Female influence over offspring paternity in the red flour beetle *Tribolium castaneum*

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In animals having internal fertilization, both sexes can potentially influence the post-copulatory processes of sperm transfer, sperm storage and sperm use for fertilization. In this experiment, we investigated whether *Tribolium castaneum* females can influence male paternity success following consecutive matings with two different males. We compared second male paternity success (P2) between females exposed to carbon dioxide (CO₂) and control females kept in air, in both cases for 30 min between two matings. CO₂ exposure inhibits muscular activity and has previously been shown to decrease sperm storage by *T. castaneum* females. Females exposed to CO₂ after their first mating showed significantly higher P2 than control females during the later portion of a one-month oviposition period. These results are consistent with reduced storage of first male sperm by CO₂-exposed females. Also, *T. castaneum* females showed considerable variation in spermathecal morphology, and P2 decreased with increasing spermathecal tube volume. These results demonstrate that *T. castaneum* females can influence male paternity success, and suggest that differential sperm storage may be an important mechanism of post-copulatory female choice.

**Keywords:** cryptic female choice; sperm precedence; spermathecal morphology; sexual selection

1. **INTRODUCTION**

In polyandrous species, males that mate with previously inseminated females show considerable variation in their paternity success (Lewis & Austad 1990; Simmons 2001). This variation may arise from male-mediated sperm competition, as well as from female-controlled processes that bias paternity (Eberhard 1996; Simmons 2001). With internal fertilization, females have the potential to influence male paternity success, a phenomenon known as post-copulatory, or cryptic, female choice (Eberhard 1996). Females may exert control during several steps leading to fertilization: they may influence the quantity of sperm transferred by mating males, the quantity and location of sperm storage within the reproductive tract, or the use of stored sperm for fertilizations. For example, muscular activity of the female reproductive tract is required for moving sperm into and/or out of storage in several insects (Davey 1958; Villavaso 1975; Tschudirein & Benz 1990; LaMunyon & Eisner 1993). Although sperm competition has been well studied (Parker 1970; Smith 1984; Birkhead & Møller 1998; Simmons 2001), considerably less is known about mechanisms of post-copulatory female choice.

Sperm precedence, defined as non-random differential fertilization success among mating males (Lewis & Austad 1990), has been particularly well studied in the red flour beetle, *Tribolium castaneum*, yielding evidence that both males and females influence sperm precedence. Lewis & Austad (1990) demonstrated consistent differences in second male sperm precedence (P2) among *T. castaneum* male pairs. Recent studies showing that male strains differ in P2 (Bernasconi & Keller 2001; Pai & Yan 2002) provide further evidence supporting male influence over fertilization success. Male fertilization success has also been found to depend upon interactions between particular male and female genotypes (Pai & Yan 2002; Nilsson et al. 2003). *Tribolium castaneum* copulatory behaviour, which includes male leg-rubbing and female quiescence (Bloch Qazi 2003), represents one arena for such interactions. Recent studies show differing results concerning how male leg-rubbing behaviour influences paternity success. Edvardsson & Arnqvist (2000) found that higher rates of male leg-rubbing (number of bouts per unit time) were associated with increased P2, but another study found no effect on P2 using other measures of male leg-rubbing behaviour (Bloch Qazi 2003). However, longer durations of female quiescence during copulation resulted in higher quantities of sperm transferred and greater fertilization success of the mating male (Bloch Qazi 2003). *Tribolium castaneum* males with higher olfactory attractiveness to females also gain higher P2 (Lewis & Austad 1994), raising the possibility of female post-copulatory choice of sperm from attractive males. Direct evidence for an active female role in the process of sperm storage was provided by Bloch Qazi et al. (1998), who found an 11-fold reduction in sperm storage in *T. castaneum* females that had been anaesthetized with carbon dioxide (CO₂) for 30 min after single mating; sperm retained normal motility when isolated female reproductive tracts were exposed to CO₂. These results suggest that one mechanism by which *T. castaneum* females might influence male paternity success is through differential sperm storage. Furthermore, the morphology of the female spermatheca (sperm storage organ) might potentially allow sperm from several males to be stored in different compartments (Eberhard 1996; Hellriegel & Ward 1998). In *T. castaneum*, the female spermatheca has been previously described as three long blind-ended tubules connected to the anterior bursa copulatrix by a short common duct (Sinha 1953).

