

Male inbreeding status affects female fitness in a seed-feeding beetle

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Abstract

Inbreeding generally reduces male mating activity such that inbred males are less successful in male–male competition. Inbred males can also have smaller accessory glands, transfer less sperm and produce sperm that are less motile, less viable or have a greater frequency of abnormalities, all of which can reduce the fertilization success and fitness of inbred males relative to outbred males. However, few studies have examined how male inbreeding status affects the fitness of females with whom they mate. In this study, we examine the effect of male inbreeding status (inbreeding coefficient $f = 0.25$ vs. $f = 0$) on the fecundity, adult longevity and the fate of eggs produced by outbred females in the seed-feeding beetle, *Callosobruchus maculatus*. Females mated to inbred males were less likely to lay eggs. Of those that laid eggs, females mated to inbred males laid 6–12% fewer eggs. Females mated to inbred males lived on average 5.4% longer than did females mated to outbred males, but this effect disappeared when lifetime fecundity was used as a covariate in the analysis. There was no effect of male inbreeding status on the proportion of a female's eggs that developed or hatched, and no evidence that inbred males produced smaller nuptial gifts. However, ejaculates of inbred males contained 17–33% fewer sperm, on average, than did ejaculates of outbred males. Our study demonstrates that mating with inbred males has significant direct consequences for the fitness of female *C. maculatus*, likely mediated by effects of inbreeding status on the number of sperm in male ejaculates. Direct effects of male inbreeding status on female fitness should be more widely considered in theoretical models and empirical studies of mate choice.

Introduction

Inbreeding can have large effects on fitness by increasing homozygosity across the genome, which increases expression of deleterious recessive alleles and reduces heterozygote advantage (Charlesworth & Charlesworth, 1999). Traits closely related to fitness are expected to be under strong directional selection, which should erode additive genetic variation and increase the influence of nonadditive effects on trait variation, including directional dominance (Crnokrak & Roff, 1995). This increase

in dominance in traits closely related to fitness should make fitness traits more susceptible to inbreeding depression, a prediction that has been upheld in many experimental and comparative studies (DeRose & Roff, 1999). Male sexual characters, both morphological (Sheridan & Pomiankowski, 1997) and behavioural (Miller *et al.*, 1993; Aspi, 2000; van Oosterhout *et al.*, 2003; Reid *et al.*, 2005; Ala-Honkola *et al.*, 2009; Bolund *et al.*, 2010), often exhibit substantial inbreeding depression. These inbreeding effects on male traits can translate into a reduction in male mating success by reducing male mating activity or by influencing male–male competition (Sharp, 1984; Meffert & Bryant, 1991; Miller *et al.*, 1993; Joron & Brakefield, 2003; Mariette *et al.*, 2006; Ala-Honkola *et al.*, 2009; Drayton *et al.*, 2010; Michalczyk *et al.*, 2010). Inbred males have been shown to have

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smaller accessory glands (and thus likely transfer less accessory gland material) (Simmons, 2011), transfer less sperm (Zajitschek *et al.*, 2009) and produce sperm that are less motile, less viable or have a greater frequency of abnormalities (Fitzpatrick & Evans, 2009). All of these changes can reduce the fertilization success of inbred males when there is sperm competition (Konior *et al.*, 2005; Zajitschek *et al.*, 2009; Michalczyk *et al.*, 2010; Simmons, 2011).

Although inbreeding effects on male reproductive success are well documented, few studies have examined how mating with an inbred male affects female fitness, especially in the absence of male–male competition. Heterozygosity can be recreated in offspring when a female mates with an unrelated male, regardless of whether that male is inbred or outbred (Frankham *et al.*, 2002). Models thus predict that the genetic costs of mating with inbred males should generally be small and occur only under limited conditions (Lehmann *et al.*, 2007). Yet a growing number of studies are demonstrating that females prefer mating with outbred males (Ilmonen *et al.*, 2009; Zajitschek & Brooks, 2010; Okada *et al.*, 2011). Given the general lack of indirect fitness benefits expected from mating with outbred rather than inbred males, the fitness consequences of these mating preferences are unclear. One possibility is that direct effects on female fitness are important. Reduced sperm numbers, reduced nuptial gift size or other changes in accessory gland materials could reduce female fitness by negatively affecting offspring production or reducing the viability of eggs. However, available results are mixed. For example, mating with an inbred male reduces female fecundity in *Drosophila simulans* (Okada *et al.*, 2011) but not in *Tribolium castaneum* (Michalczyk *et al.*, 2010).

