THE ROYAL SOCIETY

Fertilization by proxy: rival sperm removal and translocation in a beetle

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Competition between different males' sperm for the fertilization of ova has led to the evolution of a diversity of characters in male reproductive behaviour, physiology and morphology. Males may increase sperm competition success either by enhancing the success of their own sperm or by negating or eliminating the success of rival sperm. Here, we find that in the flour beetle *Tribolium castaneum*, the second male to mate gains fertilization precedence over previous males' sperm and fertilizes approximately two-thirds of the eggs. It is not known what mechanism underlies this pattern of last-male sperm precedence; however, the elongate tubules of the female sperm storage organ may encourage a 'last-in, first-out' sperm use sequence. Here we present an additional or alternative mechanism of sperm precedence whereby previously deposited sperm are removed from the female tract by the mating male's genitalia. In addition to providing evidence for sperm removal in *T. castaneum*, we also show that removed, non-self sperm may be translocated back into the reproductive tracts of new, previously unmated females, where the translocated sperm go on to gain significant fertilization success. We found that, in 45 out of 204 crosses, sperm translocation occurred and in these 45 crosses over half of the offspring were sired by spermatozoa which had been translocated between females on the male genitalia. In the natural environment of stored food, reproductively active *T. castaneum* adults aggregate in dense mating populations where copulation is frequent (we show in three naturally occurring population densities that copula duration and intermating intervals across three subsequent matings average 1–2 min). Selection upon males to remove rival sperm may have resulted in counter-selection upon spermatozoa to survive removal and be translocated into new females where they go on to fertilize in significant numbers.

Keywords: *Tribolium castaneum*; genitalia; sperm competition; mating frequency

1. INTRODUCTION

Since the conceptualization of sperm competition (Parker 1970), biologists have recognized that sexual selection can proceed beyond mating at the gametic level. Sperm competition occurs when sperm from two or more males compete for fertilization of a female's ova and this post-mating phenomenon is recognized as a cryptic but powerful force in the evolution of reproductive physiology, behaviour and morphology. Despite recognition of the importance and outcomes of sperm competition, few studies clearly demonstrate the mechanisms by which males actually achieve fertilization success within the environment of the female reproductive tract.

When sperm competition proceeds in a manner analogous to a lottery (Parker 1982) the relative number of sperm transferred to the female may be a fundamental determinant of male fertilization success (Martin *et al.* 1974; Parker 1982; Simmons 1987). However, there are other mechanisms by which males may enhance success in competition for fertilization. In dung flies, for example, males achieve fertilization precedence by using their own ejaculate to dilute and displace previous males' sperm in storage (Parker & Simmons 1991). Male staphylinid beetles *Aleochara curtula* use their spermatophore to hydraulically displace rival sperm back out of the female storage organ (Förster *et al.* 1998). Studies of odonates provide some of the clearest demonstrations of how males achieve fertilization precedence. Waage (1979) first demonstrated that male damselflies (*Calopteryx maculata*) possess specialized genitalia for removing rival sperm from the female reproductive tract before inseminating their own. Males achieve fertilization precedence by eliminating rival sperm from the competition. Studies of both Anisoptera and Zygoptera show that most species have evolved genitalia which remove or reposition rival sperm in storage (reviewed by Waage 1984; Siva-Jothy & Tsubaki 1989; Cordero & Miller 1992).

Sperm removal using modified genitalia is not restricted to the Odonata. Its occurrence, however, has only been documented in four other species. In the tree cricket *Truljalia hibinonis*, males use their own ejaculate to force around 90% of rival sperm out of storage and onto the mating male's genitalia for subsequent removal and ingestion (Ono *et al.* 1989). In another orthopteran, the bushcricket *Metaplastes ornatus*, mating males use their genitalia to evert the female's reproductive tract following which the female consumes the rival sperm in storage (Von Helversen & Von Helversen 1991). Again, significant numbers of stored sperm are removed (*ca.* 85%) prior to

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Received 18 January 1999 *Accepted* 18 February 1999

Table 1. *Paternity results for three consecutive crosses using malathion susceptibility* (Asm) *or resistance* (PRm) *as genetic markers*

