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ORIGINAL ARTICLE

Proximal traits and mechanisms for biasing paternity in the red flour beetle *Tribolium castaneum* (Coleoptera: Tenebrionidae)

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Abstract A comprehensive understanding of sexual selection requires knowledge of the traits and mechanisms responsible for increasing a male's paternity share (proportion of progeny sired) relative to that of other males mating with the same female. In this study we manipulated by starvation the expression of traits that might influence male paternity share in *Tribolium castaneum*. We then conducted experiments to examine how male starvation affects male performance during sequential episodes of sexual selection from mating to progeny production, and investigated female control over specific stages by using live vs dead females. Comparison of starved vs fed males revealed that *T. castaneum* females have control over spermatophore transfer during mating, as live females rejected inseminations by starved ("low quality") males. None of the measured male copulatory behaviors (leg-rubbing frequency, asymmetry, and percent of time spent rubbing) affected the probability of successful insemination, but the last two were positively associated with male paternity share. Spermatophore positioning within the female reproductive tract was not affected by male treatment (starved/fed), by female treatment (live/dead), or by male copulatory behaviors. Starvation, however, had a dramatic effect on male reproductive physiology, decreasing both accessory gland size and total number of sperms transferred (but not sperm viability in seminal vesicles). In addition, females who mated to starved males stored fewer sperms in their spermathecae, which, together with decreased ejaculate size, may explain the reduced paternity share of starved males compared to fed males. This study elucidates some cryptic mechanisms influencing male reproductive success and aids our understanding of trait evolution through sexual selection.

Keywords Sperm competition · Cryptic female choice · Condition-dependent selection

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Introduction

Sexual selection operates on traits that affect the reproductive success of individuals. In polygamous species, male–male competition and female choice occur not only before mating (Andersson 1994) but also during and after mating (Eberhard 1996; Simmons 2001). At these latter stages of interaction between the sexes, males struggle to increase their paternity share (also termed paternity success or sperm precedence) among a female's offspring by increasing the competitive ability of their own ejaculate relative to that of rival males. In various species, this goal is achieved by displacing sperms of previous males, by increasing sperm quantity to outnumber rival sperms, by transferring ejaculate constituents that reduce females' likelihood of remating with other males, and by guarding females to prevent remating (Parker 1970; Smith 1984; Birkhead and Möller 1998; Simmons 2001). In animals with internal fertilization, these and other male-driven processes occur inside the female's body, a situation that provides females with the opportunity to influence the processes of sperm transfer, storage, and utilization. Depending on the quality of her current mate, a female might be able to mediate the number of sperms transferred or stored, to discard sperms, or to remate with another male (Eberhard 1996).

In the red flour beetle *Tribolium castaneum*, both sexes mate multiple times over an adult life span that can exceed 1 year (Sokoloff 1974). Males transfer a spermatophore consisting of a membranous sac filled with sperms and accessory gland products into the female bursa copulatrix. About 4% of the sperms transferred is moved for long-term storage into the tubular spermatheca where it remains viable for up to 140 days (Bloch Qazi et al. 1996). Additional sperms remain in the female anterior bursa for about 1 week after a single mating. These reproductive features provide both sexes with ample opportunities to influence male paternity share.

T. castaneum males show high among-male variation in paternity success (proportion of offspring sired) when they are the last male to mate with a previously mated female. Some of this variation in paternity success is explained by male and female genotypes (strains) and by an interaction between male and female genotypes (Lewis and Austad 1990; Wilson et al. 1997; Pai and Yan 2002; Nilsson et al. 2003). However, few studies have addressed the specific mechanisms by which males and females achieve control over paternity. Among premating correlates of paternity success, male long-range olfactory attractiveness to females was positively related to proportion of progeny sired (Lewis and Austad 1994). Copulatory behaviors may also influence paternity success. Male leg-rubbing during copulation was previously studied in *T. castaneum*; however, two studies using different behavioral measures reached different conclusions about how male leg-rubbing rate is related to male paternity success (Edvardsson and Arnqvist 2000; Bloch Qazi 2003). An active female role in moving sperms into long-term storage in the spermatheca was demonstrated by Bloch Qazi et al. (1998) using carbon dioxide (CO_2) to inhibit muscle contractions after single mating. A subsequent study confirmed the potential for such differential sperm storage to affect paternity by demonstrating that females exposed to CO_2 between two matings produced a lower proportion of progeny sired by the second male (Fedina and Lewis 2004). However, the mechanisms through which females exercise this control remain poorly understood, and direct evidence of female ability to bias paternity toward higher quality males is still lacking.

