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No evidence that female bruchid beetles, *Callosobruchus maculatus*, use remating to reduce costs of inbreeding

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Despite the often dramatic negative effects of inbreeding on offspring fitness, matings between closely related individuals sometimes occur. This may be because females cannot reliably recognize related males before mating with them. As an alternative to precopulatory choice, polyandrous females may avoid inbreeding through postcopulatory mechanisms if they can assess mate relatedness during or after copulation. These mechanisms include increasing remating propensity and decreasing rate of offspring production in response to incestuous matings. Stored product pests, such as the bruchid beetle *Callosobruchus maculatus*, have an ecology that is likely to expose them to frequent risks of inbreeding when a small number of females found a new population on a previously uninfested store of beans. Using this species, we show that inbreeding has negative effects on offspring viability but that females do not appear to discriminate between brothers and unrelated males prior to mating. Furthermore, females that first mated with brothers did not increase their remating propensity or decrease their rate of offspring production relative to females that first mated with unrelated males. Our findings suggest that the costs of inbreeding have not been sufficient to drive the evolution of mating behaviour as a mechanism of inbreeding avoidance in *C. maculatus*.

Inbreeding can have dramatic negative effects on offspring fitness (Thornhill 1993; Falconer & Mackay 1996; Pusey & Wolf 1996) and preferences for unrelated mates have been documented in several species (see Pusey & Wolf 1996 for a review). The sex-biased dispersal patterns of many animals have also been interpreted as ways of avoiding inbreeding (Pusey & Wolf 1996). However, it is not known how prevalent inbreeding avoidance mechanisms are in the majority of animal taxa and matings between closely related individuals sometimes occur even when there appear to be negative effects of inbreeding depression (Thornhill 1993). This may be because the benefits to inclusive fitness associated with inbreeding mean that the negative effects of incestuous mating on offspring fitness or on male paternity success with other females must be substantial for inbreeding avoidance to evolve

(Parker 1979; Kokko & Ots 2006). However, it is also possible that it is often very difficult for females to recognize related males before mating, particularly in species lacking precopulatory courtship and especially where mating typically follows a female simply being seized by a male. Nevertheless, the close association between copulation and male ejaculate may provide females with the necessary cues. Females could discriminate between mates by utilizing a variety of postcopulatory mechanisms (see Eberhard 1996) and it has been suggested that avoidance of genetically incompatible mates, such as closely related males, has been an important factor behind the evolution of polyandry (Brooker et al. 1990; Stockley et al. 1993; Zeh & Zeh 1996, 1997; Tregenza & Wedell 2000).

Polyandrous females may favour unrelated males through at least four postcopulatory mechanisms. First, they could increase the fertilization success of unrelated males by preferential use of their sperm. There is evidence suggesting that female sand lizards, *Lacerta agilis* (Olsson et al. 1996, 2004), field crickets, *Gryllus bimaculatus* (Tregenza & Wedell 2002; Bretman et al. 2004) and

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Gryllodes supplicans (Stockley 1999), red junglefowl, *Gallus gallus* (Pizzari et al. 2004) and possibly females of the fly *Drosophila melanogaster* (Mack et al. 2002) have the ability to favour sperm from unrelated males over sperm from related males. Second, males may transfer smaller ejaculates to related females because offspring may tend to be of lower fitness and hence, if males are sperm limited, they may choose to allocate their ejaculate elsewhere (Wedell et al. 2002). Third, females could increase their remating propensity and shorten the time until they remate after having mated with related males (Eberhard 1996), thereby reducing the number of eggs fertilized by sperm from related males. Fourth, females could reduce their investment in offspring fathered by related males, allowing them to allocate more resources to the production of offspring fathered by unrelated males (Sheldon 2000; Bilde et al. 2007). These mechanisms are not mutually exclusive and have the potential to give polyandrous females a large fitness advantage that could be important for the maintenance and prevalence of polyandry. Despite this, it is not yet known whether females of any species use the latter two mechanisms to avoid inbreeding.

