



Does fertilization by proxy occur in *Tribolium* beetles? A replicated study of a novel mechanism of sperm transfer

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Sperm competition is a fundamentally important component of postcopulatory sexual selection, and direct removal of rival male sperm from the female reproductive tract has been documented in at least three insect orders: Odonata, Orthoptera and Coleoptera (e.g. Siva-Jothy 1987; Ono et al. 1989; Gage 1992; Simmons 2001). Haubruge et al. (1999) reported a novel twist on sperm removal, a process they termed fertilization by proxy, in the red flour beetle *Tribolium castaneum*. This study suggested that not only do males remove previously stored sperm from females, but that males also translocate rival males' sperm when they subsequently mate with new females. The mechanism they proposed was rival sperm removal by an array of chitinous spines located on the mating male's aedeagus, followed by translocation of these still viable sperm when this male subsequently mated with another female.

Empirical evidence for this novel phenomenon was based on a physiological paternity marker involving resistance to the insecticide malathion, which in *T. castaneum* is due to a spontaneous mutation in a carboxylesterase enzyme that confers malathion-specific resistance (Dyte & Rowlands 1968; White & Bell 1988; Haubruge et al. 2002; Arnaud et al. 2005). The experimental design included three consecutive crosses: (1) a malathion-resistant virgin male was mated with a malathion-susceptible virgin female, (2) the

same susceptible female was mated with a susceptible virgin male and (3) the same susceptible male was mated with a new susceptible virgin female. Adult progeny collected from the two mated females were subjected to an insecticide-exposure bioassay (contact with 1% malathion in acetone) for 3 h. Paternity was assigned based on whether progeny died (indicating they had been sired by the directly mating, susceptible male) or survived (presumed to be sired indirectly by the resistant male). Haubruge et al. (1999) reported that fertilization by proxy was quite common (occurring in 22% of sequential matings), and that in such cases, a surprisingly high percentage (53% on average) of offspring were sired by translocated sperm.

To date, this study by Haubruge et al. (1999) remains the only evidence for rival sperm translocation, a phenomenon that could potentially counteract any fitness advantages that males may gain from sperm removal. As pointed out recently by Kelly (2006), replication studies traditionally have been undervalued in behavioural ecology, and consequently both confirmatory and contradictory results may be difficult to publish. However, verification of key empirical results should be standard operating procedure to rigorously test any novel evolutionary hypotheses. Based on the important evolutionary implications of the phenomenon of sperm translocation, we conducted a partial replication of the study by Haubruge et al. (1999), in which we exactly duplicated most experimental procedures but used a different paternity marker to differentiate between the two potential sires.

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METHODS

Tribolium castaneum stock cultures were maintained on King Arthur enriched wheat flour and kept in the dark at 29°C and 70% relative humidity (RH). Males and females were sexed as pupae, after which groups of 20 females were kept together in 22 g of flour. Males were kept individually in 1 g of flour because isolated males mate more readily (Haubruge et al. (1999) did not specify whether beetles of either sex were kept individually or in groups).

For this replication we used wild-type beetles (+/+, rust body colour) from the Berkeley synthetic strain, and Chicago black (*b/b*, black body colour) as a morphological paternity marker; this is an autosomal, semidominant body colour allele widely used in previous studies of *Tribolium* sperm competition (e.g. Schlager 1960; Lewis & Austad 1990; Bernasconi & Keller 2001; Pai & Yan 2002; Fricke & Arnqvist 2004; Fedina & Lewis 2006). Replicating Haubruge's et al. (1999) experimental design and procedures, we conducted three consecutive crosses (Fig. 1): (1) a virgin *b/b* male was mated with a virgin *b/b* female, (2) the same *b/b* female was mated with a virgin +/+ male and (3) the same +/+ male was mated with a new virgin *b/b* female. Copulations were defined as male intromissions lasting at least 24 s, and we conducted 203 trials (each consisting of three consecutive crosses) in which copulations were observed in all three crosses. Following Haubruge et al. (1999), we ensured that intermating intervals between all crosses were less than 5 min.

Following these mating crosses, female 1 (doubly mated) and female 2 (singly mated) were kept individually to lay eggs for 12 days in 50 grams of flour, sufficient to provide excess food for developing larvae; Haubruge et al. (1999) kept females for 12 days in only 5 g of flour. Progeny were reared to adults at 29°C and 70% RH for 45 days, and paternity was determined by scoring body colour phenotypes of all adult progeny (total of 14 862 progeny were scored): brown heterozygous progeny (*b/+*) that had been sired by male 2 could be readily distinguished from any homozygous black (*b/b*) progeny that had been indirectly sired by sperm translocated from male 1. Because it has previously been established in *T. castaneum* that all copulations do not necessarily result in insemination (Bloch Qazi et al. 1996; Fedina & Lewis 2006), we further restricted our consideration to a subset of successful trials in which insemination success was verified for each of the three crosses.

Successful insemination of female 1 by males 1 and 2 was confirmed by examining progeny phenotypes following Cross 2: thus, only females showing mixed progeny were included in these results (Fig. 1). Similarly, successful insemination of female 2 during Cross 3 was confirmed by the presence of any progeny (Fig. 1).

