



Multiple mating and repeated copulations: effects on male reproductive success in red flour beetles

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In many insects, both sexes mate multiple times and females use stored sperm for fertilizations. While males frequently engage in two distinct behaviours, multiple mating (with different females) and repeated copulations (with the same female), the reproductive consequences of these behaviours for males have been quantified for only a few species. In this study, males of the red flour beetle, *Tribolium castaneum*, were found to be capable of mating with as many as seven different virgin females within 15 min. Across sequential copulations with virgin females, there was no decline in either male insemination success or average female progeny production over 48 h. However, when males copulated with previously mated females, there was a significant decline in male paternity success across sequential copulations, possibly due to male sperm depletion. In separate experiments, *T. castaneum* males were found to engage in two to six repeated copulations with the same, individually marked female. These repeated copulations did not increase male insemination success, short-term female fecundity, or male paternity success. Repeated copulations may possibly play a role in sperm defence. This study indicates that males may frequently engage in multiple matings, but these additional matings may lead to diminishing male reproductive returns.

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The occurrence of multiple mating, in which both males and females copulate with two or more partners, is now widely documented in numerous species (Ridley 1990; Keller & Reeve 1995). Many recent studies have examined material and genetic benefits accruing to females from multiple mating (Jennions & Petrie 2000). From a male perspective, considerable evidence has accumulated that supports Dewsbury's (1982) early suggestion that ejaculate production is costly. Such evidence includes a decline across multiple matings in male performance measured in terms of sperm numbers, spermatophore size, stimulation of female egg production rate, and inhibition of female remating (reviewed by Simmons 2001). Given the cost of ejaculate production, multiple matings may lead to diminishing reproductive returns for males. In particular, one important component of male reproductive success, male paternity success (also known as sperm precedence or P_2), might be predicted to decline across multiple matings. However, only a few studies (Simmons & Parker 1992; Arnqvist & Danielsson 1999) have examined how male multiple mating affects male paternity success. A distinct but related male behaviour consists of repeated copulations with the same female (Hunter et al. 1993). Costs of repeated copulations, both in terms of male ejaculate

production and reduced mating opportunities with other females, might be compensated through increased male insemination or paternity success. Studies have shown that repeated copulations can provide increased paternity assurance for males (e.g. Smith 1979; Otronen 1994), and thus, this male behaviour also merits further investigation.

This study examined how two male behaviours, multiple mating and repeated copulations, may influence male insemination success, female fecundity and paternity success in the red flour beetle, *Tribolium castaneum*. In this cosmopolitan stored-product pest, both sexes mate multiply (Sokoloff 1974; Lewis & Iannini 1995), and females can use stored sperm for several months (Bloch Qazi et al. 1996). Female benefits from multiple mating have been demonstrated in terms of increased fecundity (Lewis & Austad 1994) as well as increased paternity success of sons (Bernasconi & Keller 2001; Pai & Yan 2002). When *Tribolium* males copulate with several females in rapid succession, the number of sperm transferred generally declines (Bloch Qazi et al. 1996). This study investigated whether such sperm depletion leads to reductions in male insemination success, paternity success, or female fecundity across multiple matings.

METHODS

Experimental beetles were randomly selected from a *T. castaneum* culture derived from the Berkeley synthetic

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strain. We sexed individuals as pupae, and maintained all experimental beetles in a dark incubator at 29°C and 70% RH. Males were kept singly in 20-ml vials with 3 g of King Arthur unbleached white flour, and females were kept in 20 g of flour in groups. All beetles used in a given experiment were of similar ages (± 1 week), which are specified in the descriptions given below.