These studies reveal that in *T. castaneum* both sexes are likely to contribute to differences in paternity success among multiple males mating with the same female;
however, little is known about relevant mechanisms. In this study, we investigated whether *T. castaneum* females, previously shown to influence sperm storage, also have the potential to influence male paternity success after copulation with different males. We conducted a double-mating experiment in which females were exposed to CO$_2$ between two consecutive matings to reduce muscular contractions within the female reproductive tract. We examined the dynamics of P$_2$ in females exposed either to CO$_2$ or to air for 30 min immediately after their first mating. Based on reduced sperm storage by females exposed to CO$_2$ following a single mating (Bloch 
Qazi et al. 1998), we predicted that P$_2$ would be higher for females exposed to CO$_2$ between matings. We also examined female spermathecal morphology to see whether differences in paternity success were associated with variation in spermathecal volume.

### 2. METHODS

#### (a) Study species

The red flour beetle, *T. castaneum* (Herbst) (Coleoptera: Tenebrionidae), is an economically important pest of stored grains worldwide. Both sexes mate multiply over the adult life-span, which can exceed 1 year (Sokoloff 1974). During copulation, males transfer a spermatoophore consisting of a membranous, sperm-filled sac into the female bursa copulatrix. Within 60 min post-mating, ca. 4% of transferred sperm have been translocated to the spermatheca for long-term storage (Bloch Qazi et al. 1996). Beets used in this study originated from two *T. castaneum* strains: wild-type beetles derived from the Berkeley synthetic strain and Chicago black beetles homozygous for an autosomal semidominant black body colour allele (Sokoloff et al. 1960).

#### (b) Comparison of sperm precedence between carbon dioxide-exposed and control females

Experimental beetles were randomly chosen from stock cultures, sexed as pupae, and kept in same-sex groups of 10 beetles per 10 g of King Arthur wheat flour at 30°C and 70% relative humidity. As virgin males commonly show insemination failure during their first copulation (Bloch Qazi et al. 1996; Bloch Qazi 2003), all males used in this experiment were premated by keeping them with 8–10 black females for 2 days and then isolating the males for 4 days prior to use in the experiments. Males were two to four weeks old when used in these experiments, and females were one to three weeks old. We used CO$_2$ in this experiment because CO$_2$ exposure has previously been shown in another insect species to stop rhythmic peristaltic contractions of the female reproductive tract elicited by mating, but muscle contractions resume after removal from the treatment (Tschudi-Rein 
Benz 1990).

In this double-mating experiment, randomly picked virgin black females were first placed in a 17 mm diameter mating arena with a single wild-type male. Immediately after copulation, females were assigned to one of two treatments: (i) females kept in a CO$_2$-filled chamber for 30 min post-mating (\(n = 37\)); or (ii) control females kept in air for 30 min post-mating (\(n = 37\)). Observations were conducted over 5 days; on each day, type of treatment for the first female was chosen randomly and subsequent treatments were alternated. As CO$_2$-exposed females turn onto their backs, females from both treatments were held upside down on tape to control for possible positional effects on sperm storage. Following the 30 min treatment, each female was immediately placed in a second mating arena with a black male until the second copulation was completed. Mating with both first and second males was observed and copulation duration and time to remating after removal from the treatment were recorded; all of the 37 control females and 34 out of 37 CO$_2$-exposed females remated within 20 min after removal from treatment. Approximately half of the CO$_2$-exposed females had regained mobility before the second copulation.

