226$, $P < 0.001$), and temperature affected body mass, ($F_{1,1378} = 986$, $P < 0.001$), sexual dimorphism in body mass (see above) and the magnitude of inbreeding depression on body mass ($F_{1,678} = 8.11$, $P < 0.001$). It is thus possible that temperature and inbreeding effects on body mass could explain the temperature effect on δ for male lifespan, the sex effect on δ for adult lifespan or the sex-by-temperature interaction for δ for adult lifespan. Inbreeding depression on male lifespan was indeed positively correlated (among blocks) with inbreeding depression in body mass; blocks that showed the most inbreeding depression in male mass also showed the most inbreeding depression in male lifespan (analysis of covariance, effect of $\delta_{\text{Body mass}}$ on δ_{Lifespan}, $F_{1,330} = 48.0$, $P < 0.001$). However, including $\delta_{\text{Body mass}}$ into the analysis of variance for δ_{Lifespan} was not adequate to explain the entire sex and sex-by-temperature effects of δ_{Lifespan}, both of which remained highly significant after correcting for inbreeding depression in body mass (sex: $F_{1,659} = 6.88$, $P = 0.009$; sex-by-temperature: $F_{3,659} = 3.94$, $P = 0.008$; Figure 3b).

Change of inbreeding load with age:

As in experiment 1, the genetic load calculated from the adult mortality rate ($u(t)$) generally declined with age, in contrast
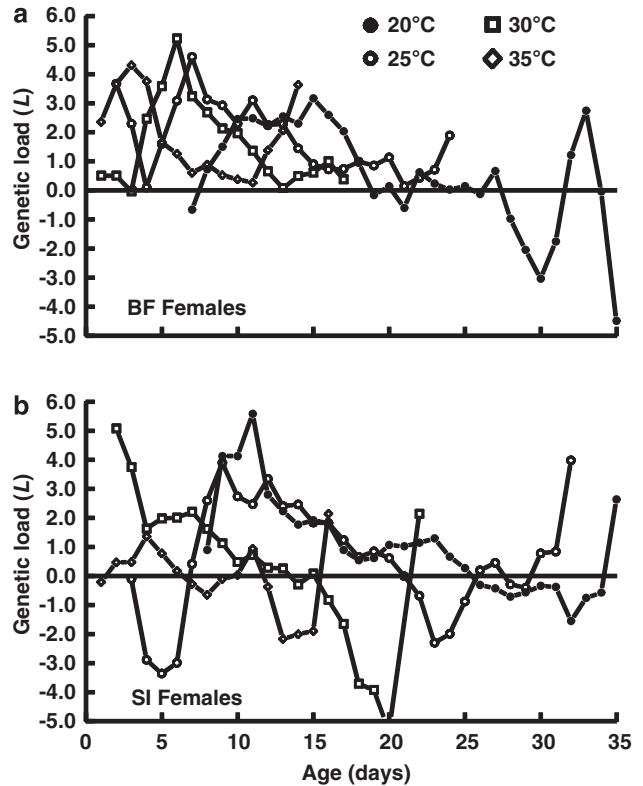

Figure 4 Age-specific inbreeding load for the mortality rate of female *Callosobruchus maculatus* reared at four temperatures. The lines are smoothed by averaging 3-day intervals centered on the age on the x axis. The Burkina Faso (BF) and South Indians (SI) are the two study populations.

to predictions from mutation accumulation theory ($F_{1,11} = 14.7$, $P = 0.003$; Figure 4). The average decline in the number of lethal equivalents expressed per day was 0.07 ± 0.03 for females and 0.20 ± 0.09 for males (this high estimate for males was driven by two particularly highly negative slopes, both for beetles reared at 30 °C; after deleting those two slopes the average decline per day is 0.09 ± 0.06). As observed for lifespan, the inbreeding load for the adult mortality rate varied among temperatures for males ($P = 0.04$), but not for females ($P = 0.49$). However, we detected no significant difference in the change in L per day between the sexes ($F_{1,11} = 0.18$ $P = 0.68$) or among temperatures ($F_{3,11} = 2.85$, $P = 0.09$).

Correlation of the genetic load across environments:

As we used a split-family design for experiment 2 (in contrast to experiment 1), with full siblings' split among the four temperatures, we can test whether the genes/alleles that contribute to inbreeding depression in one environment are the same and have similar effects on inbreeding depression in alternate environments. Specifically, we calculated Pearson correlation coefficients for the genetic load between each pair of temperatures (Supplementary Table 1). The correlations between L for pairs of temperatures, for both males and females, are all quite low (with one exception, all are $\leqslant 0.35$), and only one is significantly different from 0 ($r = 0.41$ for 20 vs 25 °C in males) after correcting for multiple comparisons with $n = 6$ estimates per sex/

population combination. We interpret this as evidence that either the specific genes contributing to the genetic load for lifespan vary substantially among temperatures, or that the allelic effects at those loci vary among temperatures, with few genes/alleles having similar effects across all temperatures.

