

Copulatory courtship and cryptic female choice in red flour beetles *Tribolium castaneum*

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Males of many animal species engage in courtship behaviours during and after copulation that appear to be solely aimed at stimulating the female. It has been suggested that these behaviours have evolved by cryptic female choice, whereby females are thought to impose biases on male postmating paternity success. Males of the red flour beetle *Tribolium castaneum* rub the lateral edges of the females' elytra with their tarsi during copulation. We manipulated female perception of this behaviour by tarsal ablation in males, thus preventing males from reaching the edge of the female elytra with their manipulated legs, and by subsequently performing a series of double-mating experiments where the copulatory behaviour was quantified. We found a positive relationship between the intensity of the copulatory courtship behaviour and relative fertilization success among unmanipulated males. This pattern, however, was absent in manipulated males, where female perception of male behaviour differed from that actually performed. Thus, female perception of male copulatory courtship behaviour, rather than male behaviour *per se*, apparently governs the fate of sperm competing over fertilizations within the female, showing that copulatory courtship is under selection by cryptic female choice.

Keywords: copulatory courtship; cryptic female choice; flour beetles; sexual selection; sperm precedence; *Tribolium castaneum*

1. INTRODUCTION

Sexual selection theory has traditionally been concerned with precopulatory processes and variance in male mating success (Andersson 1994). More interest has recently been paid to postcopulatory processes and it is becoming increasingly clear that variation in relative male fertilization success is an important source of variance in male reproductive success (Eberhard 1996; Birkhead & Møller 1998). Postmating sexual selection can arise either through sperm competition (Parker 1970) or cryptic female choice (Thornhill 1983). While the importance and the evolutionary consequences of sperm competition, or the competition between sperm from two or more males over the fertilization of eggs within a female, is now well established (e.g. Smith 1984; Birkhead & Møller 1998), the suggested significance of cryptic female choice (Eberhard 1996) is still debated (Simmons *et al.* 1996; Birkhead 1998; Telford & Jennions 1998).

Direct experimental evidence for cryptic female choice is very scarce, if present at all (Birkhead 1998; Telford & Jennions 1998), although indirect evidence suggests that it is prevalent (Eberhard 1996). Perhaps the best evidence for postmating paternity biases imposed by females comes from studies of copulatory courtship behaviour. Males of many species exhibit a multitude of different behaviours during, and in some cases even after, copulation, which cannot be aimed simply at attaining matings (Eberhard 1991, 1994). These behaviours may instead increase the relative postmating paternity success of males, by stimulating the female to preferentially use their sperm over that of other males for the fertilization of eggs (Eberhard 1996). Such copulatory courtship behaviour is very widespread in many taxa throughout the animal kingdom (Eberhard 1991, 1994) and some support for its significance has been

offered by studies where male copulatory behaviour has been related to subsequent fertilization success (see Otronen 1990; Otronen & Siva-Jothy 1991; Watson 1991, 1998; Watson & Lighton 1994; Arnqvist & Danielsson 1999). However, correlational studies such as these fail to establish the causal connection between copulatory courtship behaviour and subsequent fertilization success. In order to do so, it is necessary to avoid confounding effects of other traits involved in sperm competition (e.g. the number of sperm transferred) which could be associated with male copulatory courtship behaviour. For instance, if males that perform vigorous copulatory courtship simultaneously transfer a large number of sperm, any correlation between copulatory courtship and fertilization success could simply be the result of numerical sperm competition. Hence, to demonstrate an active role of females in generating postmating paternity biases, the ideal experiment should manipulate female perception of male copulatory courtship behaviour independently of the behaviour itself.