(Figures in parentheses show the total number of male:female pairs that were set up and apparently completed mating. For females in cross 2 giving mixed progeny (and therefore direct evidence of sperm competition), 34% of offspring are fathered by the first (resistant) male (*Prm1*); therefore the second male to mate (*Asm2*) achieves 66% fertilization precedence. Cross 3 shows that 45 of the 204 susceptible females produce both resistant and susceptible offspring. In these 45 females with mixed offspring, over half the progeny are sired by resistant males. Since in cross 3 only susceptible males mated with previously unmated susceptible females, the resistant offspring must have been sired by resistant males' sperm originally transferred in cross 1. These resistant males' sperm were removed from the recently mated females by susceptible males from cross 2 and subsequently translocated into new susceptible virgin females in cross 3.)

cross	successfully mated females (total number of pairs)	progeny per female (mean ± s.e.)	females producing susceptible progeny	females producing resistant progeny	females producing both resistant and susceptible phenotypes	percentage of resistant phenotype in mixed progenies (mean ± s.e.)
1 F *Asm*1 × M *Prm*1	176 (387)	44.9 (1.62)	0	176	0	100.0
2 F *Asm*1 × M *Asm*2	227 (356)	52.82 (1.8)	74	68	85	34.2 ± 1.9
3 F *Asm*2 × M *Asm*2	204 (324)	52.23 (1.65)	159	0	45	53.2 ± 1.7

spermatophore insemination. The aedeagus of the beetle *Psacothea hilaris* is adapted for removing rival sperm (Yokoi 1990) and the aedeagus of *Tenebrio molitor* can remove sperm (Gage 1992). However, in *T. molitor*, sperm removal may not be adaptive since males may remove their own spermatozoa (Siva-Jothy *et al*. 1996).

This study investigates fertilization precedence in the flour beetle *Tribolium castaneum* (Coleoptera: Tenebrionidae). Previous studies have demonstrated that the second male to mate in a sequence fertilizes most of the eggs (Schlager 1960; Wool & Bergerson 1979; Lewis & Austad 1990; Arnaud *et al*. 1999) and we document similar precedence in this study. Despite consistent evidence for last-male sperm precedence, the precise mechanism by which this fertilization advantage is achieved has yet to be clearly demonstrated. It has been suggested that the tubular structure of the female's sperm storage organ encourages a 'last-in, first-out' mechanism of sperm use due to sperm stratification in storage tubules (Schlager 1960). Here we show that male flour beetles are capable of removing rival sperm that has been previously deposited in the female reproductive tract. In addition, we demonstrate for the first time the translocation of these removed sperm into different females and subsequent significant fertilization success by the translocated sperm.

2. MATERIALS AND METHODS

(a) **Tribolium** *strains and culture*

We used two flour beetle strains that were either resistant or susceptible to malathion. Paternity was assigned using malathion-specific resistance which is an autosomal, semi-dominant and monofactorial genetic marker with no evidence of segregation (Beeman 1983). The resistant strain (*PRm*) was originally derived from grain stores in the Philippines. The susceptible strain (*Asm*) was derived from storage facilities in the Ivory Coast. Both strains were cultured in a dark incubator at 30 °C and 60 ± 5% relative humidity (RH) with a mixture of whole wheat flour and brewer's yeast (10/1: w/w) as rearing medium. Individuals for the mating experiments were randomly selected at the pupal stage, sexed by observing the arrangement of the genitalia and marked for identification on the elytra after adult emergence.

(b) *Experimental protocol*

We performed three consecutive crosses: (i) resistant male virgins were mated with susceptible female virgins (F *Asm*1 × M *PRm*1), (ii) the same (recently mated) susceptible females were then mated with new susceptible male virgins (F *Asm*1 × M *Asm*1), and (iii) these same (recently mated) susceptible males were remated with new susceptible female virgins (F *Asm*2 × M *Asm*1) (table 1). Only progeny from those pairs which appeared to have achieved successful intromission were screened. The maximum intermating interval between each cross was 5 min.

After their final matings, individuals from both groups of females (F *Asm*1 and F *Asm*2) were isolated singly in new vials with 5 g of wheat flour and brewer's yeast (10/1: w/w) for 12 days. Vials were stored at 27 °C and 60% RH during the entire period of egg laying and larval development. After 45 days, adult progeny of every female was sifted from each 5 g of rearing medium. Paternity of the progeny was assigned using a discriminating-dose, malathion contact bioassay: after 3 h of contact on a filter paper impregnated with an acetonic solution of malathion (1% w/v), susceptible genotypes die while resistant genotypes survive (Haubruge *et al*. 1997). Malathion-specific resistance is semi-dominant (Beeman 1983); therefore, this test enables the determination of the male parent phenotype and, hence, the degree of fertilization precedence.