Experiment 3: heat shock does not affect inbreeding depression on adult lifespan

Despite heat shock at a high enough temperature to knock down adult beetles (45 °C), heat shock had no effect on adult lifespan ($F_{1,970} = 0.47$, $P = 0.49$). As observed in the earlier experiments, the magnitude of inbreeding depression differed between the sexes (sex effect on δ: $F_{1,466} = 14.9$, $P < 0.001$); there was significant inbreeding depression on female lifespan (average $\delta = 0.07 \pm 0.03$, averaged across populations and treatments), but not on male lifespan ($\delta = -0.01 \pm 0.01$). This was equivalent to an inbreeding load of 0.38 ± 0.13 lethal equivalents affecting female lifespan and 0.04 ± 0.06 for male lifespan (this estimate for females is lower than that in the earlier experiments, but L shows the same significant sex difference).

Heat shock had no effect on the magnitude of inbreeding depression (non-significant effect of heat shock on δ; $F_{1,466} = 0.06$, $P = 0.81$), and there was no effect of heat shock on the sex difference in δ (non-significant sex-by-heat shock interaction: $F_{1,466} = 1.60$, $P = 0.21$).

Discussion

Using *Callosobruchus maculatus* as a model system for understanding the genetics underlying patterns of adult lifespan and age-specific mortality, we found that (a) the inbreeding load affecting adult male lifespan (but not female lifespan) varied with temperature; (b) neither rearing host nor adult heat shock affected the inbreeding load for adult lifespan of either sex; (c) the large sex difference in the inbreeding load observed in earlier studies (Fox et al., 2006; Bilde et al., 2009) was dependent on temperature, but not on rearing host or adult heat shock; (d) the inbreeding load for the adult mortality rate was negatively correlated with age (or uncorrelated with age), in contrast to predictions of mutation accumulation models of senescence, regardless of environmental conditions; and (e) the inbreeding load affecting adult lifespan of *C. maculatus* is influenced by different genes, or the genes affecting lifespan have very different effects, at different temperatures. We also found that, although there was detectable inbreeding depression on adult body mass, and the magnitude of this inbreeding depression on body mass varied with temperature, these effects on body mass were not adequate to explain the large temperature effect on inbreeding depression for adult lifespan, nor the sex-by-temperature interaction for the inbreeding load for adult lifespan.

Temperature affects the sex difference in inbreeding load for *C. maculatus*

Previously we showed that the magnitude of inbreeding depression for *C. maculatus* on adult lifespan differs between the sexes (Fox et al., 2006). However, that earlier study was carried out under benign laboratory condi-

tions. In this study, we found that this sex difference in the inbreeding load is highly temperature dependent. At the intermediate (benign) temperature (26–27 °C), inbreeding had a large effect on female lifespan ($\delta \sim 12\%$), but no detectable effect on male lifespan. This was found in all three experiments reported here (Fox et al., 2006). However, δ and L for male lifespan were greater at the extreme temperatures (especially at 20 °C) than at 25 °C, and did not differ between males and females at the low extreme temperature, 20 °C (in contrast to the large difference between the sexes at intermediate temperature). Thus, not only does the expression of the genetic load affecting adult lifespan vary with temperature, the sex difference in the genetic load is highly dependent on temperature.

Why is there a large difference in the inbreeding load between males and females and why does this sex difference vary with temperature? If variation in lifespan is primarily affected by sex-linked genes, and those sex-linked genes have gender-specific expression, then sex linkage could lead to greater purging of deleterious alleles, and thus a lower genetic load, in males (the heterogametic sex) than in females. This hypothesis could also explain the sex-specific temperature effect on the genetic load; as the beetle colonies are maintained at ~ 25 °C and have been in the laboratory for > 200 generations, we expect purging of alleles expressed at this temperature, but possibly the accumulation of deleterious alleles expressed at other temperatures (those temperatures not experienced in the colonies). Thus, purging of deleterious alleles at sex-linked loci that have temperature-specific expression could lead to a large sex difference in the inbreeding load in the 'natural' environment (25 °C), but substantially greater inbreeding load for males, and thus reduction of the sex difference, at other temperatures. However, line crosses between long- and short-lived populations of *C. maculatus* fail to detect any evidence of sex-linkage of long-lifespan (Fox et al., 2004b). These line crosses examine the genetic architecture of differences between populations, but do find similar patterns of sex differences as found in within-population inbreeding studies—dominance of long-life genes over short-life genes in females, but not in males. Though it is not necessarily the case that variation in lifespan within populations shares the same underlying genetic architecture as does variation among populations, the line cross results suggest that sex-linkage is not likely an explanation for the sex differences observed here. Line crosses also find no evidence for maternal inheritance, indicating that sex-specific effects of mitochondria (Tower, 2006) is also an unlikely explanation.