The cosmopolitan red flour beetle, *Tribolium castaneum*, is one of the world's most important pests of cereal products (Sokoloff 1972). Females mate multiply and studies of sperm competition in flour beetles have shown extensive and repeatable variation in the relative fertilization success of males (Lewis & Austad 1990, 1994; Wade *et al.* 1994; Bloch Qazi *et al.* 1996). In addition, the study by Bloch Qazi *et al.* (1998) provided results suggesting an active role for females in generating this variation. Red flour beetle males also perform a behaviour that has been suggested to function as copulatory courtship (Eberhard 1996). During copulation, the male mounts the female and then rubs the lateral edges of her elytra with the tarsi of his legs. This behaviour is performed in bouts of one to a few strokes with either one leg alone or with two legs simultaneously and can involve any leg. The forelegs, however, are very rarely used (but see Wojcik 1969).

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In this study, we aimed at determining whether females cause positive relationships between male copulatory courtship behaviour and relative fertilization success, as suggested by correlative studies (see above). We did this by comparing the importance of copulatory courtship behaviour for relative fertilization success in normal red flour beetle males with that in males in which the tarsi of one or two legs had been removed. The latter males perform the leg-rubbing behaviour but do not reach the target site at the edge of the female elytra with the truncated legs. Hence, we were able to manipulate female perception of male copulatory courtship behaviour independently of male behaviour *per se*.

2. METHODS

T. castaneum stocks were provided by the *Tribolium* stock centre at the US Grain Marketing Research Laboratory in Manhattan, Kansas, USA. The wild-type Georgia strain (GA-1) and the black strain were used for the experiments described below. The former strain was collected in a farmer's corn bin in Georgia (USA) in 1980, and is genetically heterogeneous (R. W. Beeman, personal communication). The latter strain carries a semi-dominant autosomal black mutation (Sokoloff *et al.* 1960), which served as a genetic marker to enable determination of paternity of offspring following double matings (see also Lewis & Austad 1990, 1994). All beetles were maintained at 30 °C and 70% relative humidity in dark incubators. A mixture of 19 parts wholewheat flour and 1 part dry brewer's yeast was used as a culture medium (Sokoloff 1972). All individuals used in the experiment were sexed as pupae. They were virgins and 10–15 days posteclosion. Mating experiments and behavioural observations were carried out at room temperature (19–23 °C) and under diffuse room lighting.

We performed a series of double-mating experiments in which wild-type females ($n = 109$) were sequentially mated to two males each. Females were first mated to a black male and then to a focal wild-type male. The intermating interval was 24.98 h (s.d. = 0.81; range: 23.4–27.9). Focal wild-type males were randomly assigned to one of three experimental treatments, of which the first (but not the second and third) had their legs manipulated. The first group of males ($n = 61$) was anaesthetized and had one or two of their legs shortened by truncation at the midpoint of the tibia. These males had either one of the mid-legs ($n = 13$), one of the hind legs ($n = 15$), both mid-legs ($n = 13$), both hind legs ($n = 12$) or one mid-leg and one hind leg truncated (on opposite sides of the body) ($n = 8$). The second group of males ($n = 15$) was anaesthetized and handled in the same way as males in the first group but they did not have any of their legs truncated, to control for any effects of anaesthetization. The third group of males ($n = 33$) was left unmanipulated. Anaesthetization was achieved by exposing males to CO₂ for 60 s.

All matings were conducted in circular arenas (10 mm high, 35 mm diameter) where the bottom was covered with a thin layer of flour–yeast medium. All individuals were preconditioned prior to matings, by keeping beetles of both sexes isolated individually in separate arenas at room temperature for 24 h. Males assigned to any of the manipulation treatments were manipulated prior to this isolation period. Males were then introduced into the females' arenas and the behaviour of the beetles was recorded. In first matings, involving black males, only copulation duration was recorded. In second matings, involving wild-type males, the behaviour of the beetles was

observed under $\times 6$ magnification in a dissecting microscope, allowing more detailed observations of the copulatory courtship behaviour. Here, we recorded not only copulation duration but also every bout of strokes of each leg performed by males. The rate of stimuli delivered to the female, i.e. the number of bouts of strokes divided by copulation duration, was later used to estimate the intensity of the copulatory courtship behaviour. In both first and second matings, only one intromission was allowed and males were removed and preserved by freezing after copulation was terminated. Pairs that had not mated within 1 h were discarded from the experiment (n above represents successful replicates). The body size of focal wild-type males was subsequently measured as the distance between the anterior edge of the pronotum and the posterior end of the last sternite (number VII) on the ventral side, by placing a digitizing tablet under a side-mounted camera lucida attached to a dissecting microscope.