(c) *Mating frequencies*

Mating frequencies of sexually mature *Asm* males were observed under three population densities that occur 'naturally' (Sokoloff 1974). Mating durations and intermating intervals of three consecutive matings were recorded for focal males in 15 cm Petri dishes that contained 6, 30 or 100 sexually mature *Asm* adults (1:1 sex ratio).

(d) *Electron microscopy*

Reproductively active males that had just mated were selected from the mass culture. Individuals were dissected and the genitalia isolated. The aedeagi were carefully separated from surrounding tissue and mounted on thin glass slides. These mounts were then freeze dried in absolute ethanol (Veltkamp

et al. 1994), critical point dried and gold coated for examination on a Philips XL30 scanning electron microscope.

3. RESULTS

(a) *Fertilization precedence*

We determined paternity for 176, 227 and 204 females in each of the three crosses, respectively (table 1).

The results for the second cross in table 1 show that, for the 85 clutches containing mixed progeny (and therefore providing direct evidence of successful double matings), last male sperm precedence was 66%, consistent with previous precedence studies (Schlager 1960; Wool & Bergerson 1979; Lewis & Austad 1990; Arnaud *et al.* 1999).

(b) *Sperm translocation*

If males transfer solely their own spermatozoa, the last cross (susceptible males × susceptible females) will produce solely susceptible progeny. However, in 45 out of 204 crosses (22% of matings), both susceptible and resistant offspring were produced; within these 45 crosses, 53% of the progeny were sired by sperm from resistant fathers (table 1). Our only conclusion is that susceptible males in the second cross had translocated resistant males' sperm (from the first cross) to virgin susceptible females in the third cross. Over half of the progeny from these females were then sired by translocated, non-self sperm.

(c) *Sperm removal*

Data providing evidence for sperm translocation (table 1) also reveal that males have evolved the potential for removing non-self sperm out of the female tract. Scanning electron microscopy examinations of the male genitalia of *T. castaneum* showed morphological characters which may enable sperm removal. Figure 1*a* shows a dorsolateral view of the aedeagus of *T. castaneum* which has a concaved furrow running along the dorsal surface. An array of chitinous spines line the proximal half of the furrow (figure 1*b*). Figure 1*c* shows a dense mass of sperm adhering to these spines on a recently mated male.

(d) *Mating frequencies*

Tribolium castaneum practise promiscuous mating patterns (table 2). Overall, the pre-mating period averages 117 s (30 males), mating duration averages 78 s (30 males, three matings per male) and the intermating intervals average 69 s (30 males, two intermating intervals per male).

4. DISCUSSION

Our results document a novel mechanism of sperm transfer: the removal of non-self spermatozoa by a mating male and the subsequent translocation of these sperm into a previously unmated female. This finding clearly demonstrates that male *T. castaneum* have evolved the capability to remove rival sperm from the female reproductive tract, a phenomenon rare in taxa except the Odonata. Removal of rival sperm may be one mechanism by which second-mating males in a sequence are able to gain subsequent fertilization precedence (66% in females giving mixed progenies, table 1). However, it seems unlikely that sperm removal will be as effective when the time interval between matings is increased and sperm migrate into less accessible spermathecal storage (Bloch-Qazi *et al.* 1996). Some sperm migrate from the bursa into the spermatheca within 10 min of spermatophore transfer and sperm migration to storage increases eightfold over the 1 h period following mating. Large numbers of sperm remain in the bursa for up to 2 h post-mating and, even

