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ORIGINAL ARTICLE

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Sperm precedence, mating interval, and a novel mechanism of paternity bias in a beetle (*Tenebrio molitor* L.)

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Abstract When females mate with more than one male, the ensuing sperm competition leads to the evolution of male mechanisms that skew paternity. Males of the yellow mealworm beetle (*Tenebrio molitor*) transfer a spermatophore to females during copulation, but sperm release and storage occur later. We investigated how the interval between two matings with different males affects sperm precedence by varying the interval between the copulations so that the second mating was either: (1) before sperm release from the first spermatophore (<5 min); (2) after sperm release but before spermatophore ejection (15–20 min); (3) after spermatophore ejection but before sperm storage (4 h), or (4) after complete sperm storage (24 h). We collected offspring over a period of 2 weeks and determined paternity by protein electrophoresis. There was second-male sperm precedence in all treatments, but when the interval was <5 min, the second male usually (86% of cases) had complete sperm precedence (i.e., P_2=1). Investigations into the mechanism of second-male sperm precedence during <5-min mating intervals indicate that sperm release from the first spermatophore is inhibited, a phenomenon which has not been previously documented.

Keywords Mating intervals · Sperm precedence mechanisms · Spermatophores · *Tenebrio molitor*

Introduction

In the last 30 years, behavioral ecologists have come to realize that when females multiply mate, the ensuing, competitive interactions between the ejaculates from two or more males influence the evolution of reproductive morphology, physiology, and behavior (Parker 1970; Birkhead 1996). Although non-random paternity in the offspring of polyandrous females has been documented in all major taxa of vertebrates and invertebrates (reviewed in Birkhead and Møller 1998), these studies rarely identify the processes inside a female that lead to paternity bias (Danielsson 1998). These mechanisms need to be characterized to complete our understanding of sperm competition and female control of paternity (Simmons and Siva-Jothy 1998). For example, there is growing evidence that females employ cryptic choice (Eberhard 1996) to influence which sperm are used in fertilization (Sakaluk and Eggert 1996) Siva-Jothy and Hooper 1996, Edvardsson and Arnqvist 2000, Ward 2000). Adequate tests for cryptic female choice can only be designed if male mechanisms of sperm competition are known (Birkhead 1996; but see also Siva-Jothy and Hadrys 1998; Birkhead 1998).

There are at least two reasons why only a few mechanisms underlying the non-random use of sperm have been documented. First, most studies of sperm competition have been conducted by behavioral ecologists who traditionally focus on the evolutionary consequences of sperm competition and not on the causal proximate mechanisms (Birkhead 1995). Second, identification of mechanisms is difficult because the processes occur inside the female, where it is difficult to obtain information on the fate of sperm (Birkhead 1995). Even for evaluation of the simplest known sperm precedence mechanisms in insects, quantification of the sperm transferred by each male and the amount stored by the female is necessary (Parker et al. 1990).

Investigations into sperm transfer and storage in insects have lead to the identification of several different sperm competition mechanisms (Waage 1979; Siva-Jothy and Tsubaki 1988; Helversen and Helversen 1991; Gack and Peschke 1994; Córdoba-Aguilar 1999); in all of these instances, interference with the transfer and storage of a rival's sperm leads to increased paternity for the copulating male. Any proposed mechanism must not only coincide with sperm transfer and storage, it must also be able to account for the outcome of sperm competition, usually measured as the proportion of offspring sired by the second of two males to mate with a female (P_2).

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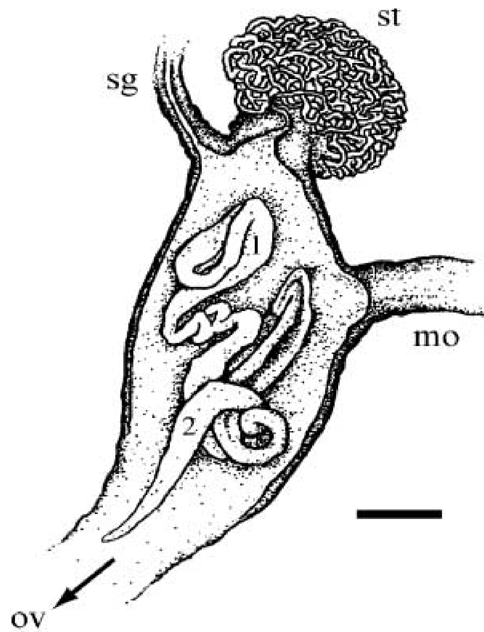