Eggs were collected from each doubly mated female for three 3-day oviposition periods (days 1–3, 13–16 and 26–29) by transferring a female into new oviposition vials containing 10 g of wheat flour. Paternity success of wild-type and black males was measured by scoring phenotypes of adult progeny. Crosses between these *T. castaneum* strains show nearly identical egg-to-adult survivorship (Sinnock 1969), so adult progeny can be used to accurately estimate male fertilization success (Lewis 
Austad 1990; Edvardsson 
Arnaqvist 2000; Nilsson et al. 2003). P$_2$ during each of the three oviposition periods was determined as the ratio of the number of homozygous black progeny to total number of progeny produced. Progeny from intermediate 10-day intervals (days 3–13 and 16–26) were also scored to confirm successful insemination by both males. To avoid confounding paternity estimates with failed insemination by either male, in our analysis we included females only when there was positive evidence of insemination by both males based on a female producing progeny sired by each male during any of three oviposition periods or during the two 10-day intervals. Also, in an attempt to standardize precision of P$_2$ estimates, a minimum of nine progeny were required for inclusion in ANOVA analysis; thus progeny numbers ranged from 9 to 55 for each of the three 3-day oviposition periods. These two restrictions yielded final sample sizes of 26 control doubly inseminated females and 22 CO$_2$-exposed doubly inseminated females; there was no female mortality but naturally declining oviposition rates resulted in reduced sample sizes during later oviposition periods.

#### (c) Effects of carbon dioxide exposure on long-term sperm viability

Previous studies on *T. castaneum* have shown that sperm motility is retained following a 30 min CO$_2$ exposure (Bloch Qazi et al. 1998). In this study, we extended the assessment period and compared the effect of CO$_2$ exposure on long-term sperm viability by using two distinct but complementary methods.

First, we indirectly assessed long-term sperm viability by comparing progeny production between CO$_2$-exposed and air-exposed females over time. Virgin black females were allowed to mate once with a single wild-type male, and then randomly assigned to be either exposed to CO$_2$ or air for 30 min immediately after mating. Following treatment, eggs were collected from each female for 40 days (in seven consecutive 5–7-day oviposition periods), and the larvae produced during each oviposition period were counted.

Second, we directly measured long-term viability of stored sperm in CO$_2$-exposed versus control females. Virgin black females were allowed to mate once with a single wild-type male. Immediately after mating they were randomly assigned to two treatments: either CO$_2$ exposure or air exposure (control), both for 30 min. Females were kept in flour at 30°C and 70% relative humidity until they were dissected at 1.5, 14.5 and 28.5 days after mating. Following methods modified from Bernasconi et al. (2002), we measured the proportion of live sperm in each.
female’s spermatheca using a Live/Dead sperm viability kit (Molecular Probes, Eugene, OR, USA). Females were dissected and the spermatheca was placed in 5 μl drop of Tribolium saline (Bloch Qazi et al. 1998) and ruptured to release sperm. Two stains, propidium iodide and 50-fold diluted SYBR-14 (2.5 μl each), were added to the sperm suspension, which was incubated in the dark for 5 min. Sperm were viewed under ×200 magnification, and all live and dead sperm found in several non-overlapping fields were counted during 8–10 min. Estimates of the percentage of viable sperm per female (live/total count) were based on total sperm counts ranging from 12 to 258 sperm (mean, 88 sperm).

(d) Female spermathecal morphology

Control females from the double-mating experiment (described above) that had been exposed to air for 30 min between two matings were preserved in 70% ethanol following their 29-day oviposition period, and then dissected to examine spermathecal morphology. For each female elytral length was measured, then the spermatheca was removed and placed in 4% methylcellulose in saline. The muscular sheath surrounding the spermatheca (see fig. 1 in Bloch Qazi et al. 1998) was removed, and spermathecal tubules were disentangled (dissection was successful in 18 out of 26 females). Length and basal width of each tubule were measured from digital photographs using Nis IMAGE v. 1.6. Measured width at the tip of each tubule was ca. 5 μm and was treated as a constant. We estimated the volume of each spermathecal tubule as a frustum of a right circular cone, and was treated as a constant. We estimated the volume of each spermathecal tubule as a frustum of a right circular cone, and the spermatheca was placed in 5