In this study, we found substantial costs of incestuous mating in the bruchid beetle *Callosobruchus maculatus* and evaluated the importance of inbreeding for the mating behaviour and reproductive output of females of this species. We tested whether females are more reluctant to mate with brothers than with unrelated males. We also investigated female remating propensity and rate of oviposition in relation to mate relatedness to test whether females use postcopulatory mechanisms to avoid inbreeding. Females of this species are polyandrous (e.g. Arnqvist et al. 2005; Edvardsson 2007) but following their first mating they typically have a refractory period when they are not receptive to courting males and reduce the amount of male-attracting pheromone they emit (Shu et al. 1996). There appears to be some genetic variation among females in the length of this refractory period (Eady et al. 2004) but it also appears to be flexible, depending on the amount of material resources provided in the large male ejaculates and female access to nutrients and water (Savalli & Fox 1999; Edvardsson 2007). Mating also carries substantial costs to females because they are harmed by the spines on the male genitalia during copulation (Crudgington & Siva-Jothy 2000). It appears that female *C. maculatus* use the flexibility of their mating behaviour to trade-off the need for additional material resources against the costs of sustaining further injuries (Edvardsson 2007). Last male

sperm precedence is prevalent in *C. maculatus* (Eady & Tubman 1996) and a female remating soon after mating with a closely related male would minimize the number of eggs fertilized by sperm of the related male efficiently, even in the absence of a sperm choice mechanism. The size of the male ejaculate, which represents up to 10% of the body weight of virgin males (Savalli & Fox 1998, unpublished) and hence male ability to induce female refractoriness, decreases significantly after the first mating, exposing males to more intense sperm competition with every mating (Savalli & Fox 1999). The decreased fertilization success with other females should therefore reduce the inclusive fitness benefits to females mating with brothers.

METHODS

Callosobruchus maculatus is a widely distributed pest on stored legumes. Females attach their eggs to beans and the larvae develop inside. We used the laboratory strains *Brazil* and *South India* in this study (e.g. Dick & Credland 1984; Mitchell 1991). Cultures were maintained on black-eyed beans, *Vigna unguiculata*, at 28°C with a 18:6 h light:dark photoperiod. All mating trials were staged in 30-mm petri dishes at 28°C. The trials were carried out by introducing a female to the petri dish by tipping her into it from the dish or Eppendorf tube in which she had been isolated immediately following adult emergence. A virgin male was subsequently added by similarly tipping him into the dish. The pair was then given 10 min to initiate copulation in all first mating and remating trials except in the remating trials of experiment VII where pairs were given 5 min (see below). Virgin males typically make vigorous attempts to mate with females as soon as they encounter them (unpublished) and this occurred at every mating trial in this study. When copulation has been achieved, males hold onto the females by their genitalia alone, making it straightforward for an observer to judge when copulation has started. Like the virgin males used, all females were 1–2 days posteclosion at the time of their first mating. All individuals used in the mating trials were the offspring of females that had mated to one male only. This enabled us to obtain pairs that were either full sibs or unrelated.

We conducted a series of experiments to test the remating propensity of ovipositing females of both strains at different times after their first mating to either a brother or an unrelated male (Table 1). We also conducted an

Table 1. Strains used and the timing of remating trials in experiments I–VII

Experiment	Strain	First mating	Remating trials	Remating trials (h)
I	*Brazil*	Unrelated or brother	Unrelated or brother	6
II	*Brazil*	Unrelated or brother	Unrelated	6, 9
III	*Brazil*	Unrelated or brother	Unrelated	12, 16, 20
IV	*Brazil*	Unrelated or brother	Unrelated or brother	24
V	*South India*	Unrelated or brother	Unrelated	6, 9
VI	*South India*	Unrelated or brother	Unrelated	12, 16, 20
VII (No beans)	*South India*	Unrelated or brother	Unrelated	26, 27, 31, 35, 40, 51, 64, 75, 98

