Female mate choice across mating stages and between sequential mates in flour beetles

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Abstract

Few studies have examined how female premating choice correlates with the outcome of copulatory and post-copulatory processes. It has been shown that polyandrous *Tribolium castaneum* females discriminate among males before mating based on olfactory cues, and also exert cryptic choice during mating through several mechanisms. This study tested whether a male's relative attractiveness predicted his insemination success during copulation. Bioassays with male olfactory cues were used to rank two males as more and less attractive to females; each female was then mated to either her more attractive male followed by less attractive male, or vice versa. Dissections immediately after second copulations revealed a significantly higher percent of successful inseminations for females that remated with more attractive males compared with those that remated with less attractive males. These results indicate that cryptic female choice during copulation reinforces precopulatory female choice in *T. castaneum*, and suggest that females could use cryptic choice to trade up to more attractive males, possibly gaining better phenotypic or genetic quality of sires.

Introduction

When investigating male–male competition and female choice of mates, studies often consider either traditional premating interactions (reviewed in Andersson, 1994) or their post-mating counterparts, sperm competition (reviewed in Simmons, 2001) and cryptic female choice (reviewed in Eberhard, 1996). However, male/female reproductive success in polyandrous species depends on both mate acquisition/choice and on paternity success/choice with each mate. Not many studies have attempted to establish how these two selection episodes interact. Thus, in polyandrous females that mate with and store sperm from multiple males, female choice can occur at any stage of reproduction, from premating choice of mates, through a number of paternity biasing mechanisms employed during copulation (pericopulatory), to post-copulatory female choice of sperm. For instance, after copulation begins females can still

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eliminate or diminish a mating male's paternity by blocking complete intromission or spermatophore transfer (Tallamy *et al.*, 2003; e.g. Tallamy *et al.*, 2002; Fedina & Lewis, 2006) or by causing a reduction in the number of sperm transferred (e.g. Sakaluk & Eggert, 1996; Bussiere *et al.*, 2006; Fedina, 2007). Previous studies in different systems have found both positive (Pizzari *et al.*, 2002; Evans *et al.*, 2003; Pilastro *et al.*, 2004; Demary & Lewis, 2007) as well as negative (Danielsson, 2001; Demary & Lewis, 2007) relationships between precopulatory and peri-/post-copulatory selection episodes. We might expect negative/no correlation between these episodes in the case of sexual conflict (e.g. if it is costly for females to avoid mating), or if females gain material benefits from multiple mating. In such situations, females may mate indiscriminately with multiple males, and then select sires during or after mating (Jennions & Petrie, 2000). Discrepancies between sexual selection episodes may also arise if females use additional information obtained about male phenotypic or genetic quality to correct previous mate choice errors. Therefore, measurements of mating success alone do not necessarily predict reproductive success during subsequent episodes. This makes it crucial to measure outcomes of

peri-/post-copulatory selection, as well as precopulatory selection, to fully understand the evolution of traits through sexual selection.

Another important and often overlooked aspect of mate choice (whether it occurs before, during or after mating) is its potential dependence on prior female experience, and in particular, on characteristics of her previous mates. Many theoretical studies have addressed the question of choice strategies among multiple mates (Parker, 1983; Real, 1990; Wiegmann *et al.*, 1996; e.g. Janetos, 1980), but experimental validations of these theories are still scarce. The trade-up hypothesis elaborated by Halliday (1983) suggests that in species with nonresource-based polyandry and last male sperm precedence, females can maximize their progeny fitness by first mating indiscriminately with any male to ensure fertilization, and then accepting future mates only if they are of higher genetic quality than previous mates. Previous studies have reported some evidence both in support of this hypothesis (Pitcher *et al.*, 2003, in guppies; Gabor & Halliday, 1997, in smooth newts; Downhower & Lank, 1994, in mottled sculpins) and against it (Klemme *et al.*, 2006, in bank voles), thus supporting the need for further investigations.