Following the two matings, females were transferred to vials containing 45 g fresh medium and were allowed to oviposit for seven days. All adult offspring were counted and scored for body colour 45 days after the first day of egg laying for each female. Offspring with a wild-type phenotype were considered to be sired by the second male and offspring with a phenotype intermediate to that of the paternal strains were considered to be sired by the first male. Females that failed to produce offspring were excluded from the analysis.

To assess whether our manipulation of male legs affected male sperm transfer, we compared the number of sperm transferred in manipulated and unmanipulated males. Fifteen wild-type males, which had been anaesthetized and had had both their mid-legs truncated, and 15 unmanipulated wild-type males, were all allowed to mate with one virgin wild-type female each. Females were frozen in liquid nitrogen immediately after copulation. The entire female reproductive tract was subsequently removed and homogenized in 0.2 ml saline. From each male, the numbers of sperm in 15 subsamples (0.004 μ l) were counted in a Bürker chamber.

Variance in the proportion of offspring fathered by the second male to mate with a female (P_2) was analysed with generalized linear models with binomial errors of the number of offspring sired by the last male to mate, using the total number of offspring per female as the binomial denominator and a logit link function (see Arnqvist & Danielsson 1999). To compensate for overdispersion (McCullagh & Nelder 1989), we implemented the method of Williams (1982) prior to statistical inference. Generalized linear models were estimated with GLIM and all other statistical evaluations were performed with SYSTAT[®].

3. RESULTS

Copulation duration ranged from 12 to 717 s with a mean of 98.8 s (s.d. = 87.4) for first and 74.2 s (s.d. = 45.3) for second males. No male with a copulation duration shorter than 36 s fathered any offspring. The total number of offspring per female varied between 20 and 156, with an average of 94.7 (s.d. = 26.3).

The leg manipulation did not seem to impair the vigour of male beetles. Truncated legs were frequently used during copulatory courtship. Instead of rubbing the lateral edge of the female's elytra, however, these legs either did not reach the female at all or touched the dorsal side of the female's elytra. The precision of our estimate of number of sperm transferred in each mating

Table 1. The results of a generalized linear model, using binomial errors and a logit link function, of the proportion of eggs fathered by the last male to mate (P_2) (d.f. = 95 in all tests of single factors)(Log-likelihood ratio test of full model: $\chi^2 = 67.44$, d.f. = 12, $p < 0.001$.)

source	estimate	s.e.	<i>t</i>	<i>p</i>
leg manipulation	145.40	47.55	3.058	0.003
anaesthetization	1.18	0.63	1.888	0.062
copulation duration first male	86.96	37.13	2.342	0.021
copulation duration second male	187.80	81.30	2.310	0.023
rate of leg rubbing	9.83	3.99	2.466	0.015
copulation duration first male ⁽²⁾	22.36	9.11	2.454	0.016
copulation duration second male ⁽²⁾	-48.13	20.91	2.302	0.024
leg manipulation × copulation duration first male	-82.43	29.59	2.786	0.006
leg manipulation × copulation duration second male	-67.63	43.06	1.571	0.119
leg manipulation × rate of leg rubbing	-5.14	2.16	2.377	0.019
leg manipulation × copulation duration of first male ⁽²⁾	21.37	7.54	2.835	0.006
leg manipulation × copulation duration of second male ⁽²⁾	17.05	11.15	1.529	0.129

was high, as revealed by the repeatability of sperm counts (repeatability = 0.843, $p < 0.001$) (Lessels & Boag 1987). However, the manipulation of male legs did not significantly affect the number of sperm transferred. Males with manipulated legs transferred, on average, 2.22×10^6 (s.d. = 1.08×10^6) sperm and unmanipulated males transferred, on average, 2.09×10^6 (s.d. = 1.24×10^6) sperm ($t = 0.308$, d.f. = 28, $p = 0.76$). We also tested if different types of leg manipulations (see § 2) affected overall P_2 in a generalized linear model including manipulated males only, by adding the following three dichotomous variables to a null model: the number of legs manipulated, whether a mid-leg was manipulated and whether a hind leg was manipulated. However, none of these variables affected P_2 ($n = 61$, $\chi^2 = 0.69$, d.f. = 3, $p = 0.876$).