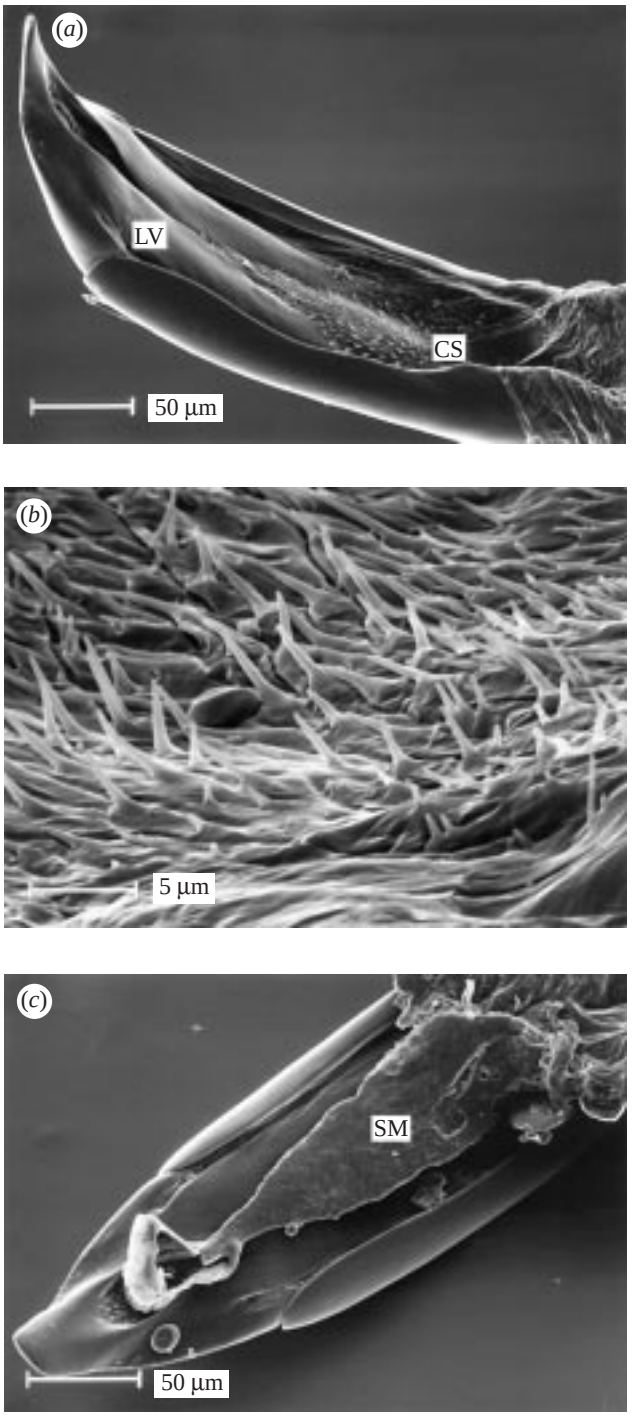

Figure 1. (*a*) A dorsolateral view of the aedeagus of *T. castaneum* which has a concave furrow running dorsally; the tip can articulate vertically where the elongate lateral valves (LV) terminate. An array of chitinous spines (CS) line the proximal half of the furrow and these are viewed in more detail in (*b*). (*c*) A large mass of sperm (SM) adheres to these spines on a recently mated male.

Table 2. *Pre-mating, mating and intermating durations for individual focal males in three different population densities*

(Mean durations are in seconds (±s.e.) and represent ten male replicates per population treatment. All individuals were sexually mature *Asm* adults and the population sex ratio was unity. Focal males were isolated for two days prior to treatment.)

population structure	pre-mating interval	first mating	intermating interval	second mating	intermating interval	third mating
6 adults	118.1 (24.9)	108.1 (21.1)	61.9 (21.5)	91.2 (29.3)	125.0 (43.2)	76.1 (21.8)
30 adults	141.6 (31.3)	86.1 (44.9)	53.2 (17.1)	84.6 (35)	60.4 (13.2)	46.6 (7.6)
100 adults	90.5 (22.7)	101.1 (30)	42.2 (9.8)	52.8 (10.1)	73.9 (35.6)	51.9 (9.8)

after 24 h, 10% of the original ejaculate persists in the bursa (Bloch-Qazi *et al.* 1996). Only 4% of the original ejaculate is accommodated in long-term storage and these sperm may remain fertile for 140 days post-mating (Bloch-Qazi *et al.* 1996). Accordingly, sperm may be removed by subsequent matings, yet the 'fertilizing set' remain inaccessible within the spermatheca soon after mating.