Theoretical and empirical studies suggest that sexually selected traits are costly to their bearer and, hence, depend on body condition (Rowe and Houle 1996; Kotiaho 2000; Kotiaho et al. 2001; Tomkins et al. 2004, but see Cotton et al. 2004). In systems with no material mating benefits, a male's body condition may signal his ability to acquire and sequester resources and is likely to correlate with male genetic quality (Hunt et al. 2004; Tomkins et al. 2004). Male body condition can be manipulated in an experimental setting to change male phenotypic quality and, potentially, his value to females. Several recent studies have investigated condition-dependence of male traits used in male–male competition and female choice (Droney 1998; Tallamy et al. 2003; Scheuber et al. 2004; Brandt and Greenfield 2004; Bonduriansky and Rowe 2005), and condition-dependence of male ejaculate characteristics (Ward and Simmons 1991; Droney 1998; Kotiaho 2000; Kotiaho et al. 2001; Simmons and Kotiaho 2002). However, few studies have investigated how male body condition affects performance at different stages of perimating (during copulation) and postmating sexual selection.

As a stored product pest, *Tribolium* naturally encounters a wide range of food quality and availability. In this study we manipulated male body condition by starvation to alter the expression of sexually selected traits that might influence paternity share. We conducted several different experiments to investigate how starvation affected male performance during sequential episodes of sexual selection between mating and progeny production, including copulatory courtship behaviors, insemination success, spermatophore positioning, sperm viability and accessory gland size, total number of sperms transferred, number of sperms stored, and ultimately, male paternity share. Our main goal was to identify male traits and mechanisms that result in certain males achieving higher paternity share. We predicted that male performance at each stage of sexual selection would be negatively affected by starvation, and also that copulatory courtship intensity and ejaculate quality would influence male paternity share through increased insemination success, spermatophore positioning, and number of sperms stored by females. We also investigated female influence over perimating and postmating processes by allowing males to mate with live vs dead females; we expected that live females would discriminate against starved males based on their low phenotypic quality.

Materials and methods

T. castaneum (Herbst) (Coleoptera: Tenebrionidae) used in our experiments originated from two strains: wild-type beetles derived from the Berkeley synthetic strain and Chicago black beetles homozygous for an autosomal, semidominant black body color allele (Sokoloff et al. 1960). Black males were used as background genotype to measure wild-type male paternity success, defined as the proportion of progeny sired by a focal wild-type male that was the last mate of a wild-type female that had been previously mated with black males. Stock cultures were maintained on King Arthur enriched wheat flour and kept in the dark at 29°C and 70% RH. For all experiments described below, male and female beetles were separated from stock cultures at the pupal stage and kept in same-sex groups of 40–60 beetles at a density of 2 beetles per 1 g of

flour. Sexually mature adults 2–4 weeks posteclosion were used in all experiments.

As a stored-product insect, *Tribolium* populations naturally experience changes in food availability as they colonize and exploit different food patches. To manipulate male body condition we randomly allocated males to one of two treatments for 7 days: (1) fed males were placed individually in glass vials containing 2 g of flour and 30 cm of coiled nylon string and (2) starved males were placed individually in glass vials containing string only. The nylon string served as a substrate for crawling and allowed males that had fallen onto their backs to turn over. Survival over 7 days was 100% for males in both treatments.

Females used in the experiments were all wild-type. They were matched by body mass (±0.03 mg) to the premanipulation mass of their last, wild-type mate. Size-assortative mating was demonstrated for *T. castaneum* (unpublished data), so this procedure experimentally controls for body size differences. Virgin females were used for some experiments, while premated females were used for other experiments. Premating of females was done by allowing each virgin female to mate ad libitum with three Chicago black males in 2 g of flour for 24 h; fertilization by black males was later confirmed by the presence of progeny developed from eggs laid during this period. This standard premating treatment was applied so that females would acquire multiply-mated status (i.e., sperms from multiple males filling their sperm storage organs). Unlike virgin females, these multiply-mated females are expected to be more selective about their subsequent mates. To further standardize sperm storage (and female state) after premating, females were isolated for 24 h before we observed their mating interactions with fed or starved wild-type males.

To distinguish female vs male influence over courtship and mating, we mated condition-manipulated males to either live or dead premated females. Females were killed by placing them in a jar with ethyl acetate vapors for 30–60 min; they were removed from the jar at least 5 min before pairing them with the focal wild-type males. For all observed matings, copulation was defined as at least one intromission (defined by continuous extrusion of male aedeagus) that lasted a minimum of 25 s (time required for male to transfer spermatophore, unpublished data).

Effect of male starvation on copulatory behaviors

Individual male–female pairs consisting of fed (n=88) or starved (n=102) males and dead (n=71) or live (n=119) premated females were observed in 1.5-cm-diameter mating arenas on a 30°C warming tray (24°C room temperature) for 15 min or until a single copulation occurred. Courtship behaviors were digitally video-recorded using a Sony DCR-TRV80 camcorder, and then analyzed frame-by-frame using iMovie software v. 5.0.2. (Apple Computer).