In the current study, we examine the effects of male inbreeding status on fitness components of his mate in the seed-feeding beetle, *Callosobruchus maculatus*. *C. maculatus* has served as a model system for studies of inbreeding depression and sexual selection. These beetles suffer substantial inbreeding depression throughout all stages of development and on most adult traits (Tran & Credland, 1995; Fox *et al.*, 2006a, 2011; Bilde *et al.*, 2009). Inbred beetles have higher larval mortality and take longer to develop to maturity, and inbred females have shorter adult lifespan and reduced fecundity compared to outbred beetles. Female survival and reproduction are also affected by the biology of their mate; male genital spines (Edvardsson & Tregenza, 2005), nuptial gifts (Fox *et al.*, 2006b; Ronn *et al.*, 2011), sperm traits (Rugman-Jones & Eady, 2008) and materials in the seminal fluid (Yamane *et al.*, 2008) all affect female adult survival and/or reproduction and may be affected by inbreeding.

This seed beetle system thus provides an excellent model to examine how inbreeding status of males affects the survival and reproduction of their mates. We tested whether mating with an inbred male (inbreeding coef-

ficient, $f = 0.25$) affects female fecundity, adult longevity and/or the proportion of a female's eggs that develop and hatch. We also test whether inbreeding status affects the size of a male's nuptial gift and/or the number of sperm transferred during mating to determine whether inbreeding effects on nuptial gift size or sperm number could explain observed inbreeding effects on female fecundity and survival.

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). 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.

We use two populations of beetles in the current study. 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, *Vigna 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 show substantial and largely similar patterns of inbreeding depression (Fox *et al.*, 2006a, 2007, 2011; Fox & Stillwell, 2009; Fox & Reed, 2010, 2011). Populations were maintained in laboratory growth chambers on seeds of *V. radiata* (SI) or *V. unguiculata* (BF) at > 1000 adults per generation for > 100 generations (BF) or > 200 generations (SI) prior to this experiment. However, all beetles were reared on a common host (*V. radiata*) for one generation before the experiment and maintained on this host for the duration of the experiment. The populations had thus been raised on a common host for three generations before we measured female reproduction and adult longevity.

Experiment 1 – Effect of male inbreeding status on female reproduction and lifespan

Our basic experimental design was to mate outbred females to a single randomly chosen nonsibling inbred or outbred male, after which we recorded female adult lifespan, lifetime fecundity and the fate of eggs (proportion development and hatching success). In the first generation, full-sib families of beetles were created by randomly pairing nonsib beetles collected from our

laboratory colonies. When beetles from this generation emerged as adults, we created a new set of families (second generation) derived either from crosses between individuals within families (full-sib matings) or between individuals that were unrelated. These matings produced families that were inbred ($f = 0.25$) or outbred ($f = 0$), respectively. Larvae from these families were allowed to develop at one egg per seed (excess eggs were scraped from the seed), one seed per dish, inside a growth chamber at light : dark 15 : 9 and either 29–30°C or 25–26°C. Dishes were checked once per day for adult beetles that emerged from a seed.

The day after emergence from the seed, outbred females were provided a randomly chosen male from their same population (SI or BF) with which to mate. Both beetles in the pair (the outbred female and her mate) were thus between 24 and 48 h post-emergence, which provides males enough time to produce a mature ejaculate (Fox *et al.*, 1995). Males were assigned to females randomly with regard to male inbreeding treatment; male treatment was blind to the researchers performing the matings. Pairs were confined for approximately 10 min, during which they nearly all mated. For the occasional pair that did not mate, the male was replaced with a fresh randomly chosen male.

Pairs were separated immediately upon completion of mating, after which females were confined solitarily in a 60-mm petri dish containing 120 mung seeds (*V. radiata*) and males were discarded. Dishes were transferred to the growth chamber where females were allowed to lay eggs until death. Dishes were checked at 24-h intervals to record female longevity. Dishes were frozen a few days later, after allowing time for all eggs to hatch but not for emergence of the next generation.

Eggs were scored in three categories: (i) there was no sign of development, (ii) the ensuing larva developed but did not bore into the underlying seed (as indicated by the presence of a dead embryo under the transparent egg cover) or (iii) the larvae hatched successfully (as indicated by the presence of frass under the egg cover and the absence of a larva).