Male Multiple Mating

We observed the mating behaviour of focal wild-type males under dim illumination (400–600 lx) at 29°C in 3.4-cm-diameter mating arenas. To determine how often males mate with different females, we placed single males ($N = 82$) in a mating arena with five virgin wild-type females, and recorded the number and duration of copulations during 15-min observation periods. Following Lewis & Iannini (1995), we used the following behaviour definitions: a mount consisted of the male dorsally mounting the female, and copulation (intromission) consisted of the male extruding his aedeagus and inserting it into the female's genital opening. Copulation duration was defined as the total length of intromission, including repeated intromissions that occasionally occur during the same mount. When contact ended following copulation, we immediately removed the mated female and replaced her with another virgin female. This procedure maintained a constant density of virgin females during the entire observation period for all males. All beetles were virgins at the outset of this experiment, and were 30–35 days posteclosion.

We measured male insemination success across sequential copulations within 30 min for 15 wild-type males, each given access to five virgin wild-type females. Mated females were removed immediately following copulation, sequentially numbered and isolated to lay eggs for 48 h. Eggs were incubated at 29°C and 70% RH for 45 days. Insemination was considered successful if mated females produced progeny (*T. castaneum* females generally produce ~10 eggs daily following a single mating, so lack of progeny reliably indicates insemination failure). The percentage of matings leading to successful inseminations was compared across multiple matings using a contingency table analysis test of homogeneity of proportions. In addition, we counted adult progeny and used one-way analysis of variance (ANOVA) to determine whether female fecundity over this 48-h period changed across male multiple matings.

Male Multiple Mating and Paternity Success

To examine how male multiple matings affect second-male paternity success (also called sperm precedence, or P_2), we allowed experimental males to mate sequentially with as many as five females that had been previously mated with males homozygous for an autosomal, semi-dominant black body colour allele. These marker genotypes have been used in previous studies of *Tribolium* sperm precedence (Schlager 1960; Lewis & Austad 1990, 1994; Bernasconi & Keller 2001; Pai & Yan 2002; Bloch Qazi 2003; Nilsson et al. 2003). In this study, we used

a standardized mating order with black first males and wild-type second males for paternity measurements to avoid the effects of strain interactions on sperm precedence (such interactions result in lower P_2 values only when black males are mated as second mates to wild-type females; Lewis & Austad 1990). Pairs consisting of a virgin wild-type female and a black male were placed in 1 g of flour for a 24-h mating period. Following this first mating, females were allowed to oviposit for 24 h to verify successful insemination by the first male (based on the presence of progeny developing from eggs laid during this first mating). Five of these previously mated females were then placed in a mating arena with a single virgin wild-type male ($N = 15$ males, all 10–15 days posteclosion) and observed for 30 min. Immediately after copulating with the second male, doubly mated females were removed, sequentially numbered and isolated to lay eggs for 48 h. Progeny were kept at 29°C and 70% RH for 45 days to develop them into adults, and adult body colour was scored to assign paternity to either the first or the second male. Differential mortality during development is unlikely to bias paternity estimates, because egg-to-adult survivorship is similar for crosses between these strains (Sinnock 1969). In this and all subsequent experiments measuring paternity success, only those females with positive evidence of successful insemination by both the first and second males were included in the analysis. Thus, P_2 values of 1 were included only if there was evidence, based on the presence of progeny from the intermating oviposition period, that the first male had successfully mated with the female; this excluded a total of six females (two for males' first matings, and one female each for a male's second, third, fourth and fifth matings). Similarly, P_2 values of 0 were excluded from analysis because failed insemination during the second mating could not be distinguished from complete first-male sperm precedence; this excluded three additional females from analysis (two for males' third matings and one for a male's fourth mating).

Male Repeated Copulations

To determine the extent to which males copulate repeatedly with the same female, wild-type males ($N = 21$) were each given access to five individually marked, wild-type females. All beetles were virgins at the outset of this experiment and were 10–12 days posteclosion. Females were individually marked with unique combinations of small pronotal and elytral paint dots. The sequence of contacts, copulations and copulation durations of the focal male with each female was recorded during 45-min observation periods.