RESULTS

We obtained 84 successful trials in which insemination was verified in all three consecutive crosses (Haubruge et al. (1999) did not report the number of trials including three successful inseminations). However, we found no evidence of sperm translocation in any of these trials (Table 1); although all the singly mated *b/b* females from Cross 3 (mated only to +/+ males) produced the expected +/b progeny, none of these females produced any *b/b* progeny, which would have represented progeny sired by translocated *b/b* male sperm (Fig. 1).

DISCUSSION

Replication of empirical studies is an important tool for behavioural ecologists to confirm and extend previous results (Kelly 2006), and to identify erroneous outcomes that may become highly influential (e.g. see Snyder & Gowaty 2007 on Bateman's 1948 results). Our results indicate that fertilization by proxy is not a common phenomenon in *T. castaneum*, as we found no evidence that viable sperm were translocated between females by mating males. This directly contradicts results obtained by Haubruge et al. (1999), who reported that sperm translocation occurred at high frequency in 22% of trials, and that the proportion of such indirectly sired progeny slightly exceeded the proportion sired directly by mating males. It is not likely that sperm translocation at these rates could have remained undetected by our study, as our sample size of 203 trials closely matched the 204 females that mated in Cross 3 reported by Haubruge et al. (1999).

Although it is difficult to reconcile these discrepant results, one possible explanation is that this phenomenon could be restricted to particular *T. castaneum* strains, perhaps reflecting differences in genital morphology. However, the strains we used show similar elaboration on the male aedeagus of the chitinous spines (unpublished data) that were suggested by Haubruge et al. to be responsible for sperm translocation.

A second explanation for differences between the two studies is the different genetic paternity markers used; visible markers, such as the black body colour marker used in our study, may be more reliable than physiological markers, such as insecticide resistance, because they are easier to detect. It is plausible that resistance alleles might occur at low frequencies within a laboratory population of susceptible beetles, and therefore remain undetected. Recent work has shown that malathion-resistant *T. castaneum* have higher female fecundity (Arnaud et al. 2002) and increased sperm competitive ability (Arnaud et al. 2005) relative to susceptible strains, and this increased reproductive fitness could allow their spread in both field and laboratory populations.

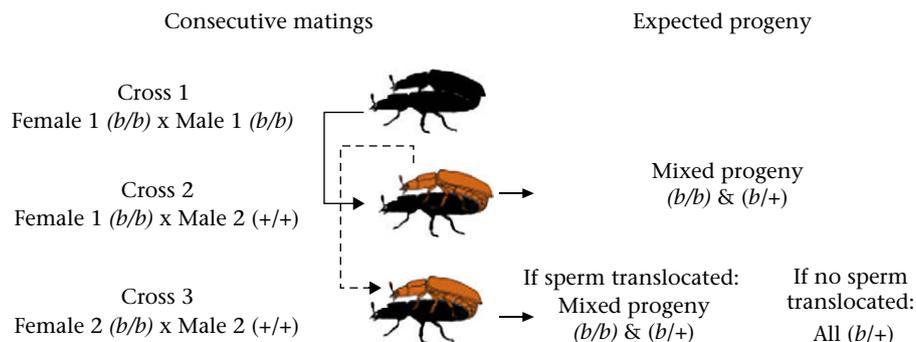


Figure 1. Experimental design showing sequential matings of two *b/b* (black) females with males of two genotypes, *b/b* male 1 and +/+ (wild-type) male 2. Successful insemination during Crosses 1 and 2 was confirmed by presence of mixed progeny (*b/b* and *b/+*). In Cross 3, evidence for sperm translocation would be the production of any *b/b* progeny by female 2.

Table 1

Experimental results showing number of trials conducted, number of successful trials (where females were successfully inseminated during all three crosses), average number of progeny produced by female 1 ($N = 84$ females mated to 2 rival males; see Fig. 1) and female 2 ($N = 84$ singly mated females) and number of trials in which female 2 produced any progeny (b/b) attributable to sperm translocation

	No. trials conducted	Successful trials	Progeny per female (mean \pm SE)	Females producing mixed progeny (b/b and $b/+$)
Female 1	–	–	85.89 (4.03)	84
Female 2	203	84	88.95 (3.67)	0

Another explanation for the incompatible results involves the high progeny densities used by Haubruge et al. (1999): *T. castaneum* females typically lay ~ 10 eggs per day, so their method of leaving individual females to oviposit for 12 days in 5 g of flour would yield larval densities of 24 beetles per gram. Such high larval densities are likely to cause substantial egg cannibalism and larval mortality (reviewed in: Sonleitner 1961; Sokoloff 1974). Also, such high rearing densities may have somehow altered the reliability of the malathion contact bioassay used to detect sperm translocation, since dominance of the malathion-resistance allele has been shown to depend on population density (Assié et al. 2007).

In conclusion, this study demonstrates the utility of replicating empirical studies in behavioural ecology, and calls into question the generality of fertilization by proxy as previously reported in *T. castaneum*. Furthermore, it is unlikely that any behavioural and anatomical features associated with sperm translocation could be maintained in the face of strong negative selection resulting from reduced fitness of males that transfer rival sperm. Extraordinary claims require extraordinary evidence (Sagan 1985), calling for further independent testing of this phenomenon.

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