Because temperature has a large effect on life history traits and the expression of the genetic load underlying inbreeding depression (Fox & Stillwell, 2009), the experiment was run at two different temperatures, once at 29–30°C and once at 25–26°C. The two replicate experiments were run sequentially rather than simultaneously, so their value is in confirming repeatability of the observed inbreeding effects; we cannot make inferences regarding effects of temperature because these may be confounded with unmeasured variables that differed between the two replicates. For replicate 1 (29–30°C), we obtained reproductive and lifespan data from a total of 914 females distributed across 67 and 66 full-sib families of the BF and SI populations, respectively. For replicate 2 (25–26°C), we measured traits of 1381 females from 69 and 54 families. Females were nearly evenly distributed

between the two mating treatments, with 49% of females mated to outbred males and 51% mated to inbred males.

Experiment 1 – Analyses

Families are the lowest level of independence in this design, and thus family means were used in all analyses of variance. Family means were calculated by averaging across sisters within each outbred family and mating treatment (mated to an inbred vs. outbred male). Most analyses are of the form: Fitness Trait = Replicate + Beetle Population + Mate Treatment + Interactions. Replicate is included only to control for a small difference in fecundity, and a large difference in lifespan, between the two replicates. The main effect of replicate reflects differences that are typical of those observed in experiments manipulating temperature (Stillwell *et al.*, 2007), so we do not report replicate effects except when treatment effects differ between replicates. Female body mass was included as a covariate in all analyses, because it can have large effects on both fecundity and lifespan in *C. maculatus*. The body mass covariate was nested within replicate due to the large difference in body size between replicates, which is expected because of known effects on rearing temperature on body mass (Stillwell *et al.*, 2007).

Experiment 2 – Nuptial gift size and sperm transfer of inbred and outbred males

This experiment was set up like Experiment 1, except we used only one female and one male per family and thus considered mated pairs rather than female families as our lowest level of independence, and males were mated at exactly 24–28 h post-emergence from their seed to reduce as much as possible the post-emergence variation among males in time available for ejaculate maturation and spermatogenesis (sperm counts and ejaculate volume of insects vary with male age; e.g. Fox *et al.*, 1995; Ponlawat & Harrington, 2007).

To measure nuptial gift size, each male was weighed at least twice before mating, on a Mettler–Toledo model AT261 balance, to a resolution of 0.01 mg (± 0.02 mg repeatability). If the two measurements of mass differed by > 0.03 mg, we weighed the beetle a third time. All measurements were averaged to obtain pre-mating mass. Males were similarly weighed immediately after mating (within 20 min). The change in male mass during mating is highly correlated with female mass gain during mating (Savalli & Fox, 1998).

Females were killed and dissected to remove their reproductive tract within 5 min of mating so that the number of sperm transferred could be counted. The ejaculate is transferred into the bursa copulatrix during mating, so this structure was highly inflated and represented the largest part of the female reproductive tract at the time of dissections. To count sperm, the female

reproductive tract was crushed and stirred in 50 μL phosphate-buffered saline solution containing 1% BSA. The sperm were stained with the nucleic acid-selective dye acridine orange. Then, 10 μL of the sperm suspension was placed on a slide. The number of sperm present was counted in five separate 2.3-mm² fields of view on each slide.

In total, we performed 174 matings and obtained sperm counts for 172 of these (41 and 46 outbred and inbred BF, respectively, and 41 and 44 outbred and inbred SI). Sperm counts were log-transformed before analysis to meet the assumptions of analysis of variance.

Results

Experiment 1 – Effect of male inbreeding status on female reproduction and lifespan

Females that mated with an inbred male were less likely to lay eggs than were females that mated with an outbred male; 6.2% of females (averaged across populations and the two replicates) failed to lay eggs when mated to an inbred male, whereas only 2.1% of females failed to lay eggs when mated to an outbred male (logistic regression, $\chi^2_1 = 23.3$, $P < 0.001$). Of females that laid eggs, females mated to inbred males laid 6–12% fewer eggs (depending on the replicate and beetle population) than did females mated to outbred males (Fig. 1; $F_{1,516} = 10.5$, $P = 0.001$). There was no evidence that this effect of male inbreeding status differed between replicates or populations; all replicate \times male treatment and population \times male treatment interactions were nonsignificant ($P > 0.72$).