Fig. 1 The appearance of a female's bursa after mating with two males within rapid succession. In this diagram, the bursa is translucent and slightly flattened. Two spermatophores (*1* and *2*) are partially intertwined. Scale bar represents 0.5 mm (*st* spermatheca, *sg* spermathecal gland, *mo* median oviduct, *ov* ovipositor)

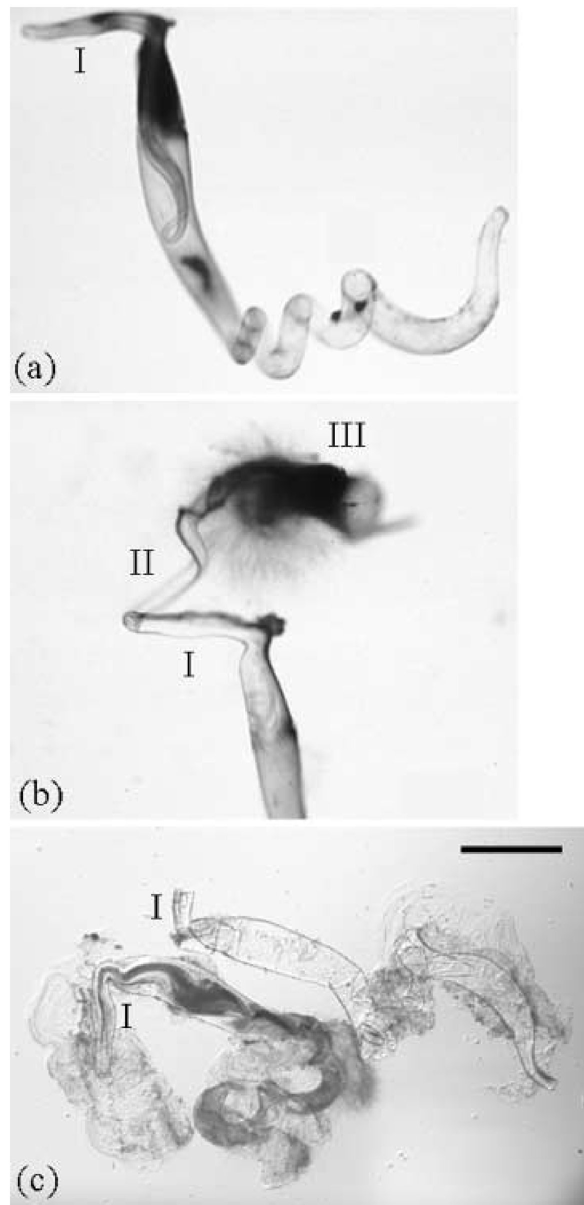

Fig. 2a–c The spermatophore of *Tenebrio molitor*. The first eversion of each spermatophore is labelled *I*. **a** A spermatophore from an interrupted copulation that has only undergone the first eversion. The dark masses are sperm. **b** The same spermatophore as in **a** after the second (*II*) and third (*III*) eversions. The sperm have moved through II to III, which has just ruptured. **c** Two spermatophores removed from a female 20 min after the second mating. The first spermatophore (*bottom left*) shows only the first eversion and sperm are still inside, whereas the second spermatophore (*top right*) has completely everted (II and III not visible) and released sperm. Scale bar represents 0.5 mm

Although the reproductive morphology and polyandrous behavior of female yellow mealworm beetles (*Tenebrio molitor* L.) has been well documented, researchers have only recently examined the fate of sperm from multiple males within the female. *T. molitor* follows the typical insect pattern of second-male precedence (P_2>0.5), but the mechanisms responsible for this are unknown (Siva-Jothy et al. 1996; unpublished data). Gage (1992) found trapped sperm under backwards-pointing spines on the male's intromittent organ and hypothesized that males remove rival sperm from the bursa of the female. However, Siva-Jothy et al. (1996) showed that this could not be the primary mechanism causing the second-male advantage because second males had the same sperm precedence when there was a short interval between matings (rival sperm still available in the bursa) as when there was a long interval between matings (rival sperm stored in the spermatheca and out of reach). Siva-Jothy and colleagues did not find a difference in P_2 values based on mating intervals, but mating interval has been identified as a factor affecting paternity in many other species (reviewed in Simmons and Siva-Jothy 1998).