Alternatively, the patterns for lifespan may be mediated by inbreeding effects on body size. Inbreeding depression on *C. maculatus* body size varies with temperature, and one study has shown that it is greater for female than male size (Tran and Credland, 1995). However, the sex-difference in inbreeding depression on body size observed by Tran and Credland (1995) has not been observed in these two study populations. Also, when considering body size in our analyses, all of our main effects (temperature, sex and the interaction) remained significant and similar in magnitude. Alternatively, the sex difference and temperature sensitivity of the inbreeding load could be due to non-random larval

mortality across temperatures. However, we found no evidence here that sex-specific mortality varied with temperature (in contrast to the results of Stillwell and Fox (2007)); sex ratios were consistently male biased (~52% male in experiment 2), but this ratio did not vary with temperature (53, 50, 53 and 51% male for 20, 25, 30 and 35 °C, respectively). This does not exclude the possibility that the specific genotypes that died varied with temperature in a sex-specific manner, but we think this explanation unlikely.

A more likely explanation for the temperature sensitivity in the sex difference in the inbreeding load is that the loci affecting adult lifespan differ between the sexes and in their sensitivity to temperature. In *Drosophila*, many loci have sex-specific effects on adult lifespan (Nuzhdin *et al.*, 1997; Pasyukova *et al.*, 2000; Vieira *et al.*, 2000; Leips and Mackay, 2002; Forbes *et al.*, 2004; Mackay *et al.*, 2005). Indeed, sex-specific expression of at least some of the genes underlying a complex trait is nearly a ubiquitous result in quantitative trait locus (QTL) studies (Mackay and Anholt, 2006; but see Curtsinger (2002) who suggests that we may commonly overestimate the frequency of sex-specific expression). Variation in allelic effects (including degree of dominance) among loci can thus generate differences in dominance and inbreeding depression between males and females. Many studies, especially of *Drosophila*, have found that the expression of genes affecting adult lifespan is environmentally dependent (including temperature-dependent) and that the degree of environmental dependence differs between the sexes (Leips and Mackay, 2000; Vieira *et al.*, 2000). For example, dietary restriction can extend adult lifespan in *Drosophila*, but the magnitude of the effect differs between the sexes (Burger and Promislow, 2004; Magwere *et al.*, 2004). The sex difference in temperature-sensitivity of the genetic load in *C. maculatus* must necessarily involve some degree of sex difference in the degree of environmental sensitivity of gene expression. The generally small correlations between the inbreeding load for adult lifespan between pairs of temperatures (Supplementary Table 1), which contrast substantially with the much higher correlations observed for the inbreeding load across temperatures for periods of larval mortality, indicate that the genes responsible for the inbreeding load affecting adult lifespan differ between temperatures. However, further research is needed to identify the mechanism underlying this temperature-by-sex interaction.

There is substantial debate in the literature about the role of stress in mediating expression of the genetic load (Armbruster and Reed, 2005; Jasnos *et al.*, 2008). Much of the variation in patterns of mortality in nature may reflect variation in the ability of organisms to respond to naturally occurring stresses (Hughes and Reynolds, 2005; Parsons, 2007; Vleck *et al.*, 2007; Kuningas *et al.*, 2008; Mangel, 2008) and gene-mediating stress responses, either by avoiding or repairing damage, have been shown to have large effects on lifespan (Schumacher *et al.*, 2008). We have no measures of oxidative or other physiological stressors (Conti, 2008), and how they vary with temperature, in *C. maculatus*. However, using temperature effects on larval survivorship as proxies for the degree of stress experienced by *C. maculatus* at the temperatures used in this experiment, our treatments range from fairly benign (25 °C) to stressful (the two