This observed reduction in average fecundity of females mated to inbred males was largely due to a

higher frequency of very low fecundities (laying 15 or fewer eggs) among females mated to inbred males (in a logistic regression, the effect of male treatment on the probability that a female lays 15 or fewer eggs was highly significant; $\chi^2_1 = 22.0$, $P < 0.001$). Across all population/treatment combinations, 7–13% of females mated to an inbred male had low fecundity (overall average = $10.2 \pm 2.9\%$), whereas only 0–6% of females mated to an outbred male had low fecundity (average = $2.4 \pm 2.7\%$). After deleting these low-fecundity females from the analysis, females mated to an inbred male laid only slightly fewer eggs (averaging just 3.1% fewer) than did females mated to an outbred male ($F_{1,508} = 3.57$, $P = 0.06$).

Females that mated to inbred males lived 1–9% longer as adults than females mated to outbred males. This difference averaged 5.4% across all treatments (Fig. 2; $F_{1,516} = 10.5$, $P = 0.001$). Although lifespan differed between the populations and replicates, the male treatment effect on lifespan did not (nonsignificant replicate \times male treatment and population \times male treatment interactions, $P > 0.34$). This large male treatment effect on lifespan is mainly due to the effect of male inbreeding status on female reproduction. When fecundity was included as a covariate in the analysis, its effect on lifespan was highly significant ($F_{2,514} = 59.1$, $P < 0.001$) and the effect of the male's inbreeding status on female lifespan became nonsignificant ($F_{1,514} = 2.15$, $P = 0.14$).

There was no evidence that male inbreeding treatment had any effect on the proportion of his mate's eggs that developed ($F_{1,512} = 2.73$, $P = 0.10$) or the proportion of developing eggs that hatched ($F_{1,512} = 0.12$, $P = 0.72$). Egg development was lower in replicate 2 ($F_{1,512} = 42.4$, $P < 0.001$), and this difference varied between populations

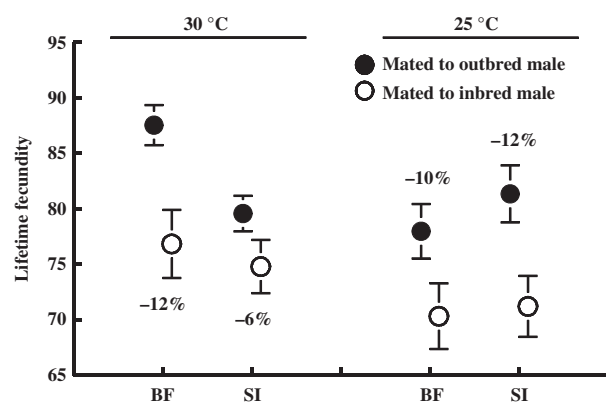


Fig. 1 Lifetime fecundity of female *Callosobruchus maculatus* mated to inbred (○) or outbred (●) males. The temperature groups reflect separate replicates of the experiments executed sequentially rather than simultaneously. The percentages reflect the relative difference in female fecundity when mated to inbred relative to outbred males for each population \times replicate combination.

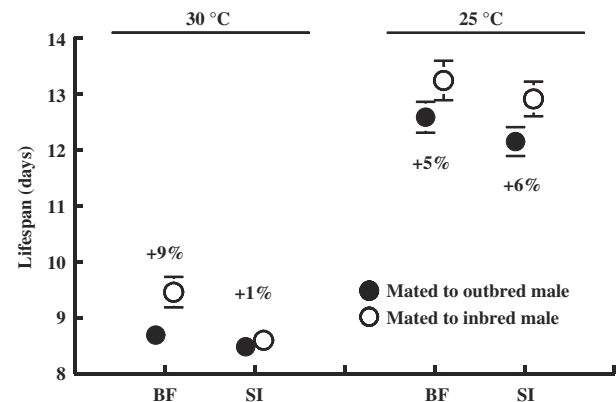


Fig. 2 Adult lifespan of reproducing female *Callosobruchus maculatus* mated to inbred (○) or outbred (●) males. The temperature groups reflect separate replicates of the experiments executed sequentially rather than simultaneously. The percentages reflect the relative difference in female lifespan when mated to inbred relative to outbred males for each population \times replicate combination.

(population \times replicate interaction; $F_{1,512} = 7.3$, $P = 0.008$), but there were no other significant main or interaction effects for the proportion of eggs developing or hatching.