To examine possible differences in insemination success and fecundity of females that had mated once or repeatedly (one, two, three or four times) with the same male, we measured the presence and number of progeny subsequently produced by each female. Following mating observations, we collected eggs from each mated female that had been isolated for three 24-h oviposition periods: day 1 (0–24 h) postmating, day 5 postmating and day 10 postmating. After 45 days, we summed the total number of adult progeny across the three oviposition periods.

These three oviposition periods were chosen to be representative of progeny production during the first 2 weeks postmating; although the multiple mating experiments used a 48-h oviposition period, both of these assays measure short-term fecundity because *T. castaneum* females can lay fertilized eggs for up to 140 days following a single mating (Bloch Qazi et al. 1996).

Insemination was considered successful if mated females produced progeny. The proportion of successfully inseminated females was compared between single copulations and repeated (two, three, or four times) copulations using a test of homogeneity of proportions. We used one-way analysis of variance (ANOVA) to examine differences in fecundity with repeated copulations for $N = 20$ females that copulated once, $N = 20$ females that copulated twice, $N = 3$ females that copulated three times, and $N = 3$ females that copulated four times, all with the same male. Females producing no progeny were excluded from this analysis.

Male Repeated Copulations and Paternity Success

To examine the effect of repeated copulations on male paternity success, we allowed wild-type males to copulate repeatedly with previously mated black females. For their initial mating, we placed each of 60 virgin black females individually in a mating arena with a virgin black male and recorded copulation durations. Following the first mating, females were allowed to oviposit for 24 h to verify that this copulation led to successful insemination. Twenty-four hours after the first mating, each female was placed in a mating arena with a virgin, wild-type male. These pairs ($N = 60$) were randomly assigned to three treatments consisting of either one, two or three copulations by the second male. Pairs were observed until the requisite number of copulations had occurred (maximum observation time = 41 min). We then removed females and collected eggs for three 24-h oviposition periods: day 1 (0–24 h) postmating, day 5 postmating and day 10 postmating. After 45 days, we determined paternity success by scoring adult progeny phenotypes as described above. As before, females in which either mating failed to result in insemination were excluded from the analysis, resulting in the following sample sizes for the three treatments: one second-male copulation, $N = 12$ females; two repeated second-male copulations, $N = 12$ females; three repeated second-male copulations, $N = 9$ females.

Relative Copulation Duration and Paternity Success

Copulation durations vary widely in *T. castaneum* (Bloch Qazi et al. 1996), so we examined how relative copulation duration by first and second males affected second-male paternity success. For 37 virgin black females, we measured the duration of their first copulation with a virgin black male and their second copulation with a virgin wild-type male. All beetles were 15–20 days posteclosion. Females were allowed to lay eggs for 24 h between matings to confirm successful insemination from the first copulation. Following the second copulation, eggs were collected

from females during three 24-h periods: day 1 (0–24 h) postmating, day 5 postmating and day 10 postmating. We examined the relationship between relative copulation duration (ratio of second to first copulation) and paternity success, measured as described above.

RESULTS

Multiple Mating and Repeated Copulation Rates

Male *T. castaneum* showed high multiple mating rates when given unlimited access to virgin females, copulating with as many as seven different virgin females per 15-min observation period (Fig. 1a), and mating with a mean \pm SE of 4.1 ± 0.15 females.

Experiments conducted with individually marked females revealed that repeated male copulations with the same female were quite common (Fig. 1b). Males engaged in repeated copulations (range 2–6) with 33% of the available females, and copulated only once with 43% of females; males did not mate with the remaining 24% of females (not shown).

Male Multiple Mating: Insemination and Paternity Success

The percentage of females successfully inseminated during copulation did not differ across *T. castaneum* males' first or subsequent matings (test of homogeneity with fourth and fifth matings pooled: $G_3 = 1.98$, $P = 0.58$; Table 1). There was also no change in the number of progeny produced during 48-h oviposition periods by females that had mated sequentially with the same male (ANOVA: $F_{4,18} = 0.95$, $P = 0.46$; Table 1). However, there was a significant decline in male paternity success across multiple matings with previously mated females (regression: $r^2 = 0.42$, $N = 19$, $P = 0.0027$; Fig. 2). The proportion of progeny sired by the second focal male in each case was high for a first mating ($P_2 = 0.893 \pm 0.08$), but by the fifth mating had declined to $P_2 = 0.151 \pm 0.03$.