Our recent investigations into sperm transfer and storage in *T. molitor* prompted us to pursue a more detailed study of the effect of mating intervals on sperm precedence. In *T. molitor*, sperm transfer and storage is a complex, multi-step process that takes place over several hours. In the first 30–60 s of copulation, a male fills a pre-formed spermatophore with sperm and transfers it into the female's bursa (Gadzama and Happ 1974; Fig. 1). Total copulation duration is between 60 and 120 s (unpublished data). The spermatophore is initially invaginated at the end positioned closest to the spermatheca; inside the female's bursa it undergoes a three-stage eversion (Fig. 2) before finally bursting and releasing sperm 7–10 min after the end of copulation (Gadzama and Happ 1974). The sperm are then stored in the spermatheca, although complete sperm storage does not occur until 6 h

after copulation (Gage and Baker 1991). The empty spermatophore is eventually ejected from the female within 4 h of copulation (Weir 1998).

To study whether a second mating during this sequence of events has an effect on sperm precedence we set up four different mating intervals. These intervals reflect the possible situations facing the second male to mate: (1) before sperm release from the first spermatophore (<5 min); (2) after sperm release but before spermatophore ejection (15–20 min); (3) after spermatophore ejection but before complete sperm storage (4 h), and (4) after complete sperm storage (24 h). If sperm release, spermatophore ejection, and/or sperm storage affected sperm precedence we expected to find between-treatment differences in P_2 values.

As we report here, a second male's precedence was highest when he mated before the first male's sperm were released from the spermatophore, i.e., when the second mating occurred within 5 min of the end of the first mating. We hypothesized that the second-male advantage could be accomplished if (1) the second male removed the previous spermatophore that had not yet released sperm, or (2) the first spermatophore failed to release its sperm. We also report the results of experiments that support hypothesis 2 as the mechanism that leads to the observed patterns of sperm precedence.

Methods

The cultures of *T. molitor* used in this experiment were originally obtained from cultures maintained by the Animal Care Facility at Arizona State University. All beetles were kept between 22–24°C and at ambient day length and humidity. Adults used in the experiment were housed in 9-cm-diameter petri dishes, separated by sex to control mating history, and supplemented with rodent diet (pellets) and apple slices. The substrate in the petri dishes was either wheat bran or, for females after mating, sifted whole-wheat flour. Females laid most of their eggs on the bottom of the petri dish but resifting the flour allowed all the eggs to be collected. We determined paternity on day-old larvae through protein electrophoresis (Murphy et al. 1996) on phosphoglucose isomerase (PGI); our cultures have two alleles designated slow (S) and fast (F) based on their migration speed. Homozygous adults (SS and FF) were obtained for this experiment from previously established monomorphic cultures; females were 5–6 days post-eclosion and males were 8–10 days post-eclosion.

Mating-interval experiment

We used reciprocal double matings with both SS and FF females to control for any affects of PGI genotype on sperm competition. Males were matched roughly by size and each pair was housed together before the matings. Genotype was denoted by marking the backs of FF males with a small spot of white paint. One day prior to the mating trials, we tested all males for the production of viable sperm in one of two ways: (1) copulation with a tester female was interrupted and the spermatophore was visually checked for moving sperm or (2) if we were unsuccessful in obtaining the spermatophore in the first attempt, we allowed the male to complete a copulation with a virgin female and looked for egg production the next day, which indicated that sperm had been transferred.

In all treatments, a virgin female was put with one male from a pair in a mating arena consisting of a petri dish bottom overturned on a piece of paper. When copulation ended, the first male was removed. We timed the mating interval between the end of the copulation with the first male and the beginning of the copulation with the second male. For the <5-min mating interval, the second male was immediately introduced into the mating arena and allowed 5 min to mate. In the 15- to 20-min interval treatment, the female was kept in the mating arena and the second male was introduced 15 min later. For the 4-h and 24-h interval treatments, after the first mating, the female was isolated in a petri dish. After the requisite time period, she was returned to the mating arena and the second male introduced and allowed 20 min to mate. In all replicates of these last two treatments, we were able to confirm that the first male successfully transferred sperm by the presence of eggs in the petri dish before the second mating. We visually confirmed all second matings, and replicates where the second male failed to mate within the specified time limit (5 of 59) were discarded.