We analysed variance in fertilization success among males in a multivariate generalized linear model. Our experimental treatment variables were included in this model, as were the copulation durations of the first and the second (focal) male, the rate at which the focal male performed copulatory courtship behaviour and their interactions (table 1). The model was highly significant and the scaled deviance ratio was 0.473 (analogous to the coefficient of determination (R^2) in a general linear model). The residuals did not differ from normality (Kolmogorov–Smirnov test, $p = 0.157$) and diagnostic residual plots did not reveal any deviant cells. The copulation duration of both males strongly affected relative paternity success and this effect was significantly nonlinear. Relatively high P_2 -values were associated with very short and very long copulation durations of the first male, whereas relatively low P_2 -values were associated with very short and very long copulation durations of the second male. Males with manipulated legs on average achieved a lower fertilization success than did males with unmanipulated legs, despite the fact that they transferred a similar number of sperm. Further, the rate at which the second male rubbed the edges of the female elytra during copulation was positively related to his subsequent fertilization success among males with unmanipulated legs. However, this relationship was absent in males with manipulated legs (figure 1). As shown by the interaction between leg manipulation and rate of leg rubbing in table 1, this difference in the importance of the copulatory courtship

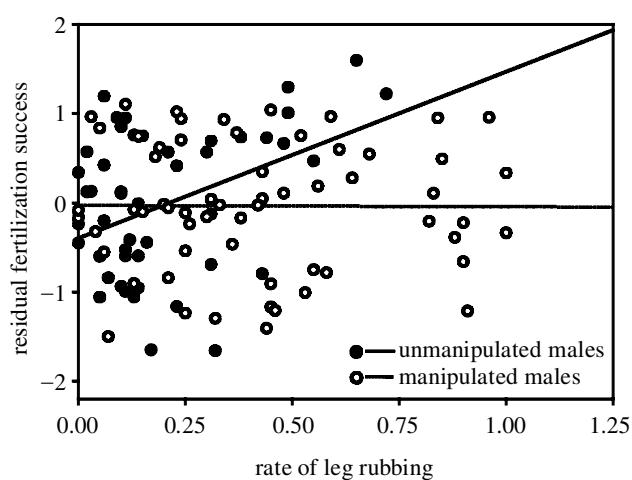


Figure 1. The relationship between male fertilization success and the rate at which males delivered courtship behaviour to females during copulation. Residual fertilization success was generated in a generalized linear model, identical to the one presented in table 1 apart from the exclusion of the rate of leg rubbing and its interaction with leg manipulation. Male fertilization success increased with the rate of leg rubbing in unmanipulated males (test of $H_0: \beta = 0$; $t = 2.75$, d.f. = 47, $p = 0.009$) but not in males with manipulated legs (test of $H_0: \beta = 0$; $t = 0.045$, d.f. = 60, $p = 0.964$). See table 1 for statistical evaluation.

behaviour for fertilization success was statistically significant.

We also assessed the importance of additional behavioural and morphological variables for male fertilization success. This was achieved by adding each of these variables separately to the model presented in table 1. However, none of these variables significantly improved model fit (intermating interval: $\chi^2 = 2.82$, d.f. = 1, $p = 0.093$; average interval between bouts of leg rubbing: $\chi^2 = 1.49$, d.f. = 1, $p = 0.222$; variance in interval between bouts of leg rubbing: $\chi^2 = 0.90$, d.f. = 1, $p = 0.343$; body size of focal male: $\chi^2 = 1.54$, d.f. = 1, $p = 0.215$).