During copulation, *T. castaneum* males rub the sides of the female's body with their legs in bouts interspersed with periods of inactivity (Bloch Qazi 2003). A leg-rubbing bout was defined as rubbing activity by any (one or more) leg that was separated from adjacent bouts by at least 0.2 s of inactivity. A leg-rubbing cycle was defined as single leg extension followed by its retraction (Bloch Qazi 2003). For each recorded copulation we selected 3–5 consecutive leg-rubbing bouts (including the same number of interbout intervals) that occurred sometime after at least three initial bouts and before female quiescence (when spermatophore transfer is most likely to take place). We measured male leg-rubbing behaviors near the beginning of copulation because these are more likely to reflect intrinsic male behaviors relatively unaffected by any female response. For each bout, we recorded the number of leg-rubbing cycles made by each of six legs and then calculated three characteristics: (1) leg-rubbing frequency (cycles/s), (2) percent of time spent leg-rubbing, and (3) leg-rubbing asymmetry. Leg-rubbing frequency was calculated by summing the number of rubbing cycles over all six legs and dividing by the actual time (in seconds) males spent rubbing. Percent of time spent rubbing was calculated as the sum of bout durations during which any leg-rubbing occurred divided by the total time (including periods of inactivity) and multiplied by 100%. Leg-rubbing asymmetry was calculated as the difference between total number of cycles summed for three right legs and total number of cycles summed for three left legs divided by the average of these two numbers. We also measured copulation duration for fed and starved males.

Effects of male starvation, female treatment, and their interaction on all four measured copulatory behaviors were estimated using two-way ANOVA with fixed effects. Copulation duration was transformed as $1/\sqrt{X}$, for this analysis to conform to normality assumptions.

Effect of male starvation on paternity share in competition with other males

We compared paternity share of fed (n=29) and starved (n=36) males by mating them to premated females and determining the proportion of progeny they subsequently sired. First, we observed and video-recorded each male–female pair in a 1.5-cm-diameter mating arena for 15 min or until copulation occurred. After mating, eggs were collected from each female over two oviposition periods (0–3 and 4–11 days postmating). Adult progeny that developed from these eggs were scored by phenotype as sired by either the focal wild-type male or by black males. Paternity share of each wild-type male was calculated as the number of his

progeny divided by the total number of a female's progeny, and multiplied by 100%. Absence of any wild-type progeny over the total 11-day oviposition period was interpreted as failure of sperm transfer by the focal male, and such females were excluded from the paternity analysis.

To analyze paternity data we used a generalized linear model (GLM) with a binomial error distribution and a logit link function in SAS (SAS Institute, NC, USA), with oviposition period as a repeated factor and courtship behaviors (measured as described in the section above) as covariates. Failures of males to mate within 15 min or transfer sperms, in addition to a few inadequate video-recordings, yielded final sample sizes of 22 fed and 22 starved males for the GLM analysis. For a subset of 20 fed males, we also measured leg-rubbing rate as defined by Edvardsson and Arnqvist (2000) who summed the number of bouts performed by all six legs over the whole copulation and divided by total copulation duration; we used linear regression analysis to examine how this measure of leg-rubbing behavior affected paternity.

Effect of male starvation on insemination success and spermatophore positioning

To examine two possible mechanisms that could generate differences in male paternity share, we dissected premated females (live or dead) immediately after they mated with the focal (fed or starved) male (matings were conducted and copulatory behaviors were measured in the same way as in the previous section). From each dissection, we recorded (1) insemination success (i.e., whether a spermatophore was transferred during copulation, n=141) and (2) spermatophore position within the female reproductive tract (n=110). The spermatophore in *T. castaneum* is similar in structure and behavior to the spermatophore of *Tenebrio molitor* (Gadzama and Happ 1974), but its expansion (eversion of a double-layered tube into a bulbous, sperm-containing sac) happens within seconds after transfer to the female (personal observation). When a spermatophore was successfully transferred, dissecting a female immediately after mating revealed two distinct spermatophore locations (Fig. 1): (a) successful positioning when the most of the spermatophore sac was located in the anterior bursa copulatrix above the level of the oviduct entrance or (b) unsuccessful positioning when the spermatophore sac was located below this level. We scored spermatophore positioning in the posterior bursa as unsuccessful because sperm released from such spermatophores is less likely to be used for fertilizations; this is due to possible displacement by either eggs coming down the oviduct or by any rival male spermatophores that might be placed in a more anterior position. *T. castaneum* females lay eggs continually at a rate of approximately one egg every 2–3 h (Sokoloff 1974, unpublished data); they also mate frequently having an average of four to six matings in 1 h (Pai and Yan 2003).