Experiment 2 – Nuptial gift size and sperm transfer of inbred and outbred males

Males of the BF population lost a larger proportion of their body mass during mating, indicating that they transferred a larger ejaculate, than did SI males ($F_{1,168} = 5.29$, $P = 0.02$), but the amount of mass that males lost during mating (absolute or relative to their body size) was not affected by male inbreeding status ($P > 0.24$ for the male inbreeding treatment effect for both dependent variables). In both populations, males produced ejaculates that averaged between 0.25 and 0.29 mg, which was 5.7% of the BF male's body mass (for both inbred and outbred beetles) and 5.0 vs. 4.9% of the SI male's body mass (inbred vs. outbred males, respectively).

Of 172 matings, seven males (four inbred and two outbred SI, one inbred BF) failed to transfer sperm. This frequency of males failing to transfer sperm did not differ significantly between populations (logistic regression, $\chi^2_1 = 3.53$, $P = 0.06$) or treatments ($\chi^2_1 = 1.07$, $P = 0.30$), but the direction and magnitude of the treatment effect are similar to those observed for the frequency of females failing to lay eggs in Experiment 1. Of matings in which males transferred sperm, females mated to inbred males had 17–33% fewer sperm (BF and SI, respectively) in their reproductive tract, on average, than did females

mated to outbred males (Fig. 3; analysis of variance, $F_{1,161} = 7.83$, $P = 0.006$). Although the figure suggests that the treatment effect might differ between populations, neither the population effect ($F_{1,161} = 1.46$, $P = 0.23$) nor the population by treatment interaction ($F_{1,161} = 0.74$, $P = 0.39$) was significant. There was no detectable relationship between the number of sperm transferred and male size, female size, the amount of ejaculate transferred (most of which is accessory gland fluids rather than sperm) or mating duration ($F_{1,157} < 1.5$, $P > 0.2$ for all variables).

In Experiment 1, we observed that the main effect of male inbreeding treatment on females was to increase the frequency of females that had very low fecundity. We tested whether this could be due to a higher frequency of inbred than outbred males transferring particularly low numbers of sperm. We arbitrarily defined a mating as a low-sperm mating if the female reproductive tract contained ≤ 1000 sperm; this is below 20% of the average number of sperm in the transferred by outbred males in both populations. The frequency of low-sperm matings was higher for SI males (population effect in logistic regression, $\chi^2_1 = 3.9$, $P = 0.049$) and higher for inbred males ($\chi^2_1 = 6.4$, $P = 0.01$); 11% and 25% of inbred males (BF and SI, respectively), vs. only 2% and 7% of outbred males, transferred < 1000 sperm.

Discussion

We found that female *C. maculatus* mated to inbred males (inbreeding coefficient, f , of 0.25) were less likely to lay eggs than were females mated to outbred males. Of females that did lay eggs, those mated to an inbred male laid fewer eggs than did those mated to an outbred male. However, there was no effect of male inbreeding status on the proportion of a female's eggs that developed or hatched. Females mated to inbred males lived longer than did females mated to outbred males, consistent with an expected trade-off between reproduction and adult lifespan. Our study is one of few that have examined the direct effects of male inbreeding status on female fitness. These studies have reached mixed conclusions, demonstrating that male inbreeding status either can reduce (Okada *et al.*, 2011) or can have no direct effect (Michalczyk *et al.*, 2010) on female fitness.

The reduced fecundity of *C. maculatus* females mated to inbred males is likely due at least in part to the reduced number of sperm in the ejaculates of inbred males. That inbred males produce ejaculates with fewer sperm has been shown in other species (e.g. Zajitschek *et al.*, 2009). Our data suggest that the fecundity of female *C. maculatus* can be sperm limited, and that this primarily occurs when females mate with males transferring particularly low numbers of sperm, a situation that is more common when the male is inbred. Of course, our experiment does not allow us to directly link sperm numbers to individual female fecundity – females needed to be killed for us to