For all treatments, after the second mating we housed the females individually in petri dishes. The first round of double matings included all four treatment groups and ran from 1 November to 6 December 1998. Siva-Jothy et al. (1996) found that P_2 values decreased over time, so we sampled offspring over a 2-week period by taking the eggs laid on the 1st and 2nd days after the second mating, on the 8th and 9th days, and on the 15th and 16th days. In the second round of double matings, conducted 5–23 May 1999, we only used a <5-min mating interval to further investigate what was occurring in this treatment. This time, we only collected eggs laid on the 1st and 2nd days. These replicates from the second round were not included in the analysis of mating interval effect but used to evaluate frequency of complete second-male sperm precedence.

Proportional data (P_2 values) were arcsine square root transformed before using appropriate parametric tests (Sokal and Rohlf 1995). All means (±1 SE) are reported using untransformed values for ease of understanding. All analyses were computed using SYSTAT 7.0.1 for Windows (SPSS Inc.).

Spermatophore removal or inhibition experiments

To see if complete second-male sperm precedence was caused by either spermatophore removal or inhibition, we dissected females that had been doubly mated within 5 min, as above. We either immediately dissected the females or waited 20–30 min after the last mating, to allow enough time for sperm release, before dissecting them. In both cases, we noted the number of spermatophores found in the bursa (Fig. 1) and, in dissections performed after 20 min, whether they had fully everted and released sperm.

To further clarify which spermatophore failed to evert and release sperm, we allowed the second male to begin copulation within 5 min of the first male, but we interrupted the copulation after 30 s and collected the second male's spermatophore before he transferred it to the female. We dissected these females after 20 min and noted the condition of the first spermatophore.

Results

Mating interval experiment

We first analyzed whether P_2 values decreased over time. Twenty females stopped laying eggs by the third sampling period, so we initially evaluated time effects for all females using the first two sampling periods (paired t_{53}=1.116, P=0.269). P_2 values did not differ between the three sampling periods for the 34 other replicates (repeated-measures ANOVA; $F_{2,88}$=0.033, P=0.968). Because P_2 values did not decline over time, we calculated a total P_2 value using all the offspring from each female (mean=38.7 offspring; range 12–72).

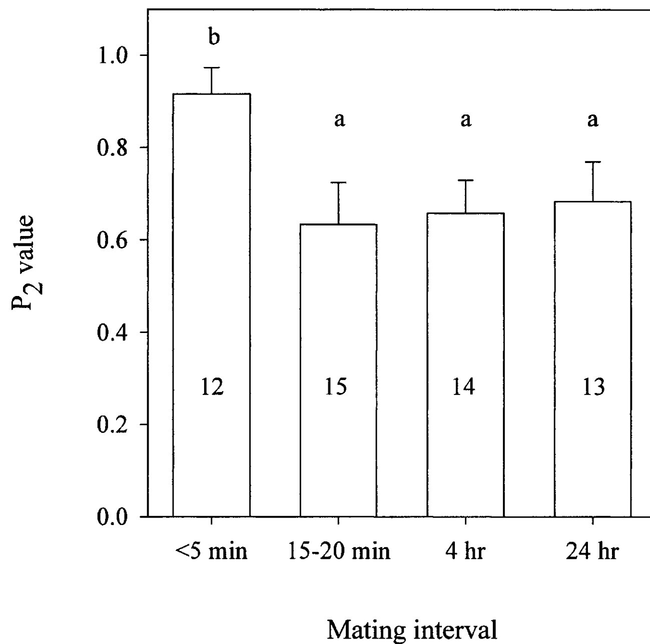

Fig. 3 Mean P_2 (±1 SE) values for all mating intervals. Treatments that are not significantly different (Tukey-Kramer method, α=0.05) are indicated by the same letter. Numbers on bars indicate sample sizes

Mating interval affected P_2 values ($F_{3,50}$=3.898, P=0.014); the <5-min treatment had significantly higher P_2 values than the other treatments (Fig. 3). Because the other three treatments were extremely similar in both mean and distribution of P_2 values, we combined them into one category (>15 min mating interval) for use in subsequent analyses. Males that mated within 5 min of the first male were also more likely to sire all the offspring; 86% of <5-min replicates had P_2=1 whereas only 12% of >15-min replicates had P_2=1 (G-test of goodness of fit; G_{adj}=62.68, df=1, P<0.001). However, the second male still had P_2>0.5 when the interval between matings was >15 min (t_{41}=2.845, P=0.007).