Male Repeated Copulations: Insemination and Paternity Success

Single copulations by *T. castaneum* males resulted in 62.2% successful insemination of females, and repeated (2, 3 or 4) copulations with the same female resulted in 76.5% insemination success. This difference was not significant (test of homogeneity: $G_1 = 1.85$, $P = 0.17$). Similarly, second-male paternity success was unaffected when males were allowed to copulate one, two or three times with a previously mated female (ANOVA: $F_{2,30} = 0.01$, $P = 0.99$; Fig. 3). When paternity success was separately analysed for oviposition days 1, 5 and 10 (data not shown), there was also no significant effect of repeated copulations (day 1: $F_{2,30} = 1.04$, $P = 0.36$; day 5: $F_{2,30} = 0.24$, $P = 0.79$; day 10: $F_{2,30} = 1.45$, $P = 0.25$). There was also no significant difference in the mean number of progeny produced by females that had copulated one, two, three or four times with the same male ($F_{3,50} = 0.28$, $P = 0.84$).

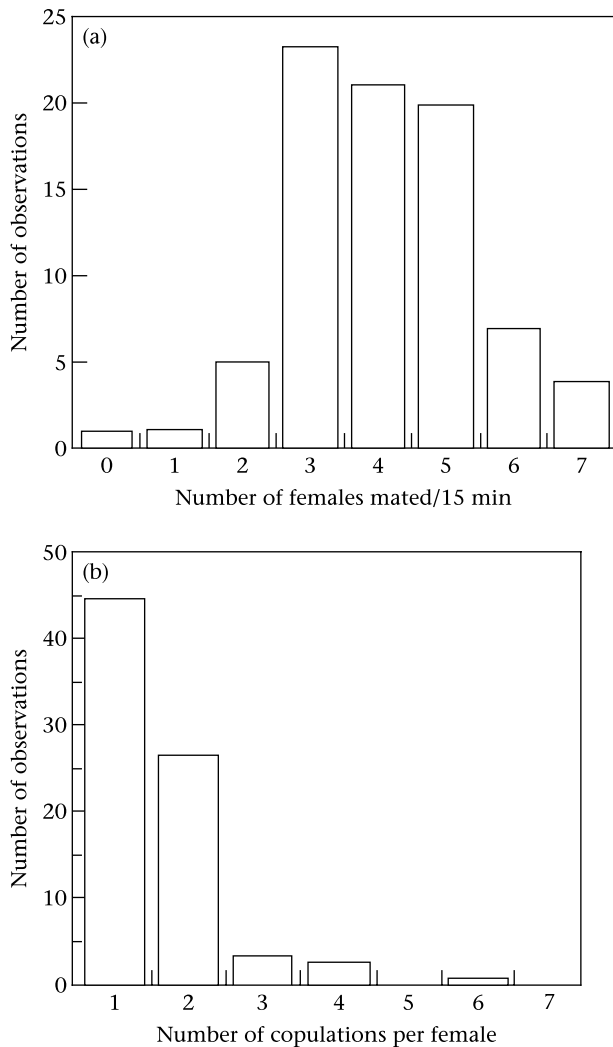


Figure 1. (a) Frequency of multiple mating by *Tribolium castaneum* wild-type males ($N=82$), each given constant access to five virgin wild-type females during 15-min observation periods; each mated female was removed immediately after copulation and replaced with a new virgin female. (b) Frequency of repeated copulations by *Tribolium castaneum* males with the same individually marked female ($N=105$ females). Twenty-one wild-type males were each given access to five wild-type females for 45-min observation periods.