Tribolium castaneum Herbst (Coleoptera: Tenebrionidae) flour beetles represent an excellent system with which to address the question of multiple levels of female mate choice. These beetles live in aggregations on a food source (Sokoloff, 1974), and their mating system is characterized by high female mating rates (Pai & Yan, 2003) and last male sperm precedence (Schlager, 1960; Lewis & Jutkiewicz, 1998). *Tribolium castaneum* males transfer sperm within a nonnutritional spermatophore that females expel soon after mating (Bloch Qazi *et al.*, 1996; Fedina & Lewis, 2006). Males also produce a volatile pheromone that attracts females from a distance (Suzuki, 1980), and females have been shown to differentiate among males based on their chemical cues (Lewis & Austad, 1994). After a male contacts a female, copulation usually follows: females relatively rarely reject male copulation attempts (personal observation). However, not all copulations in *T. castaneum* result in successful spermatophore transfer, and females have been shown to exercise cryptic choice against males in poor phenotypic conditions by rejecting spermatophore transfer during copulation (Fedina & Lewis, 2006). One indicator of female unwillingness to accept a spermatophore is longer copulation duration: previous studies have shown that longer copulations result in insemination failures and decreased paternity (Fedina & Lewis, 2006).

This study examines whether *T. castaneum* males that are more attractive to females based on male olfactory cues subsequently gain higher insemination success, and whether male insemination success depends on the relative attractiveness of a female's previous mate. Females were first allowed to choose between olfactory cues produced by two males, and were then sequentially mated with the same males in two mating orders: their more attractive male first followed by their less attractive male and vice versa. Mating behaviours and second-male insemination success (confirmed by female dissection) were compared between the two mating treatments.

Methods

Tribolium castaneum Herbst (Coleoptera: Tenebrionidae) used in this study were all wild type, derived from Berkeley synthetic strain and maintained on King Arthur enriched wheat flour under standard conditions of 29 °C and 70% RH. Experimental beetles were randomly selected from stock cultures at the pupal stage, sexed and kept in same-sex groups at low density until they were used in experiment within 1 month posteclosion.

Female choice bioassay

Individual females were allowed to choose between flour with chemical cues from two males. Male chemical cues were collected by keeping mature males for 2 weeks in 0.9 ml microfuge tubes filled with flour. A virgin female was released in the centre of a 35-mm diameter plastic Petri dish with three equidistant holes leading to two microfuge tubes containing male-conditioned flour and one control tube containing fresh flour. Each dish was covered and left in the dark at 29 °C and 70% RH for 17–18 h, after which female location was recorded. It was assumed that females located in either of the male tubes were relatively more attracted to that male. Preliminary experiments verified females' tendency to remain in the same male tube over time (78% of 18 trials, exact binomial test $P = 0.031$). Any females that did not exhibit a choice between two males, i.e. located in control tube (34%) or in arena (18%), were discarded resulting in 48 successful bioassay trials conducted over 6 days (blocks).

Mating experiment

On the same day when female choice was recorded, each female was assigned to one of two mating treatments: (1) mated sequentially to her more attractive male followed by her less attractive male (based on the above assay); or (2) mated to her less attractive male followed by her more attractive male. Each female was continuously observed in 20-mm plastic mating arena for 15 min or until she copulated with the first assigned male. Copulation was defined as at least 25 s of continuous intromission by a male's aedeagus. After this first mating, each female was left in the mating arena for 1 h, then placed in a new arena and allowed another 15 min to mate with the second male. Latency to copulation (time between first antennal contact by a male and successful copula-

tion) and copulation duration were recorded for both matings. Immediately (< 1 min) after each female's second copulation, the female was dissected in saline, and her reproductive tract examined for the presence and condition of male spermatophores and the presence of sperm in the female's spermatheca.