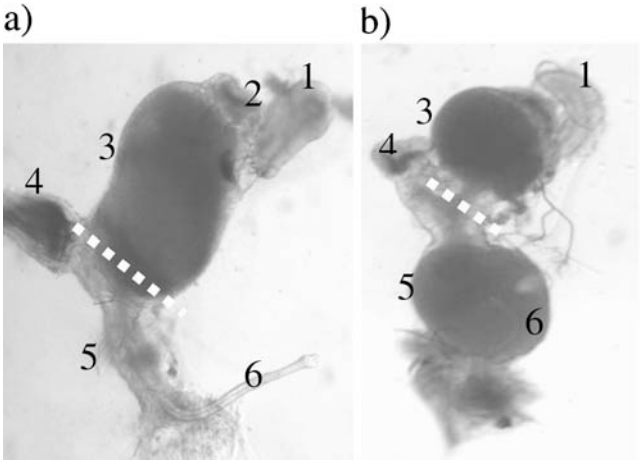

Fig. 1 Differences in male spermatophore positioning within the *T. castaneum* female reproductive tract (*white dotted line* separates anterior from posterior bursa). **a** Successful positioning: mating male's spermatophore sac is visible as a dark mass filling female anterior bursa with spermatophore tail located in posterior bursa. **b** Unsuccessful positioning: mating male's spermatophore sac and tail both located in female posterior bursa separated by muscular contraction from anterior bursa where previous males' sperm is visible as a dark mass. *1* Spermathecal gland, *2* spermatheca, *3* anterior bursa, *4* common oviduct, *5* posterior bursa, and *6* spermatophore tail

Effects of male treatment (fed vs starved) and female treatment (live vs dead) on insemination success and spermatophore positioning were analyzed using logistic regression analysis in JMP v. 5.0.1.2 (SAS Institute), with copulatory behaviors included as covariates.

Effect of male starvation on number of sperms transferred to and stored by females

Effects of starvation on male paternity share could be mediated by the number of sperms transferred to and/or stored by females. To estimate the number of sperms transferred, virgin females were mated to fed (n=12) or starved (n=11) males. Immediately after copulation, the females' reproductive tracts were dissected. All the sperms present in each female reproductive tract were pipetted with 20 μl of saline, then were thoroughly mixed (pipetting up and down ten times), and were diluted (3- to 5-step serial dilution) producing 20, 40, or 80 times diluted suspension (dilution factor was recorded for each sample and was based on visual assessment of sperm density). An 8-μl subsample was placed on a flat slide under a 22×22-mm cover slip, and the number of sperms was counted under 200× magnification in two haphazardly chosen 22×1.08-mm (equal to the width of the microscope field) vertical strips. These replicate counts were averaged, then multiplied by 20.3 (the total number of strips under each coverslip) and by the appropriate dilution factor to yield

the total number of sperms transferred. Very little sperm clumping was observed, and the repeatability of sperm counts using this method was very high (correlation r^2=0.95, repeated counts of ten subsamples, P<0.0001).

To determine the number of sperms stored, virgin females were first mated to fed (n=10) or starved (n=10) males, then kept upside down (to prevent undetected spermatophore expulsion) for 1 h to complete sperm storage in the spermatheca (Bloch Qazi et al. 1996). Females were then dissected, and the anterior bursa and spermatheca were separated into different drops of saline with minuten pins. Sperm from each storage compartment was counted as described above, and independent pooled-variance t tests were used to compare fed and starved males.

Sperm viability and accessory gland size in fed vs starved males

To examine whether male starvation affects sperm viability, we compared percent viable sperms from the seminal vesicles of starved and fed males using Live/Dead sperm-staining kit (Molecular Probes, OR, USA). Fed (n=12) and starved (n=12) males were dissected in *Tribolium* saline and a single seminal vesicle was placed into a 30-μl saline drop, ruptured, and contents were mixed by pipetting up and down 15 times. After 4–5 min from the beginning of dissection, a 4-μl subsample of sperm suspension was placed on a glass slide and 2 μl each of propidium iodide and SYBR-14 (diluted 50-fold in *Tribolium* saline) were added and incubated in the dark for 4–5 min. Sperms were viewed under 200× magnification using an Olympus BX-40 compound microscope equipped with fluorescent illumination and a Chroma 61000 triple filter set. All live (fluorescing green) and dead (red) sperms seen in several nonoverlapping fields were counted during 4–5 min, and percent of viable sperms was calculated (total sperm counts ranged from 143 to 433 sperms). Sperm viability was compared with a nonparametric Mann–Whitney test due to the presence of a single extreme outlier in the starved male group.

For the same males, digital photographs of their short (bean-shaped) accessory glands were taken; for a subset of males, photographs of their long accessory glands were also taken. Sizes of these male reproductive structures were measured using Image J v.1.240 (NIH) and compared between fed and starved males using t tests.