(e) Statistical analysis

Differences between CO2-exposed and control females in P2 were examined using repeated-measures ANOVA, with female treatment as a fixed factor and oviposition period as repeated factor. Variances were sufficiently homogenous (with a maximum ratio of 2.4) not to seriously violate ANOVA assumptions (Zar 1999). We also present, as an alternative to repeated-measures ANOVA, a statistical analysis using a generalized linear model with a binomial error distribution and a logit link function (Arnqvist & Danielsson 1999). Unlike repeated-measures ANOVA, this analysis includes all data points. It was conducted using Proc Genmod in SAS (SAS Institute, Inc., NC, USA), and the covariance structure was specified as autoregressive. Parameter estimates of effects were assessed with z-tests, and were adjusted for the moderate overdispersion of 2.61 estimated for our data. To examine differences in female-to-female variability in P2 between CO2 exposure and control treatments, we used Levene’s test (Miller 1986), consisting of an ANOVA comparing absolute deviations from each group mean between the two female treatments. The relationship between total spermathecal volume and total P2 was examined using non-parametric Spearman’s rank correlation analysis. Throughout this report, all descriptive statistics are reported as mean and standard error if not indicated otherwise.

3. RESULTS

(a) Comparison of sperm precedence between carbon dioxide-exposed and control females

Tribolium castaneum females that were exposed to CO2 for 30 min immediately after their first mating showed significantly higher mean P2 compared with control (air-

(b) Effects of carbon dioxide exposure on long-term sperm viability

To assess whether CO2 diminished the long-term viability of stored sperm, a separate experiment was

Figure 1. P2 (proportion of progeny sired by the second of two mating males) for Tribolium castaneum females exposed to either CO2 (filled bars) or air (open bars) for 30 min between two matings; means ± 1 s.e. for each of three oviposition periods are based on sample sizes of 22, 17 and 11 for CO2-exposed females and 26, 24 and 15 for control females, respectively.

exposed) females (figure 1; repeated-measures ANOVA, female treatment: F1,24 = 5.5, p = 0.027). As expected, females in both treatments showed a significant decline in P2 over one month following double mating (oviposition period: F2,48 = 14.1, p < 0.0001). Although the treatment x time interaction was not significant (F2,48 = 0.8, p = 0.442), the difference between female treatments became increasingly pronounced later during the month-long oviposition period. This was primarily a result of a slower decline in P2 in CO2-exposed females than in controls (figure 1; table 1). An alternative analysis using a generalized linear model yielded similar results, including a significant treatment effect (z = 2.08, p = 0.038), and a significant decline in P2 over time (z = 5.12, p < 0.0001). In addition, there was a marginally significant treatment x time interaction when the first versus third oviposition periods were compared (z = 1.94, p = 0.053).

When P2 dynamics over time were examined for individual females, control females (figure 2a) exhibited a fairly uniform pattern of declining P2. By contrast, CO2-exposed females showed greater variability in their P2 dynamics (figure 2b). Although P2 variation was similar during the first oviposition period (table 1; Levene’s test: F1,39 = 1.4, p = 0.238), CO2-exposed females showed greater among-female variation in P2 compared with control females during the second and third oviposition periods (table 1; Levene’s test: F1,39 = 6.2, p = 0.017 and F1,24 = 4.2, p = 0.050, respectively). The above treatment differences were not a result of fecundity differences; the numbers of progeny produced by ovipositing females did not differ between treatments for any of the three oviposition periods (table 1; three ANOVAs, all p > 0.262). Likewise, P2 variation among CO2-exposed females was not associated with differences in time to remating after removal from CO2 (regression t = 0.43, p = 0.674, n = 22, r² = 0.01).

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conducted in which singly mated females were exposed to either CO2 or air for 30 min immediately after copulation. Total number of progeny produced over 40 days did not differ between these singly mated CO2-exposed females (192 ± 27 progeny) and control females (192 ± 23 progeny) (ANOVA: F1,30 ≪ 0.1, p = 0.999). In addition, temporal patterns of offspring production (figure 3a) indicate no difference between singly-mated CO2-exposed females and control females (repeated-measures ANOVA treatment: F1,12 = 0.78, p = 0.39). As expected, progeny production declined over time (F6,72 = 2.36, p = 0.038) but there was no interaction (treatment × time: F6,72 = 0.63, p = 0.70). Finally, there was no difference between the two treatments in the percentage of females producing fertilized eggs over time (figure 3b; Fisher’s exact test: p ≫ 0.726 for each of seven sampling periods, n = 15 CO2-exposed and n = 17 control females).