Successful insemination during copulation was scored differently for female's first and second males. Successful insemination by the first male was recorded if: (1) 1-hour-'old' (deflated and crumpled) spermatophore was found inside the female bursa upon dissection (67% of cases); or (2) expelled partial spermatophore was found in the first mating arena (Fig. 2), and at the same time female spermatheca was at least half-filled with sperm (33% of cases). The second criteria could be applied because after 1 h the spermatheca is filled to approximately two-thirds of its capacity, whereas immediately after mating (i.e. if first male did not transfer spermatophore, but second male did) female spermatheca can only be filled to 0–15% capacity (Bloch Qazi *et al.*, 1996; Lewis & Jutkiewicz, 1998). In two cases, females were observed extruding spermatophores immediately after their first mating. These trials were recorded as unsuccessful inseminations because spermatophores were extruded with intact sperm sacs, and because no sperm was found inside these females' spermathecae. Successful insemination by the second male was recorded if a 'freshly' transferred spermatophore (i.e. with expanded sperm sac) was found inside the female bursa upon dissection. Similarly to the first mating, a few females ($n = 3$) extruded entire spermatophores into the arena immediately (< 1 min) after their second mating, and these trials were recorded as insemination failures by the second male.

Only females that were successfully inseminated by their first male were used in the analysis of the second-male insemination success to ensure a uniform competitive environment for all second males. This yielded final sample size of 21 females mated with the second less attractive male and 19 females mated with the second more attractive male. Effect of treatment (second male more or less attractive than the first) on second male's insemination success was analysed using a logistic regression analysis. Day of experiment (block) was also included as an independent variable. Latencies to copulation and copulation durations during each female's first and second mating were compared using paired *t*-tests (distributions of differences conformed to the test assumptions).

Results

No close-range premating discrimination by females was observed as all females copulated within 15 min with both first and second males; and in all cases latency to copulation was less than 2 min. There was no difference between latency to first and second copulations in females from either mating treatment (Fig. 1A, paired t-test for the second less attractive males, $t_{20} = 1.290$, $P = 0.212$; for the second more attractive males, $t_{18} = 0.358$, $P = 0.724$). However, the duration of second copulations relative to first copulations was significantly shorter only when the second male was more attractive (Fig. 1B, paired *t*-tests, $t_{18} = 2.139$, $P = 0.046$); no difference was observed when the second male was less attractive ($t_{20} = 1.498$, $P = 0.150$).

For virgin females across both treatments, only 16.7% of first copulations ($n = 48$ matings) resulted in insemination failure, whereas during females' second copulations a significantly higher percentage of failures occurred (50%, $n = 40$ mating; 2×2 contingency table analysis, $\chi^2 = 11.18$, d.f. = 1, $P = 0.001$). Also, in virgin females, there was no difference in the percentage of successful inseminations by more attractive males (73.1%) compared with less attractive males (95.5%; 2×2 contingency table analysis, $\chi^2 = 2.62$, d.f. = 1, $P = 0.106$). However, after females had mated once, more attractive second mates gained significantly higher percentage of successful inseminations than less attractive second mates (Fig. 2, logistic regression, treatment estimate 1.45, log-likelihood $\chi^2 = 6.45$, $P = 0.011$). Day of experiment had no effect on insemination success by the second male ($\chi^2 = 6.98$, $P = 0.222$).

Discussion

The present study demonstrates that *T. castaneum* males that are more attractive to females based on olfactory cues also have a higher likelihood of inseminating previously mated females (Fig. 2). Combined with our previous finding that insemination during copulation in *T. castaneum* is under female control (Fedina & Lewis, 2006), these results indicate consistency of female choice between premating and perimating episodes of selection. We also predicted that females who mated previously to less attractive males would be more willing to remate (i.e. show shorter latency to copulation and shorter copulation with a more attractive male). This prediction was supported by shortened copulation durations observed in females remating with more attractive males, although we did not detect longer copulations when the second male was less attractive.