Results

Effect of male starvation on copulatory behaviors

T. castaneum males starved over 7 days lost 15.3±0.4% of their initial body mass compared to 0.9±0.4% for fed males (means±SE for n=43 fed and 56 starved males; t test t=26.9 and P<0.0001). This loss of condition for starved males resulted in significant decrease in male leg-rubbing frequency during copulation (ANOVA male effect, Table 1). The other two measures of male leg-rubbing performance, percent of time spent rubbing and leg-rubbing asymmetry, were not affected by male starvation. There was no effect of female treatment (live or dead) on any measure of male leg-rubbing (ANOVA female effect, Table 1); this was expected from our design as all the measurements were taken in the beginning of copulation, presumably before female response could modify male behaviors.

Copulation duration, on the other hand, was affected by both male and female treatments, with shorter copulations for starved compared to fed males and for males mating with dead compared to live females (Table 1, means for copulation duration were backtransformed from $1/\sqrt{X}$). No interaction effect was observed for any of the four measured copulatory behaviors.

During mating behavior observations, we also noted that leg-rubbing behaviors varied among mating stages: before sperm transfer (when our measurements were taken) males rubbed more often with their middle and front legs, then slowed down (or stopped) rubbing during sperm transfer, and finally switched to rubbing mainly with their hind and middle legs after sperm transfer; they also sometimes rubbed briefly with their front legs during dismount. Leg-rubbing was also dependent on female behaviors; males appeared to intensify rubbing when females were moving, while they decreased rubbing to maintain hold of very active females.

Effect of male starvation on paternity share in competition with other males

Starved males had significantly lower paternity share compared to fed males when mating as last mates to previously mated females (Fig. 2 and Table 2). As expected for *T. castaneum*, paternity by last males declined significantly over two oviposition periods for males in both treatments, with no interaction between treatment and oviposition period (Fig. 2 and Table 2). Among behavioral traits, percent of time spent leg-rubbing and rubbing asymmetry positively affected male paternity share, while rubbing frequency had no detectable effect; copulation duration, on the other hand, had a negative effect on paternity share (Table 2). There was no relationship between the measure of male rubbing rate used by Edvardsson and Arnqvist (2000) and paternity share for a subset of fed males (linear regression $F_{1,\ 18}$=1.525 and P=0.233).

Table 1 Effect of starvation on male copulatory behaviors in *T. castaneum*

Courtship behaviors	Live females		Dead females		ANOVA F (above), *P* (below)		
	Fed males	Starved males	Fed males	Starved males	Female effect	Male effect	Interaction
Rubbing frequency (r/s)	9.6±0.4	8.2±0.3	9.8±0.4	8.9±0.5	1.44	8.48	0.43
					0.233	0.004**	0.512
% time rubbing	67.7±1.9	64.3±1.7	70.6±1.7	68.8±2.1	3.66	1.83	0.19
					0.057	0.178	0.665
Rubbing asymmetry	0.03±0.04	0.01±0.06	0.06±0.06	−0.08±0.06	0.20	1.87	1.18
					0.656	0.172	0.279
Copulation duration (s)	126.2	84.2	98.0	69.4	4.55	14.11	0.01
					0.034*	0.0002***	0.924

Trait means (±SE) are shown with ANOVA statistics (*df*=1, 186). Sample sizes (female/male treatment) are: 54 live/fed, 65 live/starved, 34 dead/fed, and 37 dead/starved

*$P<0.05$

**$P<0.01$

***$P<0.001$

Effect of male starvation on insemination success and spermatophore positioning

Starved males more often failed to transfer spermatophores to females during copulation than did fed males, but this occurred only when mating with live females (Fig. 3a and M×F interaction in Table 3). There was no main effect of either female or male treatment on male insemination success (Table 3). Among behavioral covariates, copulation duration and percent of time spent leg-rubbing were negatively correlated with insemination success (odds ratios<1, Table 3).

Spermatophore positioning was not affected by male starvation, female treatment (dead/live), or their interaction; furthermore, none of the behavioral covariates affected spermatophore positioning (Fig. 3b and Table 3).

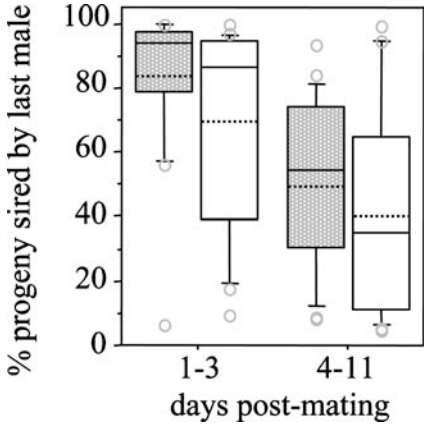

Fig. 2 Paternity share achieved by *T. castaneum* males that were fed (*filled boxes*, *n*=22) or starved (*open boxes*, *n*=22) before mating with previously mated females. Progeny were collected from each female over two oviposition periods (days postmating). *Boxed horizontal edges* represent 25th and 75th percentiles, *whiskers* indicate 10th and 90th percentiles, *solid line inside the box* is the median, *dotted line* is the mean, and *circles* represent outlying data points

Effect of male starvation on sperm quantity transferred to and stored by females

Fed males transferred on average 1.75 times more sperms than starved males (Table 4). Although the number of sperms stored in females' anterior bursae 1 h after mating did not differ between fed and starved males, the number of sperms stored in the spermatheca was significantly higher for females who mated with fed males (Table 4). Total sperm storage (anterior bursa and spermatheca combined) did not differ for females who mated with starved and fed males (*t* test $t=0.576$ and $P=0.572$).