extremes; Stillwell *et al.*, 2007), and the sex difference in the inbreeding load is greatest at the intermediate, benign, temperature and smallest at the most stressful temperatures. At least one study of *Drosophila* has found results qualitatively similar to this—that the genetic architecture underlying lifespan is different between the sexes when adults are in benign conditions (virgin flies), but converges to similar architecture for the sexes under more 'stressful' conditions (mixed-sex cages in which overall lifespan is reduced; Reiwitch and Nuzhdin, 2002). Genes that influence stress responses in *D. melanogaster* have sex-specific expression (Harbison *et al.*, 2004; Morgan and Mackay, 2006; Wang *et al.*, 2006; May, 2007) or are sex-linked (Norry *et al.*, 2007). It is thus possible that the temperature effect on the sex difference in inbreeding load for *C. maculatus* is due to a sex difference in the temperature effect on a generalized stress response. However, our finding that no effect of heat shock or rearing host on the sex difference in the inbreeding load suggests that the response is specific to temperature rather than being a generalized stress response. Unfortunately, our experiment failed to distinguish between larval and adult temperatures, so it is unclear whether the sex difference reflects late-acting consequences of changes in development in response to larval rearing temperature, or whether the sex difference reflects changes in adult gene expression in response to adult temperature.

The decline in inbreeding load with age

In our earlier study (Fox *et al.*, 2006), we found that the inbreeding load for adult mortality generally declined with age in *C. maculatus*, in contrast to the typical result observed for *Drosophila* and inconsistent with predictions of mutation-accumulation models (Hughes and Reynolds, 2005). Here, we found that a decline (or a lack of change) in inbreeding load with age is repeatable across all population/sex/treatment combinations, despite large effects of some environmental conditions (temperature) on the inbreeding load (Figures 2 and 4). We found no evidence for the increase of inbreeding load with age.

Though the accumulation of mutations must necessarily occur when selection is relaxed, the extent to which this is a major contributor to the evolution of senescence is unclear (relative to the importance of antagonistic pleiotropy between early and late fitness). Most tests of mutation accumulation models with *Drosophila* have found that the genetic load and/or genetic variance for fitness traits increase with age (Snoke and Promislow, 2003; Gong *et al.*, 2006; Swindell and Bouzat, 2006; Borash *et al.*, 2007), although results have varied among lines and/or differed between the sexes (Lesser *et al.*, 2006; Reynolds *et al.*, 2007). The evidence is more equivocal in non-*Drosophila* systems (Wilson *et al.*, 2008). Some studies have found clear increases in genetic variance and inbreeding depression for mortality rates with age (Escobar *et al.*, 2008), but others have failed to support the mutation accumulation predictions (Fox *et al.*, 2006), and yet others have found different results in different study populations or in the two sexes (Keller *et al.*, 2008). It thus appears that the significance of mutation accumulation as a driver of the evolution of senescence varies among organisms (Hughes and Reynolds, 2005).

The question yet to be answered is why we observe such variation and why the relative age-specific expression of deleterious mutations sometimes differs between the sexes. To answer these questions, we need studies on a greater diversity of organisms, including non-model species (Partridge and Gems, 2007; Wilson et al., 2008).

Our study was carried out on laboratory-adapted populations (although large outbred populations), and adaptation to laboratory conditions has been proposed as an explanation for some of the variation in results among experimental studies of senescence. For example, as beetles age, the mortality rate decelerates (Fox and Moya-Laraño, 2003), approaching a mortality plateau that is the same for inbred and outbred beetles (that is, the $u(t)$ curves converge), and the estimated inbreeding load converges on 0. The presence of mortality plateaus, or the age at which populations reach their plateau, may reflect adaptation to the laboratory (Promislow and Tatar, 1998; Snoke and Promislow, 2003; Rauser et al., 2006). However, mortality plateaus have been observed in a variety of experiments with seed beetles, including populations recently collected from the field and not adapted to the laboratory culture (Tatar et al., 1993; Tatar and Carey, 1995; Fox et al., 2003b; Fox and Moya-Laraño, 2003), that also provide no evidence for an increase in the inbreeding load with age (Fox et al., 2006).

The prediction that the inbreeding load for mortality will increase with age under mutation accumulation assumes that mutations have age-specific effects (Hughes and Reynolds, 2005; Reynolds et al., 2007). Adaptation to benign laboratory conditions can also allow the accumulation of a small number of deleterious alleles of large effect that act across all ages. These deleterious alleles can cause early mortality of those individuals with the highest mutational loads such that we would observe a decline in the inbreeding load with age, even if the expression of the load (within an individual) generally increases with age. Unfortunately, the effect of laboratory adaptation on the age-specific expression of the mutational load is still poorly understood.

Conflict of interest

The authors declare no conflict of interest.

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