When viability of sperm stored in the female spermatheca was directly assessed using a live/dead staining technique, stored sperm viability at 1.5 days following a single mating did not differ between CO2-exposed females (69 ± 6% live sperm, n = 4) and control females (60 ± 3% live sperm, n = 4; ANOVA: F1,8 = 2.0, p = 0.21). Similarly, although stored sperm viability overall had declined nearly threefold in both treatments by 28.5 days following a single mating, there was no difference between the two treatments; CO2-exposed females (n = 5) had 18 ± 7% live sperm and control females (n = 5) had 22 ± 9% live sperm (ANOVA: F1,8 = 0.1, p = 0.753).

**4. DISCUSSION**

This study provides evidence that female-mediated sperm storage can influence subsequent patterns of sperm precedence in multiply mated females. Females of *T. castaneum* flour beetles that were exposed to CO2 immediately after their first mating retained higher P2 over a one-month oviposition period. Previous work has shown that *T. castaneum* females stored significantly fewer sperm in their spermathecae after a single mating when they were...
Female influence on male paternity success  

T. Y. Fedina and S. M. Lewis

Figure 3. Fecundity dynamics of *Tribolium castaneum* females exposed to either CO$_2$ (filled symbols) or air (open symbols) following single mating: (a) daily offspring production by ovipositing females and (b) percentage of females remaining fertile over 40 days.

Figure 4. Variation in spermathecal morphology of *Tribolium castaneum* females: representative examples of (a) simple spermathecal structure with two primary tubules and (b) complex spermathecal structure with four primary tubules. Primary tubules are connected to the spermathecal duct (indicated by arrow) which opens into the anterior bursa copulatrix (not shown). Scale bar, 50 µm.

Exposed to CO$_2$ versus air for 30 min (Bloch Qazi et al. 1998). The present study demonstrates that such differences in sperm storage translate into differences in offspring paternity in doubly mated females. It is likely that reduced storage of first-male sperm by CO$_2$-exposed females leads to higher representation of second-male sperm when these females remate, and thus to higher P$_2$ than in control females.

The short intermating interval used in this study is likely to result in some sperm mixing within the bursa copulatrix, so CO$_2$ treatment effects on P$_2$ were less than what might be predicted based solely on reduced sperm storage in singly mated females (Bloch Qazi et al. 1998). In addition, two complementary approaches used here demonstrate that maintenance of high P$_2$ in CO$_2$-exposed females is not likely to be a result of any adverse effects of CO$_2$ on long-term viability of first male sperm. Thus, in natural populations, females may exert control over proportional representation of different males’ sperm in storage (and hence offspring paternity) by regulating muscular activity of the reproductive tract combined with manipulating remating time.

The present study also provides insight into some additional patterns of sperm precedence in *T. castaneum*. Our results confirm a decrease in P$_2$ over time consistent with initial sperm stratification, followed by later mixing of sperm from two males predicted to occur in species with tubular spermathecae (Walker 1980; Lewis & Jutkewicz 1998). Such sperm mixing in concert with relatively higher proportional representation of second male sperm in CO$_2$-exposed females may be responsible for the increasing gap between the two treatments in P$_2$ over time. In addition, we found greater variation in P$_2$ among CO$_2$-exposed females than among control females. This did not appear to be a result of greater variation in intermating interval, as P$_2$ was not correlated with time to remating in CO$_2$-exposed females. Another cause for greater P$_2$ variation could be a result of differences among individual females in the time required to recover from CO$_2$ anaesthesia. The resulting variation in female perception of copulatory behaviours or other stimuli produced by the second male during courtship could differentially affect sperm transfer and storage in CO$_2$-exposed females. For example, in the spotted cucumber beetle, *Diabrotica*...
undecimpunctata, females discriminate among males on the basis of male antennal stroking display during copulation, and females respond by relaxing the vaginal muscles, thus allowing successful spermatophore transfer (Tallamy et al. 2003).