Sperm viability and accessory gland size in fed vs starved males

It is surprising to note that starved males had significantly higher sperm viability in their seminal vesicles compared to fed males, although the difference was only ~5% (Fig. 4a; Mann–Whitney U test $U_{12}=25.5$, $U'_{12}=118.5$, and $P=0.007$). Accessory glands were significantly smaller when males were starved (Fig. 4b), both for short accessory gland area (*t* test $t=10.253$, $P<0.0001$) and for long accessory gland width ($t=3.81$ and $P=0.002$).

Discussion

Paternity share dependent on male phenotypic condition

When starved *T. castaneum* males were mated with previously mated females, they sired a smaller proportion of progeny compared to fed males. A similar decrease in paternity share was found for *T. castaneum* males infected with the parasitic tapeworm, *Hymenolepis diminuta*, (Yan and Stevens 1995). Our study additionally provides insight

Table 2 Results of generalized linear model for last-male paternity share as a function of *T. castaneum* male treatment (fed vs starved), oviposition period, and male copulatory behaviors (n=22 fed and 22 starved males)

Source	Estimate	SE	Z	P value
Male treatment	0.794	0.342	2.32	0.0201*
Oviposition period	1.279	0.203	6.30	<0.0001***
Treatment×oviposition period	0.506	0.352	1.44	0.1507
Rubbing frequency	−0.131	0.078	−1.68	0.0934
% time rubbing	0.042	0.0185	2.24	0.0257*
Rubbing asymmetry	0.725	0.352	2.06	0.0393*
Copulation duration	−0.0041	0.0015	−2.77	0.0056**

*P<0.05
**P<0.01
***P<0.001

into how male phenotypic condition affects male mating behavior and physiological traits and into potential mechanisms responsible for the observed paternity bias against males in low phenotypic condition.

Copulatory behavior as mechanism of paternity biasing

Male leg-rubbing frequency decreased significantly in response to starvation, while two other measures of leg-rubbing performance (percent of time rubbing and rubbing asymmetry) were not affected. Such condition-dependence of leg-rubbing frequency suggests this trait could be under sexual selection (Rowe and Houle 1996; Kotiaho 2000; Kotiaho et al. 2001; Tomkins et al. 2004). However, leg-rubbing frequency did not alter either male insemination success or paternity share. A different measure of male copulatory behavior, percent of time spent leg-rubbing, was positively associated with paternity share. This association, along with the constancy of this behavior under starvation, suggests that percent of time spent leg-rubbing may be an important behavioral trait determining male reproductive success. Other studies have also found increased allocation to sexually-selected traits under resource limitation; in both the green swordtail, *Xiphophorus helleri*, and the horned beetle, *Onthophagus acuminatus*, males reared under poor larval conditions show disproportionately greater investment to swords or horns relative to body size (Basolo 1998; Emlen 1997). Finally, positive relationships between asymmetric rubbing and male paternity share suggest there may be some bias in female sensory input and/or central processing, perhaps associated with asymmetry of the female reproductive system in *T. castaneum* as found in other insects (e.g., Cordoba-Aguilar 1999).

Previous studies looking at the relationship between male leg-rubbing behaviors and paternity in *T. castaneum* have reached different conclusions. Using frame-by-frame analysis of video-recordings, Bloch Qazi (2003) measured (for second and third leg pairs only) the number of leg-rubbing cycles, total rubbing bout duration, and rubbing endurance; she found no relationship between these variables and male paternity share. However, these measurements were taken during female quiescence (when sperm transfer occurs); during quiescence males often slow down or cease rubbing, and this could possibly explain the lack of effect. In another study, Edvardsson and Arnqvist (2000) observed male leg-rubbing directly under magnification and found a positive relationship between leg-rubbing rate (total number of bouts by all six legs divided