Similar to the present study, an agreement was observed between female premating preference for more colourful males in guppies and subsequent peri- and post-copulatory episodes when females accept more sperm from preferred males, and when these sperm gain greater fertilization success (Evans *et al.*, 2003; Pilastro *et al.*, 1994). Similarly, females of domestic fowl preferred dominant males as mates, and after mating with a dominant male, these females were less likely to eject sperm (Pizzari *et al.*, 2002). In *T. castaneum*, Lewis and Austad (1994) found that male comparative rank in long-range olfactory attractiveness to groups of females
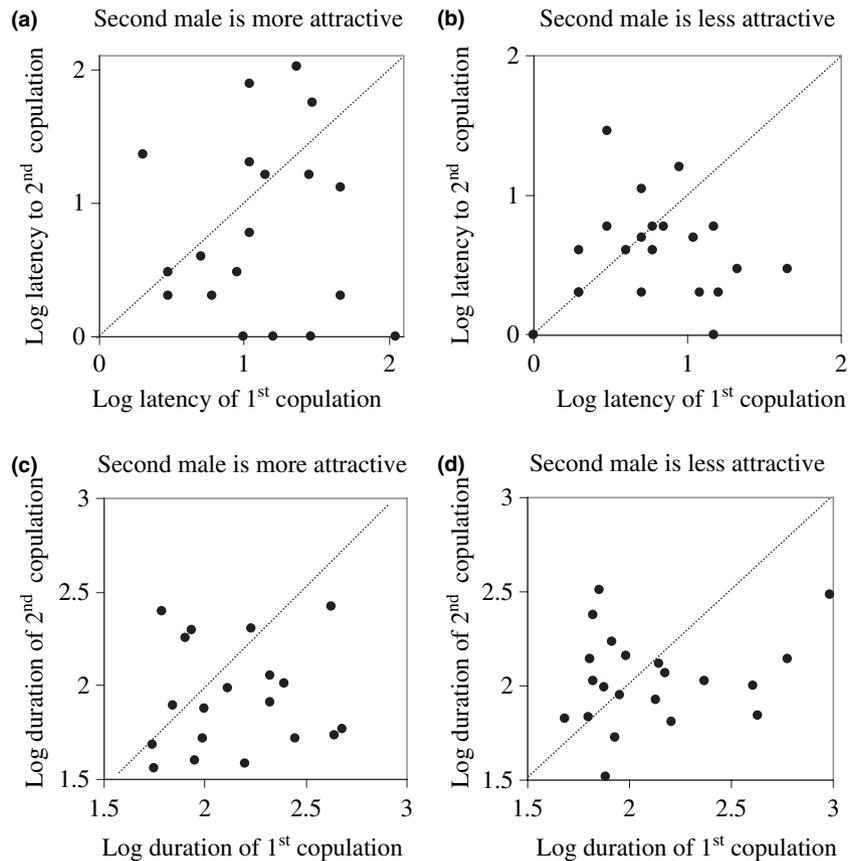
Fig. 1 Latency to copulation and copulation duration during first and second mating by individual *Tribolium castaneum* females when second male was more attractive (A, C; $n = 19$) or less attractive (B, D; $n = 21$) than female's first mate. Individual females are represented by single dots; dotted lines indicate lines of equality.
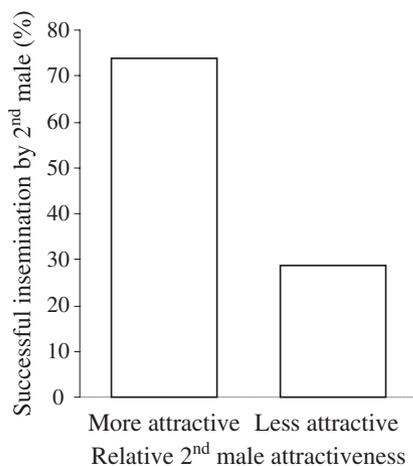

Fig. 2 Percentage of successful inseminations by *Tribolium castaneum* males when they are more attractive ($n = 19$) or less ($n = 21$) attractive to females compared with their first mates. Successful insemination defined as spermatophore transfer during copulation and its retention within 1 min after copulation.

predicted their paternity success with previously mated females. In these studies, a positive relationship between pre- and peri-/post-mating reproductive episodes may indicate female choice based on reliable male quality signals. By contrast, other studies have found negative relationships between premating and post-mating episodes of sexual selection. Thus, in water striders (*Gerris lacustris*), smaller males were inferior at acquiring mates but had higher fertilization success in post-mating competition (Danielsson, 2001). In *Photinus greeni* fireflies, males with more attractive bioluminescent displays had lower paternity success than less attractive males (Demary & Lewis, 2007). Such negative relationships between pre- and post-mating selection episodes may possibly reflect females correcting mate choice errors based on additional cues about male phenotypic and/or genetic quality obtained during mating.