Unrelated and brother refer to how the males used in the first matings and the subsequent mating trials were related to the females. Unlike in experiments I–VI, females in experiment VII were not provided with beans to oviposit on between mating trials.

experiment where females were prevented from ovipositing (Table 1). At the time of their first mating, all females were offered either a brother or an unrelated male. To test for any precopulatory discrimination against related males, we recorded the proportion of virgin females that did not mate within 10 min in all experiments. In experiments I and IV, we also tested for precopulatory discrimination against related males in females that had already mated with a brother or an unrelated male. In these experiments (I and IV) females were offered either a brother or an unrelated male at their second mating trial (after 6 h in experiment I and after 24 h in experiment IV). Following their first mating to either a brother or an unrelated male, females were presented only with unrelated males in all other experiments (II, III, V, VI and VII).

We investigated potential postcopulatory inbreeding avoidance mechanisms by assessing how mating with a brother, rather than an unrelated male, affected the remating propensity of ovipositing females (experiments I−VI) and of females prevented from ovipositing (experiment VII) at different times following their first mating. Together, these experiments covered a range of time from when a low proportion of ovipositing females normally remate to when a high proportion normally remate. In all experiments except experiment VII, females were allowed to oviposit on approximately 60 black-eyed beans in individual 90-mm petri dishes at 28°C. We recorded female rate of oviposition after mating with a brother or an unrelated male in experiment I. This was done to test whether females change their reproductive output in response to mating with a closely related male.

In experiments with more than one remating trial (II, III, V, and VI), females that did not mate were returned to the same petri dish after each mating trial. In experiment VII females were instead kept isolated at 28°C without access to beans between mating trials.

To obtain a measure of the effects of inbreeding in the *Brazil* strain, we recorded egg to adult survival of offspring produced by full sib and nonsib matings in experiment I (Table 1). It is known from other recent studies that the *South India* strain suffers substantial inbreeding depression (Fox et al. 2007). We checked the beans for emerging adults until 14 days after when outbred offspring normally emerge to make sure we did not miss any offspring with unusually slow development. This would represent a prolonged development time of over 50%. Fox et al. (2007) found that inbred offspring take on average 5% longer time to develop in the *South India* strain.

RESULTS

Inbreeding Depression and Oviposition

Females of the *Brazil* strain mated to brothers suffered a significantly reduced offspring survival in experiment I. Egg to adult survival was (mean ± SD) 0.732 ± 0.219 compared with 0.890 ± 0.124 for females mated to unrelated males, representing an 18% decrease in survival (Mann−Whitney U test: $U = 349$, $N_1 = 21$, $N_2 = 23$, $P = 0.011$; one female did not lay any eggs and was excluded from this

analysis). This is similar to the 21% reduction in egg to adult survival in the *South India* strain (Fox et al. 2007). However, females that first mated with brothers did not lay fewer eggs in the first 6 h following mating than females that first mated with unrelated males (experiment I: $\text{mean}_{\text{brother}} \pm \text{SD} = 17.09 \pm 10.15$; $\text{mean}_{\text{unrelated male}} \pm \text{SD} = 15.30 \pm 7.67$; two-sample t test: $t_{43} = 0.668$, $P = 0.51$).

Precopulatory Inbreeding Avoidance

Virgin females were not more reluctant to mate with brothers than with unrelated males in any of the experiments. Summed over all the experiments, 11 of 131 virgin *Brazil* females did not mate when offered a brother compared with 12 of 130 when offered an unrelated male (Fisher's exact test: $P = 0.83$). Similarly, in the *South India* strain four of 130 virgin females did not mate when offered a brother compared with three of 126 when offered an unrelated male (Fisher's exact test: $P > 0.99$).

Females did not appear to discriminate against their brothers as second mates in experiments I and IV where females were offered either a brother or an unrelated male in the remating trials. Only three of 45 females remated after 6 h (one with a brother and two with unrelated males; see Table 2) in experiment I. They had all mated with brothers in the first mating but the difference was not significant and the low number of remating females makes the power of the test low (Fisher's exact test: $P = 0.11$).