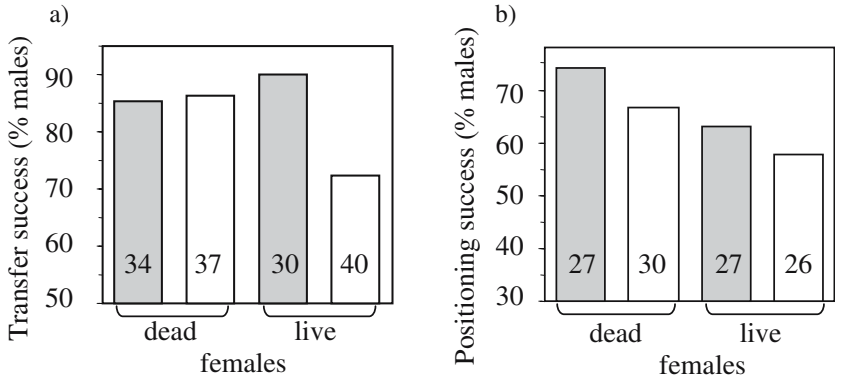

Fig. 3 Percentage of fed (*filled bars*) vs starved (*open bars*) *T. castaneum* males that **a** successfully transferred spermatophores to dead or live females and **b** successfully positioned spermatophores within female reproductive tracts. Sample sizes are given inside *bars*

Table 3 Logistic regression analyses of male insemination success and spermatophore positioning as a function of male treatment (starved vs fed), female treatment (dead vs live), and copulatory behaviors

Source	*df*	Insemination success			Spermatophore positioning		
		Odds ratio	χ^2	*P* value	Odds ratio	χ^2	*P* value
Female treatment	1	0.546	1.058	0.304	0.548	1.678	0.195
Male treatment	1	2.840	2.902	0.089	1.402	0.486	0.486
M×F interaction	1	3.971	5.794	0.016*	0.762	0.369	0.544
Day of experiment	8		9.607	0.294		12.156	0.144
Rubbing frequency	1	4.939	0.810	0.368	0.214	1.156	0.282
% time rubbing	1	0.013	4.450	0.035*	0.444	0.207	0.649
Rubbing asymmetry	1	8.659	1.871	0.171	0.295	0.774	0.379
Copulation duration	1	0.003	16.562	<0.0001**	0.390	0.268	0.604

Odds ratios for yes/no transfer, successful/unsuccessful positioning, and results of log-likelihood ratio tests are reported. Whole model log-likelihood ratio tests: for transfer χ^2=31.994 and *P*=0.006, and for positioning χ^2=17.938 and *P*=0.266. Sample sizes given in Fig. 3

*$P<0.05$

**$P<0.001$

by copulation duration) and paternity for unmanipulated males. However, they did not find any reduction in paternity when males' legs were partially ablated to decrease females' perception of leg-rubbing behaviors. We used Edvardsson and Arnqvist's (2000) measure of leg-rubbing rate (measured from video-recordings) for a subset of males in this study, but found that this measure did not predict paternity. Some of these inconsistencies within and among studies may be due to differences in strains or environmental conditions. However, the high within-mating variability in leg-rubbing behaviors we observed suggests that females might not be using these behaviors to assess male quality. An alternate interpretation is that male leg-rubbing decreases female resistance to male intromission and sperm transfer, which is supported by our finding that copulations where males spent high percentage of time leg-rubbing mostly resulted in insemination failure. Finally, it is possible that behaviors involving the first leg pair may have a different/additional purpose as these legs bear sexually dimorphic setiferous glands whose secretions were shown to attract females (Faustini et al. 1981).

Copulation duration was significantly affected by both male (fed/starved) and female (live/dead) treatments. Shorter copulations for starved compared to fed males could either reflect energetic limitations or a shorter time required to transfer smaller ejaculates. In addition, shorter copulations with dead females may indicate that live females normally resist spermatophore transfer. The previous finding that during a single mating *T. castaneum* males transfer significantly more sperms to dead females (Bloch Qazi et al. 1998) is in agreement with this interpretation. Copulation duration was strongly negatively related to both insemination success and paternity share, again suggesting that longer copulations reflect female resistance to sperm transfer.

Insemination success and spermatophore positioning

Starved *T. castaneum* males had lower insemination success than fed males when mating with live females, but were equally likely to transfer spermatophores when mating with dead females. This suggests that females play an active role in rejecting inseminations by males of low phenotypic quality. However, the cues females might use to perceive male phenotypic condition remain unknown, as none of the male leg-rubbing behaviors we measured was positively correlated with insemination success. Females might assess other leg-rubbing characteristics (e.g., amplitude of leg swing and pressure applied when rubbing) or male chemical signals (e.g., pheromones and cuticular hydrocarbons). *T. castaneum* males showed decreased pheromone production during

Table 4 Starvation effects on *T. castaneum* sperm quantity (1) transferred to virgin females during a single mating and stored by females 1 h after mating in either (2) anterior bursa (AB) or (3) spermatheca (SPT)

Sperm location	Number of sperms, $\times 10^3$ (mean±SE)		*t* test	
	Fed males	Starved males	*t*	*P* value
1. Total transferred	260.6±39.3 (11)	149.±21.9 (12)	2.416	0.025*
2. Stored in AB	44.4±7.9 (10)	54.7±10.1 (10)	−0.805	0.432
3. Stored in SPT	4.5±1.0 (10)	1.7±0.4 (10)	2.528	0.021*