Spermatophore removal or inhibition experiments

Two spermatophores were found in dissections performed immediately after the matings for all 12 females. In 22 dissections performed 20–30 min after the second mating, no spermatophores were found in 3 cases, one spermatophore was found in 1 case, and two spermatophores were found in 18 cases. In all the latter 18 cases, only one of the spermatophores had fully everted and released sperm and the other was arrested at the first eversion (Fig. 2c). When the second male was not allowed to transfer a spermatophore, the first spermatophore failed to release sperm in 16 out of 18 replicates. This result indicated the spermatophores that failed to release sperm in the dissections after complete double matings were from the first male.

Discussion

The time between two matings has a large effect on whose sperm get used for fertilization. Males that mated <5 min after the first male had higher mean P_2 values than males who mated after a longer interval and had complete sperm precedence in 86% of replicates as opposed to only 12% for all other treatments.

Our dissections indicate that complete second-male precedence likely results from the inhibition of sperm release from the first spermatophore, preventing its sperm from being stored and used for fertilization, and not from the removal of the first spermatophore before sperm release. Only 4 of 22 dissections did not have an inhibited spermatophore; one female contained only one spermatophore and three females contained no spermatophores. It is possible that one of the two males failed to transfer a spermatophore, but unlikely that both males failed in three cases. Rather, the spermatophores had already been ejected because free sperm was found in all three females.

How spermatophore evacuation is inhibited is not yet known, but the interruption of the second male during copulation suggests that the inhibition occurs within the first 30 s of the second mating. One possibility is chemical inhibition either through male accessory gland secretions or even female-derived products. Sperm release occurs due to increasing internal pressure and a simple short-term pH change can prevent sperm release in vitro (Gadzama 1972). Alternatively, inhibition could be caused directly by physical means. The male's aedeagus is covered with a spiny sheath (Gage 1992); inside the female, the spines could puncture the previous spermatophore, releasing the internal pressure but preventing most or all of its sperm from being released (M.J.G. Gage, personal communication). Regardless of the cause of inhibition, this is a novel mechanism resulting in sperm precedence, and it could be important in other species with delayed sperm release from a spermatophore.

The importance of sperm release inhibition to the mating system of *T. molitor* depends on the normal interval between matings. If females rarely remate within 5 min, then this mechanism is unlikely to cause strong selection. However, short mating intervals are probably common. Females do not exhibit a refractory period and usually accept a mating if the male is in the correct position (unpublished data); therefore female mating interval depends on the density of courting males. Local population densities often become high in pockets of stored grain, the larval food. Adult males also tend to stay on the top of the grain where mating takes place (Thompson 1995; Thompson 1998), so females are likely to encounter many courting males in a short period of time and mating twice in 5 min must be common.

Short female mating intervals create selection on males to delay female remating to avoid having their sperm release inhibited. Other traits of male *T. molitor* may have evolved in response to this selection. Gage and

Baker (1991) showed that males more often remain on or near a female for a minute or more after mating when other males are present, which constitutes post-copulatory mate guarding by males. Additionally, females attract males by a pheromone, but males also have a pheromone that makes females less attractive (Happ 1969). Short-term mate guarding and production of an anti-aphrodisiac could be counter-adaptations to sperm release inhibition.

Besides inhibition of sperm release, mate guarding, and anti-aphrodisiacs, there must be another mechanism causing sperm precedence because second males sired more than half the offspring, regardless of mating interval. Multiple mechanisms that affect which sperm are used for fertilization may be the rule, because in many species males face dual selection for mechanisms that overcome sperm of previous mates and mechanisms that prevent their own sperm from being overcome by subsequent mates (Parker 1970). Additionally, conflicts between males and females over which sperm to use can lead to female mechanisms for biasing paternity (Eberhard 1996). The end product of these interactions, the paternity of offspring, is not useful in itself for gaining an understanding of the selection caused by sperm competition and cryptic female choice. Only by identification and characterization of the many underlying mechanisms can we gain a complete understanding of the evolutionary significance of post-copulatory sexual selection.

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