More females remated after 24 h in experiment IV but, again, there was no significant difference between females that had first mated with brothers (30 of 39) and females that had first mated with unrelated males (25 of 37) (Fisher's exact test: $P = 0.45$). Relatedness of the second male did not influence female remating propensity (Fisher's exact test: $P = 0.32$). Here, females presented with a brother actually had a somewhat higher proportion of rematings (Table 2).

Remating Propensity

The proportion of once-mated ovipositing females that were willing to remate increased in both strains over the time period covered in our experiments (Tables 2−6). However, inbreeding avoidance appears to play no role in female remating because the relatedness of the first male did not have a significant effect on female remating propensity in any of the experiments (see Tables 2−6 for statistical evaluation).

Table 2. Proportion of *Brazil* females remating in the four treatments after 6 h in experiment I and after 24 h in experiment IV

Second/first mating	Unrelated male	Brother
Unrelated male 6 h	0/12	0/11
Brother 6 h	2/13	1/9
Unrelated male 24 h	11/17	14/20
Brother 24 h	13/19	17/20

Table 3. Contingency table of the number of *Brazil* females remating after 6 and 9 h and the number of females not remating at all in experiment II

First mating/remated	6 h	9 h	Did not remate	Total
Unrelated male	14	5	19	38
Brother	11	4	24	39
Total	25	9	43	77

Fisher's exact test: $P = 0.65$.

In experiment VII, females that had mated once were kept without access to oviposition substrate. Here, many females remained unreceptive to males for the full duration of the experiment (98 h) and there was no difference in remating propensity between females first mated to brothers (seven of 20 remated) and females first mated to unrelated males (six of 12 remated) (Fisher's exact test: $P = 0.47$). Furthermore, there was no significant difference in time until mating between females of the two groups that remated within 98 h (mean$_{\mathrm{brother}} = 22$ h; mean$_{\mathrm{unrelated\ male}} = 20$ h; Mann–Whitney U test: $U = 14$, $N_1 = 7$, $N_2 = 6$, $P = 0.31$).

DISCUSSION

We found substantial viability effects of inbreeding in the *Brazil* strain and another recent study found similar effects in the *South India* strain (Fox et al. 2007). In addition to the approximately 20% reduction in egg to adult survival, significant negative effects of inbreeding on development rate, female fecundity and female longevity have also been found in this species (Tran & Credland 1995; Fox et al. 2006). In all, this should be more than enough to make the net fitness consequences of incestuous matings negative for females. Despite this, the behaviour of the more than 500 females included in our study strongly suggests that female *C. maculatus* do not alter their mating behaviour to avoid inbreeding regardless of whether they have started oviposition; neither virgin nor nonvirgin females were less willing to mate with brothers than with unrelated males. Overall, virtually identical proportions of virgin females presented with brothers and virgin females presented with unrelated males mated: 94.3% and 94.1%, respectively. This suggests that there is no precopulatory discrimination against related mates in this species. Indeed, the very high proportion of virgin females of both groups mating suggests that virgin females

Table 5. Contingency table of the number of *South India* females remating after 6 and 9 h and the number of females not remating at all in experiment V

First mating/remated	6 h	9 h	Did not remate	Total
Unrelated male	16	6	18	40
Brother	20	5	13	38
Total	36	11	31	78

Fisher's exact test: $P = 0.53$.

generally mate indiscriminately with the first male they encounter. There are therefore potential benefits to females that use the apparent flexibility of their remating propensity as a postcopulatory inbreeding avoidance mechanism. Despite this, females that had mated with brothers were not more likely to remate or to remate sooner than females that had mated with unrelated males in any of the seven experiments. Taken together, our experiments represent a strong indication that females do not alter their remating propensity to avoid inbreeding.