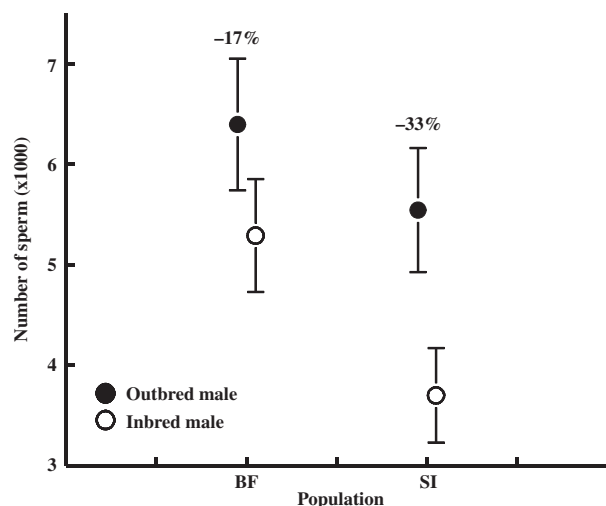


Fig. 3 Number of sperm in the reproductive tract of females mated to an inbred (○) or outbred (●) male *Callosobruchus maculatus*. The percentages reflect the relative difference in the number of sperm in females after mating with inbred relative to outbred males for each population.

count sperm, and thus fecundity and sperm numbers were recorded for different females – but the result that females mated to inbred males are more likely to have especially low fecundity matches our result that inbred males are more likely to transfer especially low numbers of sperm (e.g. < 1000 sperm). The direct link between sperm transfer and female fecundity remains to be tested, although previous studies have shown that sperm limitation is fairly common, though not universal, in insects (Cutter, 2004).

Our sperm counts (Fig. 3) are much lower than sperm numbers reported for a different population of *C. maculatus* by Eady (1994, 1995), but are of the same approximate magnitude of sperm numbers observed by Miyatake and colleagues for the congeneric beetle, *Callosobruchus chinensis* (Yamane & Miyatake, 2008; Katsuki & Miyatake, 2009). The difference between sperm counts in our study of *C. maculatus* and those of Eady may be due to differences in insemination dynamics between populations, such as evolved differences in response to the level of polyandry (Yamane & Miyatake, 2008; Gay *et al.*, 2009). Alternatively, the difference between studies may be due to differences in methodology or environmental conditions, such as differences in the age of males used in the experiment. Male age can have a substantial effect on the number of sperm in the ejaculate of insects (e.g. Ponlawat & Harrington, 2007). To control for this, we examined sperm transfer by males that were exactly 24–28 h post-emergence from their host seed (to match the lower end of the age range of beetles used in Experiment 1). In a small subsample of BF males mated > 48 h after emergence (12 matings), sperm counts were much higher than those recorded at 24 h post-emergence (approximately 26 000 sperm transferred by outbred males, approximately 12 000 by inbreds, compared to only 4000–7000 at 24 h in Fig. 3). However, the inbreeding effect remained very large (despite the very small sample sizes, it was significant; $P = 0.03$). Our results thus clearly demonstrate that the number of sperm transferred during mating differs substantially between inbred and outbred *C. maculatus*. Considering that males mate frequently, and should begin encountering females very early in adult life (especially in the storage environment to which beetles are adapted), our results should be ecologically very relevant.

In addition to reduced numbers of sperm, the frequency of abnormal sperm may be higher, or the quality of sperm lower, when males are inbred (Fitzpatrick & Evans, 2009). Sperm must migrate from the bursa copulatrix to the spermatheca (Eady, 1994, 1995) and then survive long enough in the spermatheca to be available to fertilize an egg. Sperm of inbred males may not perform as well as sperm from outbred males at either of these steps. Unfortunately, we have no data on sperm quality traits (such as sperm viability) that could affect the ability of sperm to fertilize eggs. However, our results suggest inbred males did not produce genetically

damaged sperm. If the sperm of inbred males had been genetically damaged, we would expect eggs fertilized by those males to be less likely to develop or hatch, an effect that we did not observe. Previous studies have also failed to detect an effect of mating with inbred males on egg hatch (Okada *et al.*, 2011), suggesting that inbreeding may often negatively affect sperm morphology or physiology (and thus the ability to fertilize eggs) but rarely affect sperm karyotype.

Male seed beetles produce large nuptial gifts (Fox *et al.*, 2006b), materials from which are incorporated into eggs (Huignard, 1983; Boucher & Huignard, 1987). The size of the accessory gland, and thus the amount of seminal fluid transferred to females during mating, can be affected by inbreeding in some species (Simmons, 2011). However, we found no effect of inbreeding on the size of the ejaculate in *C. maculatus*. In another experiment (C. Fox, unpublished), we likewise failed to find any evidence of a difference between inbred and outbred males in the change in mass of their ejaculate with age or additional matings. We thus have no evidence that inbreeding effects on the size of the nuptial gift contribute to explaining the observed male inbreeding effect on female fecundity. However, the relative proportions of different accessory gland proteins in the seminal fluid, many of which affect female behaviour and physiology (Avila *et al.*, 2011), might vary with male inbreeding status and thus affect female reproduction.