Female influence over paternity has been previously demonstrated for only a few insect species using various techniques (Wilson et al. 1997; Ward 1998, Simmons et al. 1999; Civetta & Clark 2000; Nilsson et al. 2003). Several studies have used reciprocal double-mating designs with different combinations of related and unrelated mates to statistically partition influence of male versus female genotypes on sperm precedence in Callosobruchus maculatus, Drosophila melanogaster and T. castaneum (Wilson et al. 1997; Clark & Begun 1998; Nilsson et al. 2003). Although these studies document significant influence of female genotype on P2, the mechanisms of female control were not addressed. Extensive research on the yellow dung fly Scathophaga stercoraria showed that females can differentially partition sperm from two males in their multiple spermathecae (Otronen et al. 1997; Hellriegel & Bernasconi 2000), that these females can mediate male sperm displacement (Simmons et al. 1999; Hosken & Ward 2000) and that these females can strategically adjust sperm use from two males according to the male’s phos- phoglucomutase genotype (Ward 1998). To our knowledge, Hellriegel & Bernasconi (2000) is the only previous study that used anaesthesia to investigate the effect of female muscular control on sperm storage after mating with multiple males. Although this study demonstrated an active female role in partitioning sperm from two males into storage, it did not address how observed differences in storage might translate into subsequent offspring paternity.

Other studies have focused on the physiological and morphological basis of female control over sperm storage in several arthropod species (Gschwentner & Tadler 2000; Clark & Lange 2002; Fritz 2002; Burger et al. 2003). Thus, in a haplopygine spider there is a nail-like structure with attached muscles that locks a sclerotized spermathecal opening (Burger et al. 2003). In the seed bug Lygaeus similans, a muscular valve controls access to the sperm storage organ (Gschwentner & Tadler 2000). Multiple innervation of spermathecal compartments has been found in the fly Anastrepha suspensa (Fritz 2002) as well as in Locusta migratoria (Clark & Lange 2001). In L. migratoria females, endogenous neuropeptides were found to increase or decrease neurally evoked spermathecal muscle contractions in a dose-dependent manner (Clark & Lange 2002). Although these examples may be naturally selected adaptations for efficient sperm storage and use, they may also represent mechanisms for cryptic female choice allowing females to differentially use sperm from two or more males.

In our study spermathecal morphology was found to be highly variable among T. castaneum females, as has been reported for some other insects. Intraspecific variation in the number and size of spermathecal compartments has been documented in some dipterans, where different sper- mathecal compartments are used to partition sperm from multiple males and/or have different priority of sperm use (Otronen et al. 1997; Pitnick et al. 1999; Hellriegel & Bernasconi 2000; Ward 2000). The functional significance of variation in T. castaneum spermathecal morphology remains an open question. This type of spermatheca may allow for spatial partitioning of sperm from different males. However, tubular spermathecae may have evolved simply to reduce sperm loss, with variation in tube number and positioning being a by-product of branching during organogenesis. In dipteran females, for example, there are long muscular ducts leading to separate storage compartments and a valve at the entrance to each spermatheca (Lachmann 1997; Fritz & Turner 2002) that may allow for the observed non-random sperm distribution (Otronen et al. 1997; Hellriegel & Bernasconi 2000; Taylor et al. 2000). In T. castaneum, there are no valves separating individual tubules; however, sperm partitioning in this species could be achieved through other mechanisms, for example, through non-uniform contrac- tion of the muscular membrane surrounding spermatheca. The relationship observed in our study for larger sperma- thecal volume to be associated with lower overall P2 may be a result of acceptance of more sperm from the first mate and/or increased sperm mixing within larger sperma- thecae. It may also be harder for the second male to dis- place first male’s sperm from longer tubules by a process similar to one suggested for the yellow dung fly, Scathophaga stercoraria (Simmons et al. 1999).

This study has demonstrated that T. castaneum females can influence offspring paternity. Our results suggest that differential sperm storage may be an important mech- anism of post-copulatory female choice among multiple males mating with the same female. Further studies of how male and female behaviour and morphology interact to affect sperm storage and use will contribute to understanding the mechanisms generating differential paternity among mating males.

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REFERENCES


Sinnock, P. 1969 Components of fitness at the black locus in *Tribolium castaneum*. PhD thesis, University of California, Berkeley, CA, USA.


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