When drawing inferences from negative results it is important to consider the statistical power of the tests used (e.g. Thomas & Juanes 1996). In this case, the statistical power is the probability of obtaining a significant result if mating with a brother has a certain effect on female remating propensity. Overall, statistical power was high in our experiments. If females had increased their probability of remating by, for instance, 25% (e.g. from 10 to 35%) in response to incestuous matings, our power to detect this at the first remating opportunity using Fisher's exact test, given our sample sizes and the proportion of females that had mated with unrelated males that remated, would have been 84, 67, 33, 88, 73 and 83%, respectively, in the first six experiments. Power was relatively low in experiment VII due to the limited sample sizes. The results nevertheless indicate that many females remain unreceptive to males if they are prevented from ovipositing regardless of the relatedness of their first mates.

Furthermore, considering each experiment individually, more females that had mated with unrelated males remated in three experiments (II, VI and VII), more females that had mated with brothers remated in three experiments (I, IV and V) and in one experiment the proportions of females remating were identical (III). This is also a strong indication that there really is no effect of male relatedness.

Females did not seem to reduce their production of offspring fathered by brothers, the other postcopulatory

Table 4. Contingency table of the number of *Brazil* females remating after 12, 16 and 20 h and the number of females not remating at all in experiment III

First mating/remated	12 h	16 h	20 h	Did not remate	Total
Unrelated male	7	5	1	7	20
Brother	6	5	2	7	20
Total	13	10	3	14	40

Fisher's exact test: $P > 0.99$.

Table 6. Contingency table of the number of *South India* females remating after 12, 16 and 20 h and the number of females not remating at all in experiment VI

First mating/remated	12 h	16 h	20 h	Did not remate	Total
Unrelated male	34	24	3	4	65
Brother	40	17	3	4	64
Total	74	41	6	8	129

Fisher's exact test: $P = 0.63$.

inbreeding avoidance mechanism that we investigated. Incestuous matings did not cause females to reduce their rate of oviposition. Once-mated females mated to brothers even had a slightly higher rate of oviposition than once-mated females mated to unrelated males but the difference was far from significant.

Female *C. maculatus*, like females of the field cricket *G. bimaculatus* (Tregenza & Wedell 2002; Bretman et al. 2004), may use sperm choice as a way to avoid inbreeding. Wilson et al. (1997) showed that the outcome of sperm competition is influenced by an interaction between male and female genotype in *C. maculatus*. This is what would be expected if females were able to discriminate against the sperm of brothers and other genetically incompatible males. However, females would still benefit from changing their mating behaviour in response to incestuous matings. A substantial proportion of a female's lifetime oviposition occurs while she is still unreceptive to males following her first mating and only has the sperm from her first mate to use for fertilization. For instance, the results of the current study show that a majority of females are still unreceptive to courting males after 6 h and females had at that time on average laid close to a fifth of the eggs they would normally lay over their entire lifetime. Virgin females that mate with brothers will therefore fertilize a substantial proportion of their eggs with sperm from brothers regardless of any sperm choice mechanism.

Emerging virgin female *C. maculatus* may risk encountering brothers in their natural environment because females lay eggs in pods of beans in the field, on collections of seed in stores or, in the case of our strains, in the laboratory. In their evolutionary past one would expect that discrete patches of food, such as a sack of beans, would typically be located by only a few individuals, which would oviposit rapidly, such that emerging individuals would find themselves surrounded by a mixture of full sibling and unrelated potential mates. However, it appears either that the evolution of kin discrimination has been prevented by unknown constraints or that other factors are more important to female mating behaviour than inbreeding avoidance. *Callosobruchus maculatus* has a short reproductive life and, at least in laboratory populations, competition over beans among ovipositing females and developing larvae can be severe. This may have created selection for females that mate and begin oviposition rapidly, explaining why virgin females mate indiscriminately with the first male that courts them.

More research is needed to establish whether postmating choice of compatible sperm is widespread in insects including *C. maculatus*. It is still uncertain how important inbreeding avoidance has been in the evolution of insect behaviour in general. Our results suggest that it has not been a major driving force in the evolution of the mating behaviour of *C. maculatus*.

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