Experimental results presented here also suggest that *T. castaneum* females might be employing a trade-up mating strategy (Halliday, 1983; Gabor & Halliday, 1997) to select among multiple sequential mates through cryptic choice during copulation. Thus, compared with mated females, virgin females had lower discrimination against spermatophore transfer by less attractive mates, which agrees with the fertilization assurance prediction of the trade-up mating strategy (Halliday, 1983; Gabor & Halliday, 1997). The second

prediction of the model is also supported, as *T. castaneum* females much more readily accepted spermatophores of more attractive males after being inseminated by less attractive males, compared with the reversed mating treatment. Several previous studies have also found confirmation for the trade-up model of sequential mate choice. Thus, in guppies, *Poecilia reticulata* females were more responsive to second males and produced a higher percentage of progeny sired by second males if these males had increased orange colouration relative to females' first mates (Pitcher *et al.*, 2003); orange colouration predicts male genetic quality (Houde, 1992; Pilastro *et al.*, 2004). Similarly, in newts, *Triturus vulgaris*, virgin females were nondifferentiating, whereas previously mated females selected their next mate depending on his relative crest size (Gabor & Halliday, 1997). As an alternative explanation to our results, it is also possible that *T. castaneum* females first mate indiscriminately and then use a threshold strategy to select additional mates.

It is unknown whether male olfactory attractiveness reflects male genetic or phenotypic quality in *T. castaneum*, although such correlations were shown in insects, fishes and humans (Wedekind *et al.*, 1995; Rikowski & Grammer, 1999; Barber *et al.*, 2001; Pilastro *et al.*, 2004; reviewed in Jennions & Petrie, 2000; Moore *et al.*, 2003). Previous studies have identified several genetic benefits to *T. castaneum* females of mating with multiple males, including higher larval viability of offspring (Pai *et al.*, 2005), higher insemination success and paternity success of sons, as well as higher egg viability for F1 offspring (Bernasconi & Keller, 2001; Pai & Yan, 2002). However, these studies did not investigate whether females were selective in allowing fertilizations by males of higher genetic quality, or what cues females might use to make such choices.

In our experiment, all females copulated with both their first and second mates, probably in part due to limited escape options in the mating arenas. Similar conditions may exist at high *T. castaneum* population densities, where females might be unable to avoid copulations with undesirable males. In this case, females may be selected to use cryptic choice mechanisms, including blocking spermatophore transfer (Fedina & Lewis, 2006) or immediate expulsion of the spermatophore after copulation (observed in this study). Sperm ejection after mating was demonstrated for other female insects (Otronen *et al.*, 1997; Snook & Hosken, 2004; Cordoba-Aguilar, 2006; reviewed in Eberhard, 1996), and longer retention of the externally attached spermatophore in crickets increases both sperm quantity transferred to the female and subsequent male paternity share (Sakaluk & Eggert, 1996; Simmons, 1987). By design, we neither timed spermatophore expulsion nor differentiated amounts of sperm expelled/retained by females. However, female control over these processes deserves further study as a potential

additional mechanism of cryptic female choice in *T. castaneum*.

In polyandrous animals, reproductive success is determined not only by the number and quality of mates, but also by paternity share achieved with each mate. This paternity share can be influenced by a number of cryptic mechanisms of sperm competition and female choice. An integrative approach looking at these sequential stages can help elucidate the evolution of sexually selected traits. The present study demonstrates a positive correlation in the direction of sexual selection between two sequential reproductive episodes: female mate choice and insemination success during copulation. It also suggests that females might be improving on the attractiveness (and may be quality) of their previous mates by accepting spermatophores only from more attractive partners. To completely validate the theory, further studies must demonstrate a correlation between male attractiveness and genetic benefits for the offspring.

Acknowledgments

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