The overall mean P_2 , disregarding other sources of variance in P_2 , was 0.47 (s.d. = 0.40) for males with manipulated legs, 0.70 (s.d. = 0.37) for males that were

anaesthetized only and 0.49 (s.d. = 0.40) for unmanipulated males.

4. DISCUSSION

The current study presents the first experimental evidence that cryptic female choice selects for male copulatory courtship behaviour. Relative fertilization success in red flour beetles was positively related to copulatory courtship performance when females could perceive the stimuli provided by males in a normal way but unrelated to copulatory courtship performance when females could not. This demonstrates that it is female perception of male behaviour, rather than male behaviour *per se*, that is important for fertilization success. Hence, females apparently impose biases on male postmating paternity success according to behavioural stimuli provided by males during copulation.

Male copulatory courtship behaviour in red flour beetles apparently elicits female responses that increase male postmating paternity success. This could be achieved if appropriate tactile stimulation of female elytra somehow induces females to aid in sperm transport from the site of ejaculate deposition (bursa copulatrix) to the site of sperm storage (spermatheca) (see Eberhard 1996). In keeping with this suggestion, Bloch Qazi *et al.* (1998) presented experimental evidence for an active role of female red flour beetles in such movement of sperm within the female reproductive tract. Since the rate of stimuli provided to the female was important for male fertilization success (figure 1), constraints on stimulation rate must limit the expression of this behaviour. Watson (1991, 1998) and Watson & Lighton (1994) demonstrated that male copulatory courtship behaviour is positively related to fertilization success in the sierra dome spider and that this behaviour is constrained by high energetic costs. Similar energetic costs may be important in red flour beetles. Trade-offs between copulatory courtship behaviour and other behaviours, such as maintaining a secure position on top of the female during copulation, may also be involved.

When phenotypic manipulations are used in experimental studies, one must always consider whether the results could be mere artefacts generated by abnormal behaviour of the manipulated individuals. Our leg manipulation clearly did not prevent red flour beetle males from performing seemingly normal leg movements during copulation, a finding which is in line with the fact that leg ablation does not interfere with other behaviours in insects (such as grooming; e.g. Zack 1978). In our case, however, one remote possibility is that sensory stimulation of male tarsi somehow is related to the quantity of sperm transferred by the male. Two lines of evidence show that this is not the case. First, manipulated and unmanipulated males did not differ in the amount of sperm transferred to the female during copulation. Second, while such a mechanism would be expected to generate differences between manipulated and unmanipulated males in their overall fertilization ability, it would not cause differences between unmanipulated and manipulated males with regard to the slope between copulatory courtship behaviour and fertilization success.

Our results show that short and long copulations were relatively unsuccessful in red flour beetles. This is indicated by the nonlinear relationship between copulation duration and P_2 for both the first and the second males. The relative failure of short copulations is most probably due to a minimum time requirement for successful spermatophore transfer (cf. Bloch Qazi *et al.* 1996). It is more difficult to see why extended copulations should be unsuccessful. Whatever the causes may be, dome-shaped relationships between copulation duration and fertilization success in insects may be more common than previously believed (see also Arnqvist & Danielsson 1999) and monotonously increasing functions (cf. Parker & Simmons 1994) may actually be relatively rare. We also found that the relationship between the copulation duration of the first male and the fertilization success of the second and focal male described a deeper concave function for manipulated males than for unmanipulated males (see interactions in table 1). This implies that manipulated males had a relatively low fertilization success as second mates in cases where the copulation duration of the first male was close to optimal, which is in line with our conclusion that appropriate stimulation of the female during copulation is important in order for the male to achieve a high degree of sperm precedence.

In conclusion, our results show that females impose biases on male postmating paternity success based on courtship stimuli received during copulation. If this is true for most species exhibiting copulatory courtship behaviour, cryptic female choice is indeed a consequential evolutionary mechanism (Eberhard 1996).

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