Copulation Duration and Paternity Success

Observed copulations varied widely in duration, ranging from 17 s to 9.3 min with a median duration of 101 s. Male paternity success was not affected by either the ratio of second to first copulation duration (regression: $r^2 = 0.00$, $N = 37$, $P = 0.41$), or the duration of the second male's copulation ($r^2 = 0.07$, $N = 37$, $P = 0.10$).

DISCUSSION

Costs of male ejaculate production suggest that males should engage in ejaculation strategies that maximize their reproductive success (Dewsbury 1982). Numerous studies of strategic ejaculation have shown that males of

Table 1. Percentage of *Tribolium castaneum* females inseminated and female 48-h progeny production across male multiple matings (as many as five sequential matings within 30 min)

Male's mating sequence	% Inseminated	Number of progeny per female (N)
First mating	76.7	20.5 ± 3.1 (6)
Second mating	75.0	35.4 ± 11.2 (5)
Third mating	58.8	23.6 ± 3.5 (8)
Fourth mating	72.7	22.5 ± 1.5 (2)
Fifth mating	66.7	31.0 ± 2.0 (2)

Female progeny production (reported as $\bar{X} \pm SE$) measured 0–48 h postmating; number of females for progeny counts in parentheses.

many species can adjust sperm numbers or spermatophore size according to the proximal cues of female age, size, or mating status, as well as population-level cues relating to risk of sperm competition (reviewed by Simmons 2001). This study focused on the effects of male multiple mating and repeated copulations on *T. castaneum* male insemination and paternity success, and female egg production. Previous studies on *T. castaneum* have shown that males can successfully inseminate as many as three different females in rapidly sequential copulations, and that the number of sperm transferred generally declines across consecutive matings (Bloch Qazi et al. 1996). The present study shows that *T. castaneum* males are capable of mating with as many as seven different females within 15 min. Across these rapidly sequential copulations, no reduction was found in either male insemination success or the number of progeny produced by females within 2 days postmating. However, male paternity success (P_2) showed a significant decline across multiple matings. This decline may be due to depletion of male sperm or accessory gland products, or perhaps may reflect declining male

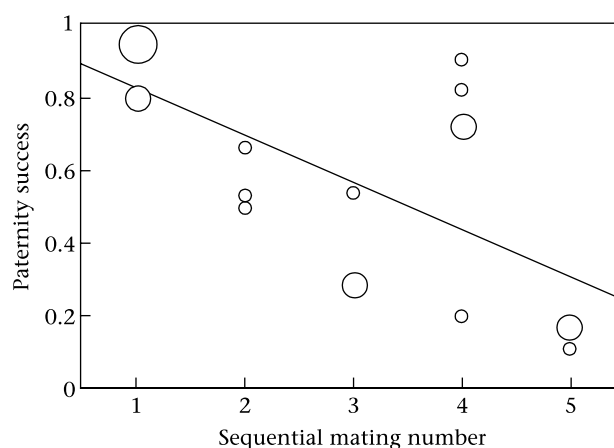


Figure 2. Changes in paternity success (proportion of doubly mated female's progeny fertilized) of wild-type second males across multiple matings (as many as five sequential matings within 30 min). Increased symbol size represents overlapping data points. Fifteen *Tribolium castaneum* males were each given access to five wild-type females previously mated with black first males. Paternity success is given only for confirmed double matings: $N=5$ first matings, $N=3$ second matings, $N=3$ third matings, $N=5$ fourth matings, and $N=3$ fifth matings.