Female *C. maculatus* mated to inbred males lived longer as adults than did females mated to outbred males. This likely reflects a well-known trade-off between reproduction and adult survival – females mated to inbred males lived longer because they allocated fewer resources to reproduction and thus had more resources available for somatic maintenance (Tatar *et al.*, 1993; Messina & Fry, 2003). This hypothesis is supported by our data – when we include lifetime fecundity as a covariate in our statistical model, the effect of male inbreeding status on female fecundity disappears and most of the variance among females in lifespan is explained by lifetime fecundity, which is our proxy for total reproductive allocation.

Our experiment was performed using only once-mated females in a controlled laboratory environment. We thus must consider variables in nature that will mediate female mating behaviour and inbreeding effects on male fertility. For example, inbreeding depression commonly increases with the stressfulness of the environment (Fox & Reed, 2011), and thus inbreeding effects on spermatogenesis could be much greater in more heterogeneous and stressful natural conditions than in our benign laboratory conditions. This has been demonstrated for sperm production in guppies – males produce fewer sperm, and show much greater inbreeding depression in sperm production, when reared in semi-natural greenhouse conditions compared to the laboratory (Zajitschek & Brooks, 2010). If similar effects occur for *C. maculatus*, we would expect the consequences of male inbreeding

for both male and female fitness to be much greater under natural conditions. Also, *C. maculatus* are polyandrous, with females commonly mating multiple times (Fricke & Maklakov, 2007; Fox & Moya-Larano, 2009). Multiple mating creates sperm competition, during which sperm from inbred males may perform poorly, further reducing the fitness of inbred males in nature relative to the monandrous conditions of the laboratory experiment (Konior *et al.*, 2005; Zajitschek *et al.*, 2009; Michalczyk *et al.*, 2010; Simmons, 2011). However, multiple mating also provides females with additional sperm that may ameliorate the negative effects of having previously mated with an inbred male. Further studies are needed to test hypotheses about how mating frequency and plasticity in sperm and/or reproductive investment in response to social environment affect the fitness consequences of matings involving inbred males.

Broader implications: direct effects of male inbreeding status on the evolution of female mating preferences

The large effects of inbreeding on offspring fitness have led to the evolution of a diversity of inbreeding-avoidance mechanisms (Pusey & Wolf, 1996). In contrast to the effects of mating with relatives, however, mating with inbred partners does not necessarily reduce offspring fitness; as long as the parents are unrelated, heterozygosity can be re-created in their offspring, restoring outbred fitness (Frankham *et al.*, 2002). Models thus predict there to be fitness costs of mating with inbred males only under limited conditions (Lehmann *et al.*, 2007). Likely because of this, there has been little theoretical attention given to the evolution of mate choice when mates vary in inbreeding status (but see Reinhold, 2002), and effects of male inbreeding status on female mate choice have been examined in only a few contexts. For example, female guppies prefer courtships performed by outbred males (Zajitschek & Brooks, 2010), females of wild-derived house mice prefer the scent of outbred males (Ilmonen *et al.*, 2009) and female fruit flies mate more quickly (an index of preference) with outbred males (Okada *et al.*, 2011), but neither female flour beetles (Michalczyk *et al.*, 2010) or crickets (Drayton *et al.*, 2010) prefer outbred males.

Our study and a few others now demonstrate that focusing on indirect benefits may underestimate the costs of mating with inbred males (Ilmonen *et al.*, 2009). In seed beetles (this study) and fruit flies (Okada *et al.*, 2011), females have higher fecundity when mating with outbred males. In house mice, outbred males defend territories better than do inbred males, likely reducing the risk of infanticide (Ilmonen *et al.*, 2009). Survival (Keller *et al.*, 2008), immunocompetence (Hale & Briskie, 2007) and other traits that likely affect parental care performance in birds are subject to inbreeding depression such that females mating with inbred males may fledge

fewer offspring. The consequences of mating with inbred males may thus commonly be underestimated. Direct effects of male inbreeding status on female fitness should be more widely considered in theoretical models and empirical studies of mate choice.

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