We also demonstrate that, within a maximum 5 min intermating interval, non-self sperm are able to survive removal and are translocated back into new females. What, therefore, are the natural selection pressures generated by such sperm removal and translocation? Although the intervals between matings were brief (a maximum of 5 min), non-self spermatozoa were clearly able to survive removal from the female tract on the chitinous aedeagus, migrate back into new females, locate the sperm storage site and, subsequently, fertilize significant numbers of ova. Behavioural observations of mating in *T. castaneum* (table 2) show that mating is frequent and brief. Even in the lower population density of six adults, focal males remate within 1–2 min suggesting that sperm removal and translocation occurs frequently. *T. castaneum* is a pest of stored products and its natural environment is in dried stored foods, particularly cereal products. In such stores reproductively active adult *T. castaneum* aggregate extremely densely at the grain surface, usually in population densities exceeding those (table 2) we examined mating frequency under (White 1988) and we therefore suggest that sperm removal and/or translocation are not simply artefacts of high mating frequencies in laboratory cultures. Female *T. castaneum* mate very frequently with many different males and store sperm (Sokoloff 1974) and, therefore, it is possible that selection upon males to remove rival sperm may have resulted in counter-selection upon sperm to survive translocation. Removed sperm are subsequently able to outcompete rival sperm in new females for fertilization. The fertilization precedence that we observe of translocated sperm would exert a significant evolutionary force as an alternative mechanism for the spread of genes such as insecticide resistance in natural populations. It is counter-intuitive that males remove rival sperm and confer significant fertilization success upon these through translocation. However, *T. castaneum* ejaculates are much greater than can be accommodated by the spermatheca (*ca.* 4% is stored; Bloch-Qazi *et al.* 1996) and perhaps bursal filling is one strategy by which mating males counteract subsequent sperm removal in the short term.

Our observations of the male genitalia of *T. castaneum* suggest a mechanism for sperm removal and translocation. Figure 1*a* shows that the aedeagus has a concave furrow and, in the ventral base of the furrow (figure 1*b*), there is an array of chitinous spines where sperm may adhere (figure 1*c*). The dimensions of the female tract show that sperm can only be removed from the female's bursa copulatrix where they are deposited at mating. Sperm move from the bursa into the tubular spermatheca (Bloch-Qazi *et al.* 1996) and the aedeagus is too large to enter the narrow spermathecal ducts.

Recent work shows that differential larval survival may confound paternity studies (Gilchrist & Partridge 1997). Under our standard, non-competitive culture conditions, it is unlikely that *Asm* and *PRm* strains differ significantly in survival; there is no evidence that insecticide resistance generates significant larval survival differences in other species in the absence of insecticide challenge (McKenzie & O'Farrell 1993; Baker *et al.* 1998) and our P2 (per cent fertilizations by the second male to mate) result is consistent with previous studies using different paternity determination techniques (Schlager 1960; Wool & Bergerson 1979; Lewis & Austad 1990). Similarly, it is not likely that resistance gene mutations confound the results: in the absence of mutagens, the mutation rate for insecticide resistance is relatively rare (e.g. <1 in 10^6 in *Lucilia cuprina* (Smyth *et al.* 1992)). Our findings suggest that further work is possible to understand the precise dynamics and evolutionary significance of sperm removal and translocation in *T. castaneum* and other species. For example, it is not known whether or what proportion of a male's own sperm is removed and possibly translocated (self-sperm removal may occur in *T. molitor*; Siva-Jothy *et al.* 1996) or how previously deposited sperm are forced onto the chitinous aedeagal spines. It is also not clear whether sperm removal is the primary adaptation of these spines or whether removal is an indirect effect of the spines being used in anchorage and precedence studies will be necessary to determine this. We have demonstrated that spermatozoa can survive on the aedeagus of a non-self male for up to 5 min between matings; however, it is not yet known what is the maximum period that removed sperm can remain viable on male genitalia. Sperm translocation generates a conflict of interests for a male when he has mated with a recently mated female and encounters a new receptive female. There are clear benefits to mating with the new female but, in doing so, males may suffer costs in translocating rival sperm from recent previous matings. Analyses of male mate choice will demonstrate whether translocation has generated selection on male mating behaviour. Finally, our translocation results also illustrate

that studies of fertilization precedence between only two males can profoundly underestimate the complexity of sperm competition and fertilization dynamics at this ultimate stage in reproduction (see e.g. Zeh & Zeh 1994).

We are very grateful to Kess Veltkamp for scanning electron microscopy expertise. Scott Pitnick, Nina Wedell and Mike Siva-Jothy provided very helpful criticism. This research was funded by the FRIA (L.A.) and the Royal Society (M.J.G.G.). Collaboration was made possible by the FNRS/CGRI and the British Council.

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