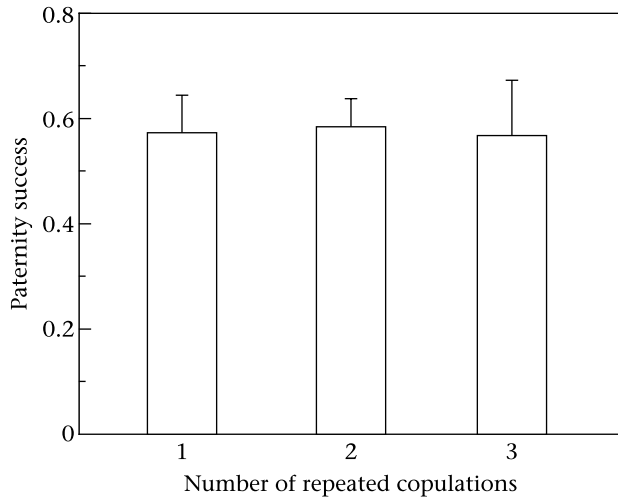


Figure 3. Effect on second-male paternity success of repeated copulations by wild-type *Tribolium castaneum* second males with the same female ($\bar{X} \pm SE$ proportion of progeny fertilized by second male). Previously mated black strain females were randomly assigned to mating treatments in which they copulated either one time ($N=12$), two times ($N=12$) or three times ($N=9$) with the wild-type second male. Paternity success was estimated from progeny produced during three 24-h oviposition periods at 1, 5 and 10 days postmating; only females with positive evidence of successful insemination by both males were included in the analysis.

performance in copulatory courtship, which has the potential to influence paternity success (see Edvardsson & Arnqvist 2000; Bloch Qazi 2003).

These results contrast with those of two previous studies that have examined effects of male multiple mating on paternity success. In the dung fly, *Scathophaga stercoraria*, male mating history (0–5 previous matings in rapid succession) did not influence male sperm precedence (Simmons & Parker 1992). Male remating interval (2 h versus 48 h) was manipulated in Arnqvist & Danielsson's (1999) study of *Gerris lateralis* water striders, and although males with shorter remating intervals transferred fewer sperm to females, there was no significant reduction in male sperm precedence. The present results in *T. castaneum* show that, although multiple matings are clearly reproductively advantageous to males, these additional matings do not generate a linear increase in male reproductive success. The patterns of *T. castaneum* male mating behaviour and paternity success described here may be important determinants of male reproductive success under conditions of high population density in this stored-product pest.

This study also indicates that *T. castaneum* males often copulate repeatedly (as many as six times) with the same female, even when virgin females are available. Given the costs of repeated copulations, both in terms of male ejaculate production and lost mating opportunities with other females, some compensatory reproductive advantage might be expected. Although in several other species repeated copulations with the same female have been shown to increase male paternity success (e.g. Smith 1979; Muller & Eggert 1989; Birkhead & Møller 1992; Otronen 1994), no such advantage was found in the present study.

Repeated copulations with the same female also did not increase *T. castaneum* male insemination success or the number of progeny produced over 10 days by females. These repeated copulations are unlikely to represent male failure to discern female reproductive status, because *T. castaneum* males can discriminate virgins from previously mated females (Graur & Wool 1982; Lewis & Iannini 1995) and males gain a reproductive advantage by preferentially copulating with virgin females (Lewis & Iannini 1995). It is possible that repeated copulations might increase sperm defence, limiting paternity success of males that later mate with the female. Previous work on *T. castaneum* has shown that the female sperm storage organ is filled to only two-thirds capacity by the first mating (Lewis & Jutkiewicz 1998); repeated copulations might serve to monopolize space in the female spermatheca, thus limiting sperm storage from later mates (Simmons & Siva-Jothy 1998). Repeated copulations may also increase transfer of male accessory gland products, some of which have been shown to inhibit female remating in other insects (Eberhard 1996; Simmons 2001).

In *T. castaneum*, copulation duration does not appear to influence either the number of progeny subsequently produced by females or the paternity success of males mating with previously mated females. The biological significance of the wide range of copulation durations observed in this species is currently unknown, although longer copulations might prevent female remating during the critical 60-min postmating period when sperm storage occurs (Bloch Qazi et al. 1996). In polygamous species with long-lived adults such as *Tribolium*, further studies of male mating behaviours will provide insight into the dynamics of male insemination and paternity success over the course of a male's life span.

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