Pooled-variance *t* test results are shown, sample sizes are in parentheses

*$P<0.05$

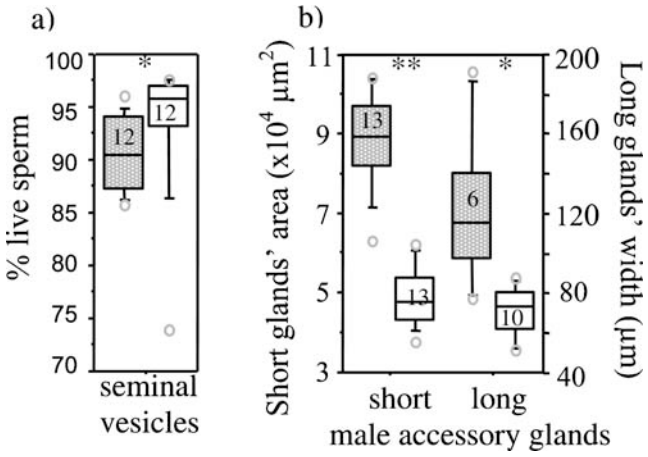

Fig. 4 Ejaculatory components of fed (*filled boxes*) and starved (*open boxes*) *T. castaneum* males. **a** Sperm viability in male seminal vesicles and **b** size of accessory glands. Sample sizes are inside *boxes*. *Asterisks* denote significant differences between male treatments (see text for statistical results). *Boxed horizontal edges* represent 25th and 75th percentiles, *whiskers* indicate 10th and 90th percentiles, *solid line inside the box* is the median, and *circles* represent outlying data points

starvation (Hussain 1994), and in another tenebrionid beetle, *Tenebrio molitor*, chemical signals collected from starved males were less attractive to females (Rantala et al. 2003).

Another possible mechanism through which females could influence male reproductive success is by controlling male spermatophore placement during copulation (Eberhard 1996; Simmons 2001). *T. castaneum* females could potentially affect spermatophore positioning by contracting musculature located at the junction between the anterior and posterior bursa copulatrix, thus preventing males' spermatophores from reaching the anterior bursa (close to the sperm storage site). Although we found that spermatophore placement was independent of male phenotypic condition and copulatory behaviors, or whether the females were alive or dead, spermatophore positioning might become more important when two or more males inseminate a female in rapid succession. However, in our experiment females had at least 24 h between matings so prior spermatophores and most of the sperms had already been expelled.

Ejaculate properties and number of sperms stored by females

Additional mechanisms that increase male paternity share were well-established for many organisms, and they include males transferring more sperms to females or having a higher proportion of their sperms stored and used by females (Ward 1993; Eberhard 1996; Simmons 2001). In our experiment, starved males transferred significantly (1.75 times) fewer sperms to virgin females than did fed males. This difference likely resulted from male physiological limitations on sperm production, but we cannot currently determine how much of this difference was due to virgin females discriminating against starved males. In addition, starved males had fewer sperms stored in females' spermathecae 1 h postmating. Although these sperm counts were necessarily made using virgin females, such differences could be even more pronounced in natural populations if mated females show greater discrimination than virgin females against low quality males (Eberhard 1996; Simmons 2001). Thus, the lower paternity share gained by starved males in our experiment can be explained at least in part by reduced number of sperms transferred and stored. Although starved males had higher sperm viability in their seminal vesicles compared to fed males (perhaps due to higher oxidative stress produced by metabolic processes), these relatively small viability differences did not appear to influence paternity share.

Fed and starved *T. castaneum* males also showed pronounced differences in accessory gland sizes that may have also contributed to differences in paternity share. In other insect species, male accessory gland proteins are known to incapacitate stored sperms from previous matings (Price et al. 2000, but see Snook and Hosken 2004) and to affect several aspects of female physiology (e.g., time to remating and oviposition rate) that in turn affect male paternity share (Simmons 2001; Wolfner 2002; Fry and Wilkinson 2004). In the red flour beetle, two paired glands (long and short) both contribute to spermatophore production. Histological studies of the short accessory glands in *Tribolium freemani* identified at least five cell types involved in manufacturing secretory products (Roberts and Grimnes 1994). Hence, decreased accessory gland sizes in starved *T. castaneum* males are likely to result in smaller spermatophores containing reduced quantities of accessory gland products, with potential effects on sperm competitive ability and female physiology.

In summary, this study presents evidence for several mechanisms through which both sexes can influence male paternity share when a female mates with multiple males. This study indicates that *T. castaneum* females have control over spermatophore transfer during mating, as they discriminate against inseminations by low quality males. In addition, male paternity share was influenced by the number of sperms transferred to and stored by females. Starvation had a prominent effect on male reproductive physiology, dramatically decreasing these numbers, and reducing male accessory gland size. Additional studies of the proximate mechanisms and traits responsible for biasing paternity will provide a better understanding of how sexual selection drives trait evolution.

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